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Effect of temperature on in vitro proliferative activity of human umbilical vein endothelial cells

Q. R. Yang and D. Vanden Berghe*

Laboratory of Microbiology and Hygiene, Faculty of Medicine and Pharmaceutical Sciences, University of Antwerp (UIA), B-2610-Wilrijk (Belgium)
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Abstract. Human umbilical vein endothelial cells, skin fibroblasts, and retinal pigment epithelial cells are cultivated in medium supplemented with 15 to 20% serum in our laboratory. The effects of various incubation temperatures on the proliferation of these cells was examined. Our study shows that the mitogenic response of the endothelial cells to a change of temperature differed markedly from that of the fibroblasts and epithelial cells. Cultivation of human umbilical vein endothelial at 37 °C required seeding densities as high as $1-2 \times 10^4$ cells/cm^2, and yet resulted in a low growth rate and premature senescence. However, under the same culture conditions, but at 33 °C, the proliferative capacity of these endothelial cells was potentiated. The results were striking; at 33 °C the cells grew actively and the life span was extended. The number of cumulative population doublings increased fourfold compared with that for the same cells cultivated at 37 °C. The inoculum size could be reduced, since at 33 °C the endothelial cells were able to replicate at seeding densities as low as 20 cells/cm^2. The cells serially subcultured at 33 °C retained morphological features and specific immunological markers of endothelial cells.
Key words. Human umbilical vein endothelial cells; human retinal pigment epithelial cells; human skin fibroblasts; incubation temperature; cell growth.

Endothelial cells derived from human umbilical vein (HUVEC) have been employed extensively in a large number of laboratories to study vascular physiology and pathology, because of the availability of the tissue, the relative ease of isolation of the cells, and their human origin.

However, there are some difficulties in propagating HUVEC in vitro. HUVEC in culture exhibit a fastidious nature; their proliferative activity is very dependent on high seeding densities, and even rich media containing a high concentration of serum can only support their growth for a short period[1-3]. These observations, together with the finding that HUVEC respond poorly to the mitogenic influences of serum[4,5], have led to an assumption that something which is essential to the growth of HUVEC in culture must be lacking or insufficient in serum. A variety of growth substances have therefore been added to serum-supplemented culture medium, for example fibroblast growth factor[6], epidermal growth factor[6], or partially purified endothelial cell growth factor from bovine brain[7] or bovine hypothalamus[8,9]. Some other additives, for potentiating the mitogenic effect of the growth factors, or enhancing cell attachment, such as thrombin[6], human fibronectin[8] and heparin[9], have also been reported to be important for the growth and maintenance of HUVEC in culture. Although the addition of growth factors to the cells has been reported to be effective in making long-term culture of HUVEC possible, in our opinion it does have disadvantages: 1) the purified growth factors are expensive, 2) the crude tissue extracts contain many undefined growth substances, and 3) a long-term exposure of HUVEC to the additional exogenous angiogenic stimuli may create problems when the role of endothelial cell mitogens in regulating the growth of these cells is being assessed.

We have discovered that a moderately low incubation temperature, i.e. 33 °C, can dramatically enhance the proliferative capacity of cultured HUVEC. In this study, HUVEC were subcultured in medium containing 20% human serum without additional growth and attachment substances. Various experiments were designed to determine the cells' proliferative response to the change of temperature.

To our knowledge, there have been no previous reports in the literature on the effect of temperature on the in vitro growth of HUVEC.

Materials and methods

Materials. Medium 199 (M199), fetal calf serum (FCS), human adult serum (HAS), antibiotics, trypsin-EDTA (0.05%–0.02%) solution and buffers were purchased from Gibco, Scotland. Murine monoclonal anti-human von Willebrand's Factor (vWF) antibody was from Dakopatts, Belgium. Murine monoclonal anti-human endothelium clone EN-4 (EN-4) was from

* To whom correspondence should be addressed.

Sanbio, The Netherlands. FITC-conjugated $F(ab')_2$ fragments of a goat anti-mouse IgG antibody and collagenase (*Clostridium histolyticum*, type IA) were obtained from Sigma, USA. Tissue culture flasks, i.e. 75 cm^2 and 25 cm^2 tissue flasks (T-75 and T-25 flasks) and 48-well plates were from Costar, USA.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were prepared by an adaptation[10] of the method described by Jaffe et al.[1,11]. The cells were cultivated in M199 supplemented with 20% heat-inactivated (56 °C, 30 min) HAS, 100 U/ml penicillin and 20 µg/ml gentamicin (growth medium). Primary cultures were incubated at 37 °C, while the subcultures were routinely incubated at 33 °C.

Human retinal pigment epithelial (HRPE) cells were isolated and routinely cultured in our laboratory as previously described[12,13]. Human fibroblasts (HFIB) derived from normal skin obtained from the Laboratory of Genetics, University of Antwerp[13]. HFIB were subcultured with the same method used for HRPE cells. Both HFIB and HRPE cell strains routinely grew in M199 containing 15% FCS and 100 U/ml penicillin at 37 °C. However, in the following experiments 15% HAS was used.

