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OPTIMIZED MEDIUM FOR CLONAL GROWTH OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS WITH MINIMAL SERUM

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SUMMARY

An optimized basal nutrient medium, MCDB 131, has been developed that supports clonal growth of human microvascular endothelial cells (HMVEC) with as little as 0.7% dialyzed fetal bovine serum (dFBS) when also supplemented with 10 ng/ml epidermal growth factor (EGF) and 1 μg/ml hydrocortisone. An extensive initial survey of available media showed that MCDB 402, a medium optimized for low-serum growth of Swiss 3T3 cells, supported the best clonal growth of HMVEC with 10% dFBS. Quantitative adjustment of the composition of MCDB 402 for improved clonal growth of HMVEC with reduced amounts of dFBS resulted in development of MCDB 131. Although many different adjustments contributed to the optimal properties of MCDB 131 for growth of HMVEC, the most unusual feature of this medium is its high magnesium concentration. A major benefit was achieved by increasing Mg^{2+} from 0.8 m*M* in MCDB 402 to 10.0 m*M* in MCDB 131. In the absence of defined supplements, MCDB 131 supports good clonal growth of HMVEC with 2% dFBS. This can be reduced to 0.7% by adding EGF and hydrocortisone, which act synergistically to improve growth with low levels of dFBS.

Key words: magnesium; epidermal growth factor; hydrocortisone; MCDB 131; dialyzed serum.

INTRODUCTION

For some years it has been possible to obtain long-term cultures of endothelial cells from large vessels such as bovine aorta (5,53), human umbilical vein (13,32,37), and adult human veins and arteries (31). Culture of endothelial cells from microvessels is a more recently acquired skill and, until now, a more difficult one. Microvascular endothelial cells have been established from rat epididymal fat pad (4,52), rat brain (6,10), bovine brain (14), kitten retinal capillaries (12), human foreskin (9,11,36,47), and human omentum (34). When grown in conventional media such as M199 or Dulbecco's modified Eagle's (DME), microvascular endothelial cells from each of these sources require large amounts of whole animal sera (9,36) and often other undefined supplements such as extracellular matrix or tumor cell conditioned medium (11).

Microvascular endothelial cells have been difficult to culture for two reasons: (a) primary cultures are difficult to prepare, with small cell yields and frequent overgrowth by fibroblasts; and (b) conventional nutrient media do not fully satisfy their growth requirements. Kern et al. (34) have recently cultured human microvascular endothelial cells (HMVEC) from omentum. Many of the problems described above are greatly reduced with cells from this source. Confluent primary cultures of HMVEC can be obtained quickly and easily with little or no fibroblast contamination. Although HMVEC from omentum grow quite well in M199 supplemented with 10% whole fetal bovine serum (wFBS), they do not grow at all in M199 or other conventional media, such as DME or RPMI 1640, when supplemented with dialyzed fetal bovine serum (dFBS).

We now report an optimized nutrient medium, MCDB 131, that will support clonal growth of HMVEC from human omentum with as little as 0.7% dFBS when also supplemented with 10 ng/ml epidermal growth factor (EGF) and 1 μg/ml hydrocortisone. Medium MCDB 131 is not qualitatively different from most of the other optimized media that have been developed in this laboratory. Instead, it is the quantitative differences that give MCDB 131 its unique ability to support growth of HMVEC with minimal amounts of dialyzed serum.

MATERIALS AND METHODS

Water. Deionized water used for large-scale operations, such as dialysis of serum, was obtained from a mixed bed deionization system with charcoal filtration (Continental

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Water Conditioning Corp. of Colorado, Denver). Deionized water used for preparation of medium and all other solutions that were directly used for cell culture was obtained by passing tap water first through a reverse-osmosis system (R04) and then through a mixed bed deionizer (Milli-Q), both from Millipore Corp., Bedford, MA. Water treated in this fashion is referred to below as high-purity deionized water.

Chemicals, hormones, and growth factors. All biochemicals used for preparation of media and solutions were from Sigma Chemical Co., St. Louis, MO. Inorganic salts were from Fisher Scientific, Pittsburgh, PA. Trace elements were Specpure grade from Johnson-Matthey, London, England. Hydrocortisone, dexamethasone, triiodothyronine, estradiol, insulin, prostaglandins E_1 and $F_2\alpha$, transferrin, poly-D-lysine, phosphoethanolamine, ethanolamine, dithiothreitol, and glutathione were from Sigma. Epidermal growth factor, fibroblast growth factor (FGF), fibronectin, and endothelial cell growth supplement (ECGS) were from KOR Biochemicals, Cambridge, MA. Collagen and laminin were from Bethesda Research Laboratories, Gaithersburg, MD. Thrombin was from Boehringer-Mannheim Biochemcials, Indianapolis, IN. Platelet-derived growth factor (PDGF) was from Collaborative Research, Waltham, MA. Chicken embryo extract was from GIBCO, Santa Clara, CA. Bovine pituitary extract was prepared as previously described (49). Crude somatomedin was prepared as described by Weinstein et al. (54). Brain extract was prepared as a crude homogenate of brains from female Sprague-Dawley rats in an equal volume of 0.15 *M* NaCl, centrifuged at 4340 × *g* for 20 min, and filter sterilized. Antibiotics used for primary cultures were obtained from GIBCO as a 100× mixture of penicillin, streptomycin, and Fungizone, and diluted 1:100 into the medium to yield a final concentration of 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone. EGF was dissolved as instructed by KOR Biochemicals at 10 μg/ml. Hydrocortisone was dissolved in absolute ethanol at 1 mg/ml and filter sterilized. Both EGF and hydrocortisone were stored at −20° C in small aliquots.

Media. The following commercial media were included in the initial survey: M199; Eagle's minimum essential medium (MEM); DME; Waymouth's MB 752/1; McCoy's 5A; and RPMI 1640 (all obtained from GIBCO in powdered form); and F12 (powdered, Flow Laboratories, Inglewood, CA). All media containing bicarbonate were adjusted to pH 7.40 before addition of the bicarbonate, and all were prepared with 14 m*M* (1.176 g/liter) sodium bicarbonate for use with 5% CO_2 (48). MCDB 110 (3), MCDB 153 (8), MCDB 202 (40), and MCDB 402 (48) were prepared in this laboratory as described in the cited references. A commercially prepared medium conditioned by human leukocytes, Condimed® , was purchased from Boehringer-Mannheim (Indianapolis, IN).

Many individual adjustments were made in the composition of MCDB 402, leading ultimately to the formulation of medium MCDB 131, as described in Results. The composition of MCDB 131 is listed in Table 1, both in weight and molar units, together with the molar

TABLE 1

COMPOSITION OF MCDB 131 AND MCDB 402

	MCDB 131,		MCDB 402,
	Mg/liter	M/liter[a]	M/liter[a]
Amino acids			
L-Alanine	2.67	3.00E−5	—
L-Arginine·HCl	63.20	3.00E−4	3.0E−4
L-Asparagine·H_2O	15.01	1.00E−4	1.0E−4
L-Aspartic acid	13.31	1.00E−4	1.0E−5
L-Cysteine·HCl·H_2O	35.13	2.00E−4	2.0E−4[b]
L-Glutamic acid	4.41	3.00E−5	1.0E−5
L-Glutamine	1461.50	1.00E−2	5.0E−3
Glycine	2.25	3.00E−5	1.0E−4
L-Histidine·HCl·H_2O	41.93	2.00E−4	2.0E−3
L-Isoleucine	65.58	5.00E−4	1.0E−3
L-Leucine	131.27	1.00E−3	2.0E−3
L-Lysine·HCl	181.65	1.00E−3	8.0E−4
L-Methionine	14.92	1.00E−4	2.0E−4
L-Phenylalanine	33.04	2.00E−4	3.0E−4
L-Proline	11.51	1.00E−4	—
L-Serine	31.53	3.00E−4	1.0E−4
L-Threonine	11.91	1.00E−4	5.0E−4
L-Tryptophan	4.08	2.00E−5	1.0E−5
L-Tyrosine	18.12	1.00E−4	2.0E−4
L-Valine	117.15	1.00E−3	2.0E−3
Vitamins			
D-Biotin	0.00733	3.00E−8	3.0E−8
Calcium folinate·$5H_2O$	0.602	1.00E−6	1.0E−6
α-Lipoic acid	0.002063	1.00E−8	1.0E−8
Nicotinamide	6.11	5.00E−5	5.0E−5
D-Pantothenic acid (hemi-Ca salt)	11.91	5.00E−5	5.0E−5
Pyridoxine·HCl	2.056	1.00E−5	1.0E−4
Riboflavin	0.003764	1.00E−8	1.0E−6
Thiamine·HCl	3.373	1.00E−5	1.0E−4
Vitamin B_{12}	0.01355	1.00E−8	1.0E−8
Other organic compounds			
Adenine	0.1351	1.00E−6	1.0E−6
Choline chloride	13.96	1.00E−4	1.0E−4
D-Glucose	1000.00	5.55E−3	5.5E−3
i-Inositol	7.21	4.00E−5	4.0E−5
Putrescine	0.0001611	1.00E−9	1.0E−9
Sodium pyruvate	110.04	1.00E−3	1.0E−3
Thymidine	0.02422	1.00E−7	1.0E−6
Major inorganic salts			
$CaCl_2·2H_2O$	235.23	1.60E−3	1.6E−3
KCl	298.20	4.00E−3	4.0E−3
$MgSO_4·7H_2O$	2463.8	1.00E−2	8.0E−4
NaCl	6430.0	1.10E−1	1.1E−1
$Na_2HPO_4·7H_2O$	134.04	5.00E−4	5.0E−4
Trace elements			
$CuSO_4·5H_2O$	0.001248	5.00E−9	5.0E−9
$FeSO_4·7H_2O$	0.2780	1.00E−6	1.0E−6
$MnSO_4·5H_2O$	0.000241	1.00E−9	1.0E−9
$(NH_4)_6Mo_7O_{24}·4H_2O$	0.00371	3.00E−9[c]	1.0E−9
$NiCl_2·6H_2O$	0.0000713	3.00E−10	3.0E−10
H_2SeO_3	0.00387	3.00E−8	1.0E−8
$Na_2SiO_3·9H_2O$	2.842	1.00E−5	1.0E−5
NH_4VO_3	0.000585	5.00E−9	5.0E−9
$ZnSO_4·7H_2O$	0.0002875	1.00E−9	1.0E−6
Buffers and indicators			
$NaHCO_3$	1176.0	1.40E−2	1.4E−2
Phenol red (Sodium salt)	12.42	3.30E−5	3.3E−5
Carbon dioxide		5%	5%

[a] Computer-style exponential notation is used: 3.00E-4 means 3.00×10^{-4}.

[b] MCDB 402 contains L-cystine. Molar concentration of half-cystine residues is given for easy comparison.

[c] Concentration is for complex molecule containing 7 atoms of molybdenum. Concentration of molybdenum is 2.10E-8 *M*.

composition of MCDB 402 for easy comparison. Formulations intermediate between MCDB 402 and MCDB 131 were used for some of the experiments reported in this paper. Medium AK-1 consisted of MCDB 402 with the amino acid composition of MCDB 131. Medium MCDB 130 was AK-1 with magnesium sulfate increased to 1.0×10^{-2} *M*.

Current procedures for preparation of MCDB 131 are described in Tables 2 and 3. MCDB 131 is defined in terms of molar concentrations, which are listed in Tables 1 and 2 to three significant figures. Weights are listed with sufficient accuracy to yield molar concentrations accurate to three figures when divided by molecular weight. However, in practice, it is doubtful that differences in nutrient concentration of less than ± 1% have a significant effect on cellular growth. MCDB 131 prepared in this laboratory has a final osmolality of 275 ± 5 mOSM/kg (measured with a model 3DII Digimatic osmometer, Advanced Instruments, Needham Heights, MA). MCDB 131 is designed for use with a 5% CO_2 atmosphere.

Solution A. Solution A is a HEPES-buffered saline solution with the following composition: Glucose, 10 m*M*; HEPES, 30 m*M*; KCl, 3.0 m*M*; NaCl 130 m*M*; $Na_2HPO_4 \cdot 7H_2O$, 1.0 m*M*; Phenol Red, 0.0033 m*M*. The final solution was adjusted to pH 7.60 with 1 *N* NaOH, filter sterilized through a 0.22-μm, detergent-free filter (Nalgene), and stored at 4° C.

Dialyzed fetal bovine serum. A tris-citrate-borate buffer containing 2.0 *M* Tris, 0.6 *M* sodium citrate, and 0.4 *M* sodium borate, with a final pH of 7.40, was diluted 1:40 (25 ml/liter) into wFBS (Flow Laboratories) and stirred on ice for 30 min. The buffered serum was then dialyzed at 4° C against 100 vol of deionized water for 3 d in 12,000 to 14,000 molecular weight cutoff dialysis tubing (Van Waters and Rogers, Denver, CO), with two changes to yield a final theoretical dilution of $1:10^6$ inside the tubing. The dialyzed serum was then frozen, lyophilized, and stored at −20° C. Lyophilized serum was reconstituted at 50 mg/ml (100% dFBS) in solution A (pH 7.60), filtered sequentially through 0.8-, 0.45-, and 0.22-μm filters, aliquoted, and stored frozen until used.

Trypsin. Crystalline trypsin (Sigma) was dissolved into solution A (pH 7.60) at 0.05% (wt/vol), filter sterilized, and stored frozen at −20° C in small aliquots until used.

Culture flasks and petri dishes. Monolayer cultures of HMVEC were grown in 25-cm^2 tissue culture flasks (Corning, Corning, NY), and clonal growth assays were performed in 60-mm Lux tissue culture dishes (#5220, Miles Laboratories, Naperille, IL). Lab-Tek tissue culture chamber slides were used for cell characterization studies involving fluorescence microscopy.