All cells were cultured in a humidified atmosphere, 95% air:5% CO_2.

Immunofluorescent staining for cytoplasmic vWF and membrane EN-4. HUVEC were identified by indirect immunofluorescent staining with murine anti-human vWF and EN-4 antibodies. The staining methods were adapted from Jaffe et al.[14], Cui et al.[15], and Berneman et al.[16]. HUVEC from primary cultures, early subcultures (passages 1 to 4) and late passages 15 to 20 were examined for cytoplasmic vWF and membrane EN-4 antigens.

Proliferative activity of HUVEC, HFIB and HRPE cells in the change of temperature environment. The subcultures (passages 1 to 4) of HUVEC, HFIB and HRPE cells were used for the experiments. Briefly, the HUVEC, HFIB and HRPE cells were plated into T-25 flasks at a seed density of 2×10^4 cells/cm^2 in M199 supplemented with 20% HAS for HUVEC and 15% HAS for both HFIB and HRPE cells. The cell suspensions were first incubated at 37 °C for 2 h to allow attachment of the cells to the culture flasks. Then, the cultures of each cell type were divided into four groups which were further incubated at 30 °C, 33 °C, 37 °C and 40 °C respectively. The HUVEC cultures were fed every 3 or 4 days, while the growth medium for cultures of HRPE and HFIB was not recruited between the passages. The cultures were observed daily with an inverted microscope. Trypsinization was carried out when the cells at an optimal incubation temperature reached confluence. Cell number was counted in a haemocytometer. For succeeding cultures, cells were plated into T-75 or T-25 flasks, or 48-well plates, depending upon the number of cells harvested, at seed density identical to that of the initial inoculum. To minimize the possible influence of different temperatures on cell attachment, the newly-plated cultures at each passage were incubated at 37 °C for 2 h before being transferred into incubators at 30 °C, 33 °C, 37 °C and 40 °C. The experiment lasted for 30 days; during that time the cell growth was determined and recorded by cell count upon each passage. The assay was performed in triplicate and repeated three times.

Serial subcultivation of HUVEC and determination of life span. HUVEC were subcultured by trypsinization of confluent primary cultures. The cells were seeded at a density of 2×10^4 cells/cm^2 into T-25 flasks containing 8 ml growth medium. Half of the cultures were then incubated at 37 °C and the other half at 33 °C. Seeding efficiency was determined 16 h after each passage. Growth medium was replenished every 3–4 days and the cultures were passaged every 7–10 days. The end of the life span was defined as the time when the cells could no longer replicate, i.e. cell number at harvest was equal to or less than the cell number of the inoculum × seeding efficiency, after three weeks of refeeding. The number of population doublings (PD) that occurred in primary culture and at each subculture was calculated using the formula[17,18]: $PD = \log_{10}$ [cell harvest number] $- \log_{10}$ [cell inoculum number × seeding efficiency] × 3.33. Cumulative population doubling level (CPDL) was the sum of all previous PD. The experiment was performed in triplicate. Six randomly selected HUVEC cell strains were investigated in this study.

Proliferative activity of HUVEC at low seeding densities. For cell growth assays initiated at low seeding densities, HUVEC strains at passages 1 to 3 were tested. Viability of the cells was 90 to 95%, determined by the trypan blue dye exclusion test. The viable cells were plated into T-25 flasks concentrations of 2×10^3, 200 and 20 cells/cm^2 in 6 ml growth medium. The cultures with different seeding densities were divided into two groups; one group was incubated at 37 °C and the other at 33 °C. The cultures were fed every 4 days with fresh growth medium. After 14 days incubation at 37 °C or 33 °C, half of the cultures with an initial inoculum of 2×10^3 cells/cm^2 were terminated by fixation in methanol and then stained with Giemsa. The rest were cultured for 14 more days. Then all cultures were fixed and stained. The experiment was performed in duplicate and repeated three times.

Results

Characterization of cultured HUVEC. The primary cultures initiated at inocula of 10^4 cells/cm^2 or greater were cultivated at 37 °C and grew to confluence by 3–6 days. If the initial cell inoculum was less than 10^4 cells/cm^2, at 12 h the culture was placed in a 33 °C incubator, and