Cells. Initially, primary cultures of HMVEC were prepared as previously described (34). After development of MCDB 131, primary cultures were initiated in MCDB 131 supplemented with 5% dFBS, 10 ng/ml EGF, 1 μg/ml hydrocortisone, and penicillin-streptomycin-Fungizone. Primary cultures were grown to approximately 75% confluency, trypsinized, and stored frozen in liquid nitrogen in MCDB 131 with 10% wFBS, 10% dimethylsul-

TABLE 2

STOCK SOLUTIONS FOR PREPARATION OF MCDB 131

Stock[a]		Component	Stock M/L	Stock G/L
1	(100×)	L-Alanine	3.00E−3	0.267
		L-Arginine·HCl	3.00E−2	6.320
		Glycine	3.00E−3	0.225
		L-Histidine·HCl·H_2O	2.00E−2	4.193
		L-Phenylalanine	2.00E−2	3.304
		L-Proline	1.00E−2	1.151
		L-Serine	3.00E−2	3.153
2	(100×)	L-Asparagine·H_2O	1.00E−2	1.501
		L-Aspartic acid	1.00E−2	1.331
		L-Glutamic acid	3.00E−3	0.441
		L-Tryptophan	2.00E−3	0.408
		L-Tyrosine[b]	1.00E−2	1.812
3	(100×)	L-Cysteine·HCl·H_2O[c]	2.00E−2	3.513
		L-Isoleucine	5.00E−2	6.558
		L-Leucine	1.00E−1	13.117
		L-Lysine·HCl	1.00E−1	18.165
		L-Methionine	1.00E−2	1.492
		L-Threonine	1.00E−2	1.191
		L-Valine	1.00E−1	11.715
4	(100×)	Folinate·Ca·$5H_2O$	1.00E−4	0.0602
		Pantothenate·1/2Ca	5.00E−3	1.191
		Niacinamide	5.00E−3	0.611
		Pyridoxine·HCl	1.00E−3	0.2056
		Riboflavin	1.00E−6	0.0003764
		Thiamine·HCl	1.00E−3	0.3373
		Vitamin B_{12}	1.00E−6	0.001355
		Choline chloride	1.00E−2	1.396
		myo-Inositol	4.00E−3	0.721
		Thymidine	1.00E−5	0.002422
5[d]	(100×)	D-Biotin	3.00E−6	0.000733
		DL-α-Lipoic acid	1.00E−6	0.0002063
		Adenine	1.00E−4	0.01351
		Putrescine·2HCl	1.00E−7	0.00001611
6	(50×)	$CaCl_2 \cdot 2H20$	8.00E−2	11.76
7	(100×)	$MgSO_4 \cdot 7H_2O$	1.00E+0	246.38
8[e]	(1000×)	$CuSO_4 \cdot 5H_2O$	5.00E−6	0.001248
		$FeSO_4 \cdot 7H_2O$	1.00E−3	0.2780
		$MnSO_4 \cdot 5H_2O$	1.00E−6	0.000241
		$ZnSO_4 \cdot 7H_2O$	1.00E−6	0.0002875
9[e]	(1000×)	H_2SeO_3	3.00E−5	0.00387
		$Na_2SiO_3 \cdot 9H_2O$	1.00E−2	2.842
		$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	3.00E−6	0.00371
		NH_4VO_3	5.00E−6	0.000585
		$NiCl_2 \cdot 6H_2O$	3.00E−7	0.0000713

[a] All stocks were prepared by dissolving the components in high purity deionized water, except where indicated otherwise by footnotes. Stocks 1, 2, 3, and 5 are stored at −20° C for 6 mo. or until thawed five times. Stock 4 is stored in dark bottles at −20° C for 6 mo. or until thawed five times. Stocks 6, 7, 8, and 9 are filter sterilized and stored at room temperature indefinitely.

[b] Tyrosine in stock 2 is initially dissolved in 25 ml of 4 *N* NaOH and then added to the stock containing the other components.

[c] Cysteine easily becomes oxidized to form cystine, which still supports growth of HMVEC, but tends to precipitate out of the stock solution. If a precipitate forms the entire stock 3 must be replaced. Alternatively, cysteine·HCl·H_2O can be placed in a separate stock solution so that the other components of stock 3 do not have to be replaced when the cysteine becomes oxidized.

[d] We have been able to prepare stock 5 by dissolving all of the components together in water. However, if separate (or more concentrated) stocks of the components are prepared, the lipoic acid should first be dissolved in a few drops of 1.0 *N* NaOH and the adenine should be dissolved by heating.

[e] Two drops of 4 *N* HCl (ca. 0.10 ml) are added per liter of stocks 8 and 9 to retard precipitation of components. If a precipitate occurs after storage, the stocks must be discarded.

foxide, and antibiotics. Ampules of HMVEC were thawed in 70% ethanol at 37° C, and the cells were inoculated into MCDB 131 with 10% wFBS and antibiotics. Cultures were then washed 24 h later with solution A to remove dead and unattached cells, and refed with fresh MCDB 131, 5% dFBS, 10 ng/ml EGF, 1 μg/ml hydrocortisone. Antibiotics were not used beyond this point. These secondary cultures (first passage) were refed every 2 or 3 d and were used in clonal assays before 50% confluency was reached. If allowed, the cultures would reach confluency in 5 to 7 d. Periodic tests for mycoplasma by the Hoechst 33258 stain fluorescence method were consistently negative.

Trypsinization. Trypsinization of a subconfluent, 25-cm² flask of HMVEC was as follows: The medium was aspirated off, the cells were washed twice with solution A at 4° C to remove residual serum-containing medium, and 2 ml of 0.05% (wt/vol) trypsin in solution A at 4° C was added to the flask. The trypsin solution was aspirated off 10 s after its addition, leaving only a thin film of fluid over the cells. After 1 to 3 min at room temperature, when the cells were rounded up and beginning to detach from the surface of the flask, 5 ml of MCDB 131 plus 5% dFBS was added and the cells were harvested from the culture surface by gentle pipetting. The suspension was then centrifuged at 220 $\times g$ for 5 min, and the pellet was resuspended in a small amount of serum-free MCDB 131 (or solution A if the cells were to be used in a nutrient growth-response assay). A sample was counted with a hemacytometer, and the suspension was diluted as needed for inoculation into clonal growth assays.

Clonal growth assay. Clonal growth assays were performed as described previously (48). The cellular inoculum was 2000 cells/60-mm tissue culture dish, and assays were incubated at 37° C for 14 d in an atmosphere of 5° CO_2 in air, saturated with water vapor, without medium change. At the end of the assay period, the medium was discarded and the cells were fixed with 2% (wt/vol) glutaraldehyde in 0.05 *M* sodium cacodylate (pH 7.0) and stained with 1% crystal violet. Growth responses were visually evident from the stained dishes and were quantified by measurement with an Artek colony counter (model 880, Artek Systems, Farmingdale, NY) to determine the area of the dish covered by colonies. Colony area was then plotted against concentration on semilog graph paper, and the graph was used to identify deficient and inhibitory concentrations on either side of an optimal plateau. Each medium component was then adjusted to a concentration near the center of its optimal plateau on a log scale (26).

Cellular characterization. Cellular characterization studies were undertaken on second or later passage cells that had been initiated as primary cultures and subcultured either in MCDB 131 + 10 ng/ml EGF + 1.0 μg/ml hydrocortisone + 2% wFBS (MCDB 131 + EHS2), or in MCDB 131 + 10% wFBS, as indicated. Assays for angiotensin-converting enzyme and ultrastructural studies were performed as previously described (34). Assays for Factor VIII antigen were done by indirect immunofluorescence as previously described (34), except that goat antihuman Factor VIII antibody (Atlantic Antibodies, Scarborough, ME) was used as the primary antibody and fluorescein isothiocyanate conjugated rabbit antigoat IgG was used as the secondary antibody.

Uptake of fluorescently labeled acetylated low-density lipoprotein was detected as described by Voyta et al. (51). Acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine perchlorate (DiI-Ac-LDL) was obtained from Biomedical Technologies Inc. (Cambridge, MA) at a concentration of 200 μg/ml, and was diluted to 30 μg/ml in MCDB 131 + EHS2. Second passage HMVEC were grown to confluence on Lab-Tek culture chamber slides in MCDB 131 + EHS2 and then incubated for 4 h at 37° C in MCDB 131 + EHS2 with 30 μg/ml diI-Ac-LDL. The slides were washed twice in Hanks' balanced salts solution, fixed with 2% paraformaldehyde, and rinsed twice more with Hanks' solution. Cover slips were then mounted on the slides with paraphenylene diamine (Sigma), and the cells were observed with a fluorescence microscope equipped with a rhodamine

TABLE 3

PREPARATION OF MCDB 131 FROM STOCK SOLUTIONS

1. For the preparation of 1 liter of MCDB 131, the following are added (ml) to approximately 800-ml high-purity, deionized water:

Stock 1	10.0
2	10.0
3	10.0
4	10.0
5	10.0
6	20.0
7	10.0
8	1.0
9	1.0

2. The following dry components (g/liter) are then added and dissolved:

L-Glutamine	1.4615
D-Glucose	1.000
KCl	0.2982
NaCl	6.400
Na-pyruvate	0.11004
$Na_2HPO_4 \cdot 7H_2O$	0.1314
Phenol red	0.01242

3. The volume is adjusted to approximately 950 ml with deionized water.

4. The pH is adjusted to 7.40 with 4 *N* NaOH.

5. 1.176 g $NaHCO_3$ is added and dissolved.

6. The final volume is adjusted to precisely 1 liter.

7. Osmolarity should measure 275 ± 5 mOsM/kg.

If large amounts of medium are to be made and frozen in aliquots, stocks 6, 7, 8, and 9 should be omitted because they tend to precipitate upon thawing if included. Also, $NaHCO_3$ should not be added until after the thawed medium has been completed with stocks 6, 7, 8, and 9, and adjusted to pH 7.4 with 4 *N* NaOH.

The final complete medium is filtered through a detergent-free 0.22-μm filter and stored at 4° C for up to 2 wk.

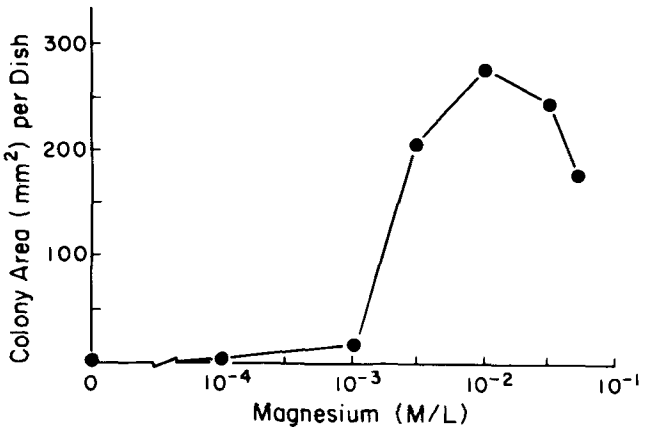

FIG. 1. Growth response of HMVEC to magnesium sulfate in medium AK-1 with 5% dFBS.

excitation-emission filter. Monolayers of human fibroblasts derived from omental adipose tissue, grown in DME + 10% wFBS and labeled with 30 μg/ml diI-Ac-LDL in DME + 10% wFBS, were used as a negative control. Monolayers of bovine pulmonary artery endothelial cells, grown in MEM (D-valine) + 10% wFBS and labeled with 30 μg/ml diI-Ac-LDL in MEM + 10% wFBS, were used as a positive control (51).

RESULTS

Growth of HMVEC in dialyzed fetal bovine serum. As previously described by Kern et al. (34), primary and secondary cultures of HMVEC grew well in M199 supplemented with 10% wFBS. However, when whole serum was replaced with dialyzed serum, M199 was unable to support growth of HMVEC. A survey was therefore conducted to determine whether an alternative medium could be found that would support clonal growth of HMVEC with 10% dFBS. Commercial media tested included M199, DME, MB 752/1, 5A, F12, and RPMI 1640. Several media that had been optimized in this laboratory for other cell types were also tested, including MCDB 153, which supports growth of human epidermal keratinocytes with defined supplements (8,49); MCDB 110, which supports growth of human diploid fibroblasts with defined supplements (3); MCDB 202 (40), which supports growth of chick embryo fibroblasts with defined supplements (18); and MCDB 402, which was optimized for growth of Swiss 3T3 cells with a small amount of dFBS (48). The survey revealed that with 10% dFBS (5 mg/ml dialyzed solids), MCDB 402 supported the best clonal growth of HMVEC (data not shown, but *see* Fig. 2 for a similar experiment done later with less dFBS).

A survey of hormones, growth factors, and extracts, including EGF, FGF, PDGF, ECGS, hydrocortisone, dexamethasone, triiodothyronine, estradiol, insulin, prostaglandins E_1 and $F_2\alpha$, transferrin, phosphoethanolamine, ethanolamine, liposome B (3), bovine pituitary extract, chick embryo extract, brain extract, and crude somatomedin, failed to augment growth above that achieved with 10% dFBS, suggesting that the composition of the basal medium was rate limiting for growth of HMVEC.

Development of MCDB 131. A series of growth-response titrations was then undertaken to improve the basal medium composition for clonal growth of HMVEC. The experimental approach to optimization has been described in detail for other cell types (1,19,29,30,33,41,42,48) and in review papers (20–28). Therefore only a few important points in the development of MCDB 131 will be presented here. Each of the components of MCDB 402 was titrated over a seven-log concentration range to determine the deficient, optimal, and inhibitory levels for growth of HMVEC. Initial experiments were done with clonal growth assays in a background of 10% dFBS, as described in Materials and Methods.

Amino Acids. MCDB 402 contains all of the amino acids involved in protein synthesis except alanine and proline. Although neither of these was found to be essential for growth of HMVEC, both were added at reasonable physiologic levels to reduce the biosynthetic load on the cells. In addition, all of the amino acids in MCDB 402 were titrated and their concentrations were adjusted to the midpoints on a log scale of their plateaus of optimum clonal growth of HMVEC in 10% dFBS. This resulted in substantial changes in amino acid concentrations, but did not significantly improve clonal growth of HMVEC with 10% dFBS. At this point the concentration of dFBS was reduced to 5% to increase the sensitivity of the clonal assays for subsequent medium component titrations. Medium MCDB 402 with the adjusted amino acid profile (which has been retained in MCDB 131) was renamed medium AK-1.

Major inorganic salts. Titrations of inorganic salts were carried out in AK-1 in a background of 5% dFBS. No adjustments were necessary for calcium chloride, potassium chloride, sodium chloride, or sodium phosphate. However, the magnesium titration revealed an unexpected and dramatic result. A 12.5-fold increase in magnesium sulfate from the MCDB 402 level of 0.8 to 10.0

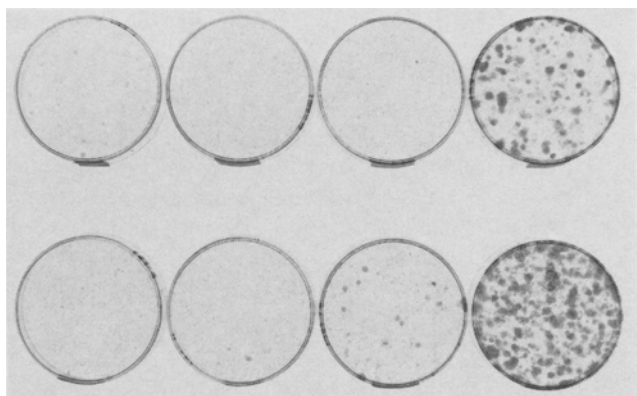

FIG. 2. Growth of HMVEC in various media with 2% dFBS. *Left to right*: M199; DME; MCDB 402; MCDB 131. *Top row*: standard Mg^{2+} levels (1×10^{-3} *M* in MCDB 131). *Bottom row*: 1×10^{-2} *M* Mg^{2+} in all media (normal level for MCDB 131).

m*M* resulted in a major increase in growth (Fig. 1). AK-1 with 10.0 m*M* magnesium sulfate was designated MCDB 130. Control experiments with magnesium chloride and sodium sulfate showed clearly that the effect was due to the concentration of magnesium ion in the medium (data not shown). The improved growth of HMVEC that resulted from increasing Mg^{2+} to 10.0 m*M* allowed dFBS to be reduced to 2% for subsequent titrations.

The effects of conventional and high magnesium concentrations in several different media (including MCDB 131, whose development is described below) are shown in Fig. 2. High Mg^{2+} levels are effective in augmenting the growth of HMVEC only in MCDB 402, MCDB 131, and intermediate formulations such as AK-1 and MCDB 130.

Vitamins. MCDB 130 contained nine water-soluble vitamins at the orginal concentrations from MCDB 402. None of these could be shown to be strictly essential for colony formation by HMVEC previously grown in vitamin-enriched media. However, riboflavin, thiamine, and pyridoxine were found to be somewhat inhibitory at the concentrations present in MCDB 402 and a distinct benefit to growth was seen when concentrations of all three were lowered. All of the vitamins were retained in the medium at levels adjusted away from toxicity in anticipation of future requirements in the absence of serum supplementation or after longer periods of depletion.

Other organic compounds. Seven additional organic compounds were present in MCDB 130: adenine, choline, glucose, inositol, putrescine, pyruvate, and thymidine. In single deletion experiments without prior depletion, inositol was the only compound for which a definite requirement could be shown. The lowest level of glucose tested was 5.5×10^{-5} *M* because of glucose introduced with the cellular inoculum. A slight benefit to growth was seen when thymidine was reduced from 1.0×10^{-6} to 1.0×10^{-7} *M*. No other changes in concentrations were needed, and all of the compounds in this group were retained in the medium at noninhibitory levels, to be tested again in the future under serum-free conditions and in experiments involving more extensive depletion.

Trace elements. A set of nine trace elements was supplied by MCDB 130. HMVEC did not stringently require any of these in the presence of 2% dFBS. However, growth was markedly enhanced by the combination of selenium at 3.0×10^{-8} *M* and iron at 1.0×10^{-6} *M*. Zinc was found to be slightly inhibitory at the MCDB 402 level of 1.0×10^{-6} *M* and was reduced to 1.0×10^{-9} *M*. The remaining trace elements were adjusted to levels well below inhibitory and retained in the medium on the basis of their importance in whole animal nutrition (50). It is possible that even under serum-free conditions, deliberate addition of these remaining trace elements may not be strictly necessary because they may be supplied in sufficient amounts as contaminants of other components. However, it is desirable to add them routinely to avoid possible effects of uncontrolled variations in the level of contamination.

The adjustments of vitamins, other organic compounds, and trace elements described above gave rise to MCDB 131, the final medium generated by a complete first round of growth-response titrations. Good clonal growth of HMVEC could be achieved in MCDB 131 with 2% dFBS (Fig. 2). However, when dFBS was reduced below that level, growth became poor. No growth was seen in serum-free MCDB 131, although the cells would attach and spread in the complete absence of supplementation. High magnesium continued to be beneficial, but MCDB 131 also supported improved growth of HMVEC in 2% dFBS with its magnesium level reduced to 1.0 m*M* (Fig. 2).

Growth factors and hormones. Growth factors and hormones that were initially found to be ineffective with 10% dFBS in MCDB 402 were tested again with dFBS reduced to 2% or less in MCDB 131. Under these conditions we observed a dramatic proliferative response to EGF at 10 ng/ml (Fig. 3). At concentrations greater than 10 mg/ml, EGF also caused a loosening of colony morphology that seemed to be due to increased random migration of HMVEC. This response has not yet been studied in detail but is of major interest for future studies. Hydrocortisone was only moderately stimulatory at its optimal level of 1.0 μg/ml, but when tested with EGF it worked synergistically to elicit a response greater than that found with EGF alone (Fig. 3). Insulin, which is beneficial in many serum-free culture systems, had little effect in the presence of 2% dFBS (Fig. 3).

Factors that seemed to have a weak positive effect in a background of 2% dFBS include transferrin, ECGS, prostaglandin E_1, bovine pituitary extract, phosphoethanolamine, ethanolamine, dithiothreitol, and glutathione. These factors will be studied further as we continue to reduce the serum level progressively. Factors and supplements for which no response was seen include FGF, PDGF, prostaglandin $F_2\alpha$, triiodothyronine, estradiol, thrombin, ceruloplasmin, chicken embryo extract, crude somatomedin, and a liposome preparation (3) composed of soy lecithin, sphingomyelin, cholesterol, vitamin E, and vitamin E acetate. As a result of the improvement in growth of HMVEC in the presence of 1

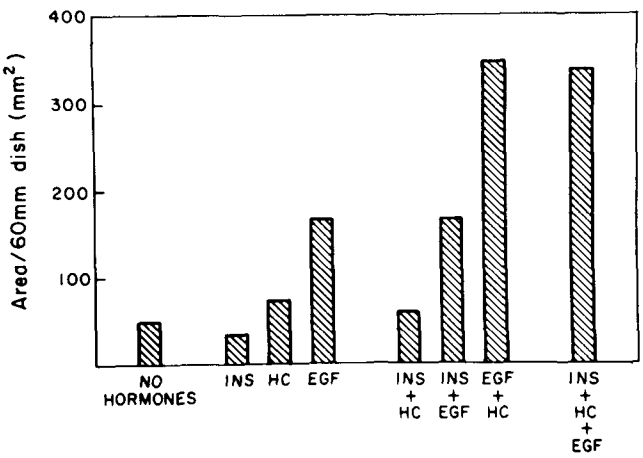

FIG. 3. Synergistic effect of EGF and hydrocortisone. *Left to right:* no hormones; 5 μg/ml insulin (INS); 1 μg/ml hydrocortisone (HC); 10 ng/ml EGF; INS + HC; INS + EGF; HC + EGF; INS + HC + EGF. All were tested in MCDB 131 plus 2% dFBS.

μg/ml hydrocortisone and 10 ng/ml EGF, the dFBS concentration has been reduced to 0.7% for future clonal growth assays.

Conditioned medium. A commercially prepared medium conditioned by human leukocytes (Condimed®) was highly stimulatory to growth of HMVEC when added at concentrations of 10 to 50% to MCDB 131 with or without dFBS (data not shown). However, because of the extreme complexity of this preparation, which contained both serum and conditioning factors, we did not pursue its growth-promoting properties further.

Extracellular matrices. Attachment factors and components of extracellular matrices were tested for possible effects on attachment and growth of HMVEC under low-serum conditions. Fibronectin, laminin, collagen type IV, and poly-L-lysine tested alone and in combination all failed to improve either attachment or growth in MCDB 131 supplemented with 2% dFBS.

Cellular characterization. HMVEC grown in MCDB 131 + EHS2 exhibited a flattened morphology (Fig. 4) characteristic of endothelial cells and very similar to that previously observed with larger amounts of whole or dialyzed serum (34). Under these conditions, the cells stained positively for Factor VIII both in primary and secondary cultures. Cultures grown in MCDB 131 plus 10% wFBS remained positive for Factor VIII through at least 4 passages with 1:4 splits (Fig. 5). Cultures of HMVEC in MCDB 131 were also consistently positive for angiotensin-converting enzyme (data not shown). They have also been found by other investigators to retain the ability to synthesize prostaglandin I_2 (2).

Secondary cultures of HMVEC exhibited bright punctate fluorescent staining with dil-Ac-LDL in the perinuclear region (Fig. 6). A similar pattern of staining was observed in bovine pulmonary artery endothelial cells, which served as positive controls, and essentially no fluorescence was observed in fibroblastic cultures derived from human omentum, which served as negative controls. To date, this pattern of staining has been observed only for endothelial cells (51) and for mononuclear phagocytes (43).

Transmission electron micrographs of secondary cultures of HMVEC did not reveal the presence of Weibel-Palade bodies. This result is consistent with numerous other reports that Weibel-Palade bodies are absent or greatly reduced in cultures derived from microvascular as opposed to large vessel endothelium (4,6,9,47,52).

Preliminary studies in another laboratory in this department indicate that monolayer cultures of HMVEC in MCDB 131 with 10% wFBS + 10 ng/ml EGF + 1.0 μg/ml hydrocortisone have a finite proliferative life span of 7 to 22 population doublings (mean of 11 population doublings) beyond initial confluency, with substantial variation from one primary culture to another (G. Stein and J. St. Clair, personal communication). In a single

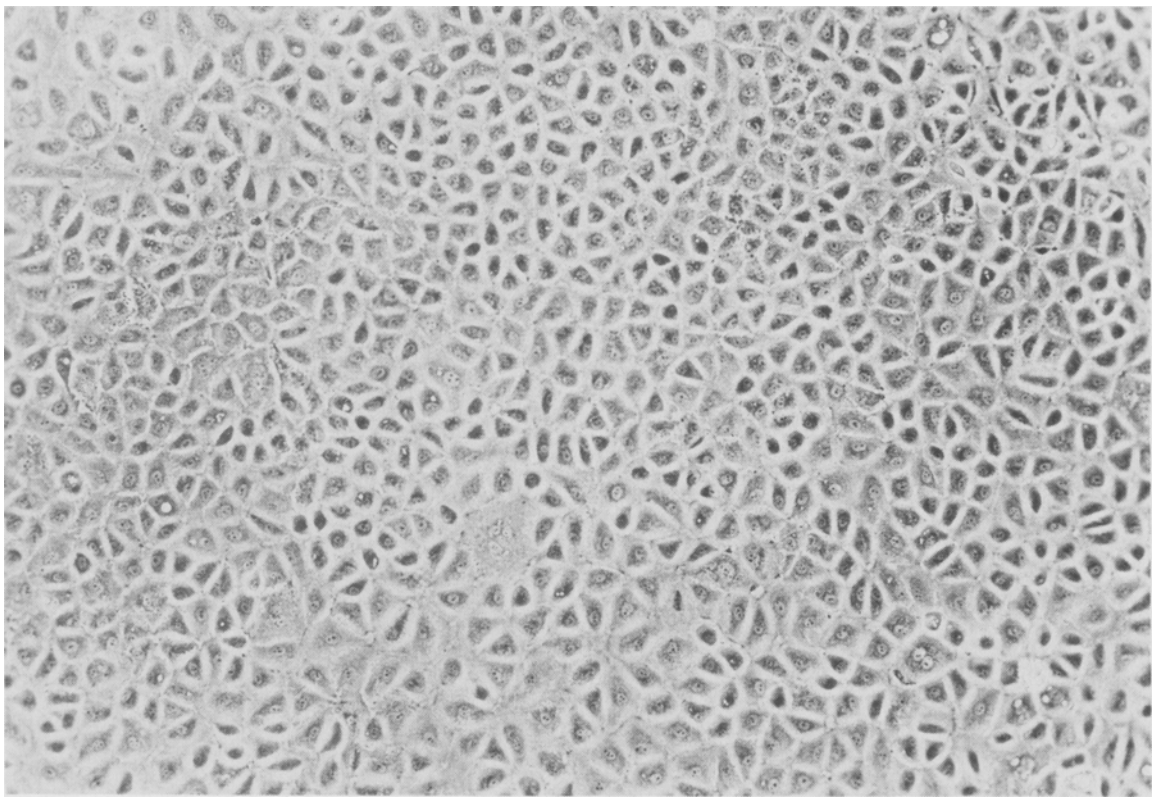

FIG. 4. Phase contrast photograph of confluent primary culture of HMVEC. The culture was established in MCDB 131 + EHS2, as described in Materials and Methods, and photographed live after 7 d. ×118.

experiment, reducing the serum to 2% (either dFBS or wFBS) reduced the proliferative life span by 3 to 4 population doublings.

Discussion

Microvascular endothelial cells from any species, and especially from humans, have traditionally been difficult to grow. Prior attempts to culture microvascular endothelial cells have always been done in conventional media that were designed for growth of fibroblastic cells with serum (DME, MEM), maintenance of fibroblasts in a nongrowing state without serum (M199), or protein-free growth of adapted permanent cell lines (RPMI 1640). Such media generally do not support growth of any types of normal cells without generous supplementation. In addition, the growth requirements of nonfibroblastic cell types are so diverse that conventional media often do not work well for them even with serum (20,21,24,25).

We have achieved good clonal growth of HMVEC with low levels of defined and undefined supplements through use of a basal nutrient medium, MCDB 131, optimized specifically for their growth. There is nothing qualitatively unique about MCDB 131 or the supplements used with it. All of the benefits are due to quantitative adjustments in nutrient concentrations.

The procedure of Kern et al. (34) for growth of HMVEC from omentum in M199 + 10% wFBS was an important first step toward the results described in this paper. The reasons why endothelial cells of omental origin grow so well in culture are not clear. However, a recent report of a lipid angiogenic factor from omentum (15) suggests that an inoculum of endothelial cells derived from the omentum could carry with it the growth factors needed for better initial growth in culture.

The only medium in our initial survey that supported reasonable growth of HMVEC with dialyzed serum was MCDB 402, which was originally optimized for low-serum growth of Swiss 3T3 cells (48). As an aside, it is interesting to note that some investigators consider 3T3 cells to be of endothelial origin (13), although this possibility remains controversial. The poor performance of the other media was not unexpected inasmuch as previous experience had shown that media designed for particular cell types often do not support satisfactory growth of other cell types (24,25,30,42,49).

Medium MCDB 402 contains unusually high concentrations of a number of nutrients, which is probably why it supported growth of HMVEC with dialyzed serum. Benefits were achieved by reducing the levels of some amino acids and vitamins, but the levels of most nutrients in the optimized medium, MCDB 131, are still quite high.

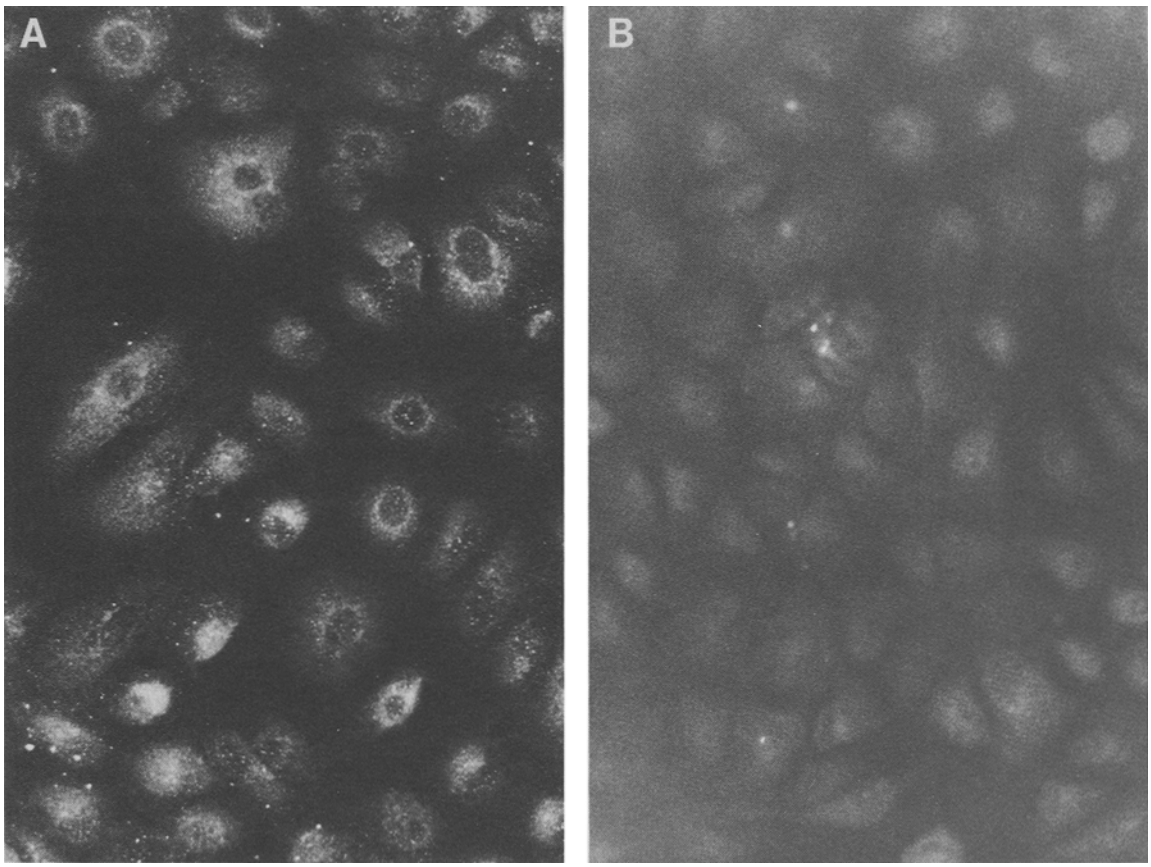

FIG. 5. Indirect immunofluorescent staining for Factor VIII. *A*, a first passage subculture (1:4 split ratio) of HMVEC in MCDB 131 with 2% wFBS + 10 ng/ml EGF + 1.0 μg/ml hydrocortisone was prepared for immunofluorescent detection of Factor VIII antigen as described in Materials and Methods. *B*, control culture prepared identically except that nonimmunized goat serum was used in place of Factor VIII antiserum. ×286.

One of the important features of MCDB 131 is its very high magnesium concentration, which was particularly beneficial in partially optimized media such as AK-1 (Fig. 1). Magnesium is known to play important roles in many aspects of metabolism, including uridine uptake, transphosphorylation, and phosphohydrolysis (7,44), but the specific reasons for its beneficial effects on HMVEC have not been investigated. Rubin et al. (45,46) reported that exposure to high levels of extracellular Mg^{2+} (20 m*M*) stimulates quiescent 3T3 cells to progress through G_1 and S. They have also suggested that the processes leading to the onset of DNA synthesis are regulated by intracellular magnesium levels. The amount of magnesium in MCDB 131 (10.0 m*M*) is approximately 10 times that found in most other culture media, and could be bypassing normal controls and providing an "artificial" stimulus for proliferation of HMVEC.

This possibility is reinforced by the observation that the stimulation caused by elevated Mg^{2+} became less dramatic as other aspects of the nutrient medium were improved. The overall effect of adjustment of all other components to their optimal levels proved to be as beneficial to clonal growth of HMVEC as the effect of increasing the magnesium. Thus, MCDB 131 with its Mg^{2+} reduced to 1.0 m*M* still supports better growth of HMVEC with 2% dFBS than that obtained in any previous medium (Fig. 2 *A*), but not as good as that in MCDB 131 with 10 mM Mg^{2+} (Fig. 2 *B*). It should also be noted that simply raising the level of Mg^{2+} in conventional media such as M199 or DME is not sufficient for clonal growth of HMVEC with 2% dFBS.

During the process of quantitative adjustment that generated MCDB 131, the dFBS concentration was reduced in steps as the basal nutrient medium was improved. In MCDB 402, HMVEC required 10% dFBS for satisfactory clonal growth, whereas in MCDB 131 that requirement was reduced to 2% dFBS. Furthermore, in MCDB 402 with 10% dFBS, HMVEC did not respond to any hormones or growth factors, whereas in MCDB 131 with 2% dFBS there was a major growth response to EGF and hydrocortisone. In MCDB 131 with 2% dFBS plus 10 ng/ml EGF and 1 μg/ml hydrocortisone, an inoculum of 2000 HMVEC/60-mm petri dish approached confluency after 14 d. To retain the sensitivity of the clonal assay it became necessary to reduce the dFBS concentration to 0.7% and the cellular inoculum to 1000 cells/60-mm dish.

The use of large vessel endothelial cells as models for investigation of microvascular phenomena is questionable because of possible differences between the two cell types. For example, there may be substantial differences

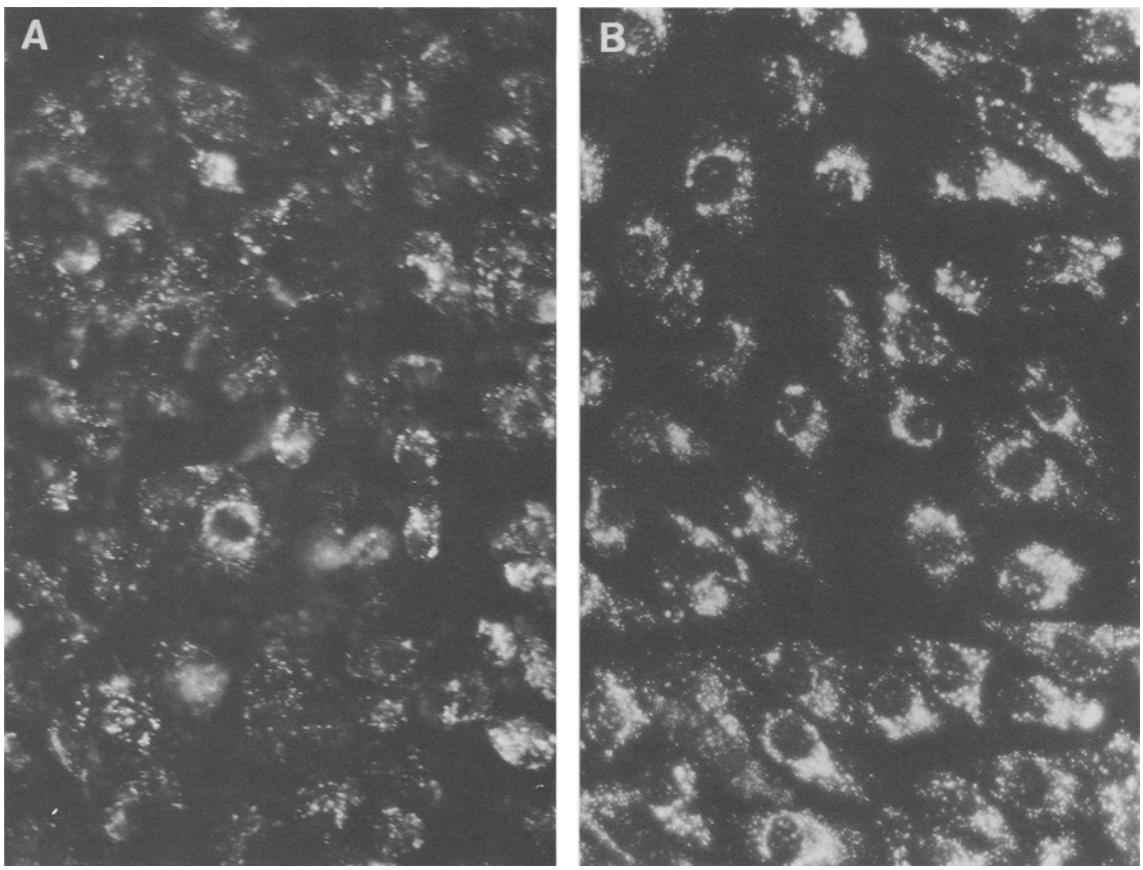

FIG. 6. Uptake of fluorescently labeled acetylated low-density lipoprotein. *A*, 2nd passage culture (1:4 split ratio) of HMVEC in MCDB 131 + EHS2 was treated with dil-Ac-LDL and processed for fluorescence microscopy as described in Materials and Methods. *B*, positive control, prepared identically, except that 4th passage bovine pulmonary artery cells grown in MEM (D-valine) + 10% wFBS were used in place of HMVEC. ×276.

in growth requirements. Gospodarowicz et al. (16) have reported that human umbilical vein endothelial cells respond much more strongly to FGF than to EGF. Davison et al. (9), on the other hand, found that HMVEC from foreskin tissue were not responsive to FGF, in agreement with our results with omental HMVEC. One possible explanation is that other aspects of the culture system may alter relative responsiveness to EGF and FGF, as has been reported for corneal epithelial cells (17). However, it seems likely that these results may reflect actual differences between microvascular and large vessel endothelial cells, which are also suggested by other studies. For example, McAuslan et al. (38,39) have observed differences between bovine retinal microvascular endothelial cells and bovine aortic endothelial cells, both in their responsiveness to EGF and to seleno compounds. Similarly, human endothelial cells from large vessels are responsive to brain-derived endothelial cell growth factors in the presence of small amounts of serum (31), whereas in the current study omental HMVEC exhibit at most only a very slight response.

The ease with which HMVEC can be cultured from human adipose tissue (34) and the availability of optimized growth conditions, as described in this paper, have now provided a model system that will allow human cells of microvascular origin to be used to study microvascular phenomena such as tumor angiogenesis, microangiopathies, and inflammatory events. Recent preliminary studies have shown that this model system can also be used with rabbit microvascular endothelial cells, thus permitting direct correlation between in vitro and in vivo studies (35).

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