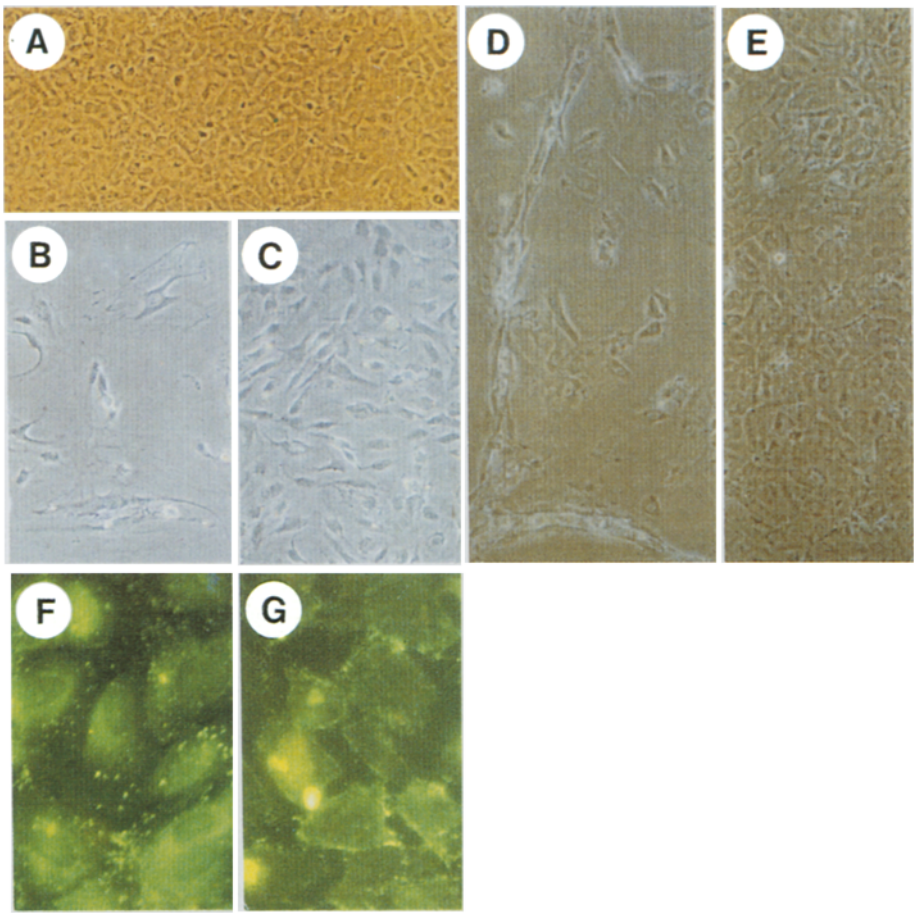

Figure 1. *A–E* Photomicrographs (× 100), demonstrating the morphology of HUVEC in culture and the organizational behaviour of HUVEC cultures: *A*) 5 days in culture; a primary culture of HUVEC reached a dense cell monolayer. *B*) Serial subcultivation at 37 °C, passage 7. Note the enlarged veil-like cells. *C*) Serial subcultivation at 33 °C, passage 15. *D*) 37 °C culture, passage 4; the culture was kept at this passage for 3 weeks. Note the formation of tubule-like structures. *E*) 33 °C culture (a pair to D), passage 4; the culture was retained at this passage for 3 weeks. *F–G* Microscopy (× 500) of immunofluorescent stained HUVEC which were serially subcultivated at 33 °C, passage 18. *F*) Immunolocalization of vWF antigen showed a positive granular cytoplasmic pattern. *G*) Immunolocalization of EN-4 antigen showed a positive granular membrane pattern.

the cells were able to reach a confluent monolayer by 10–20 days. These cells were homogeneous, and formed a closely-packed monolayer with a 'cobblestone' morphology (fig. 1A). Observation of cultured HUVEC for the entire life span has revealed no evidence that the cells transformed to spindle-shaped cells, e.g. fibroblasts or smooth muscle cells, nor that they overgrew one another. However, with increasing numbers of passages, the cells gradually became larger in size and the cultures showed a decrease in confluent cell density accordingly (fig. 3). There was also an increased frequency of giant, multinucleated cells with broad and veil-like cytoplasm and a decreased tendency to form a confluent monolayer. These microscopic changes were more striking when subcultures remained at 37 °C (fig. 1B) than when the same cells were subcultivated at 33 °C (fig. 1C). Furthermore, at 37 °C, the cells had a high tendency to migrate into clusters and, after 3–4 weeks in culture, often formed a complex network of tubule-like structures (fig. 1D). Once the cells had organized themselves into such a structure, they became mitotically inactive, despite the frequent renewal of growth medium. In contrast, this organizing behaviour was not apparent in the 33 °C subcultures (fig. 1E).

Indirect immunofluorescent staining with antisera to vWF and EN-4 revealed the presence of both antigens in the late passages of HUVEC continuously cultivated at 33 °C (fig. 1F and 1G). Some primary cultures and early subcultures of HUVEC cultivated at 37 °C and 33 °C were also examined for these two antigens. All HUVEC tested were positively stained (data not shown).

Effect of temperature on the proliferative activities of HUVEC, HFIB and HRPE cells. Of the three kinds of human cell types investigated, HFIB and HRPE exhibited a similar pattern of proliferative response to the four temperatures tested (fig. 2B and 2C). 37 °C was the optimal temperature for the propagation of HFIB and HRPE cells, and at 40 °C they were able to grow

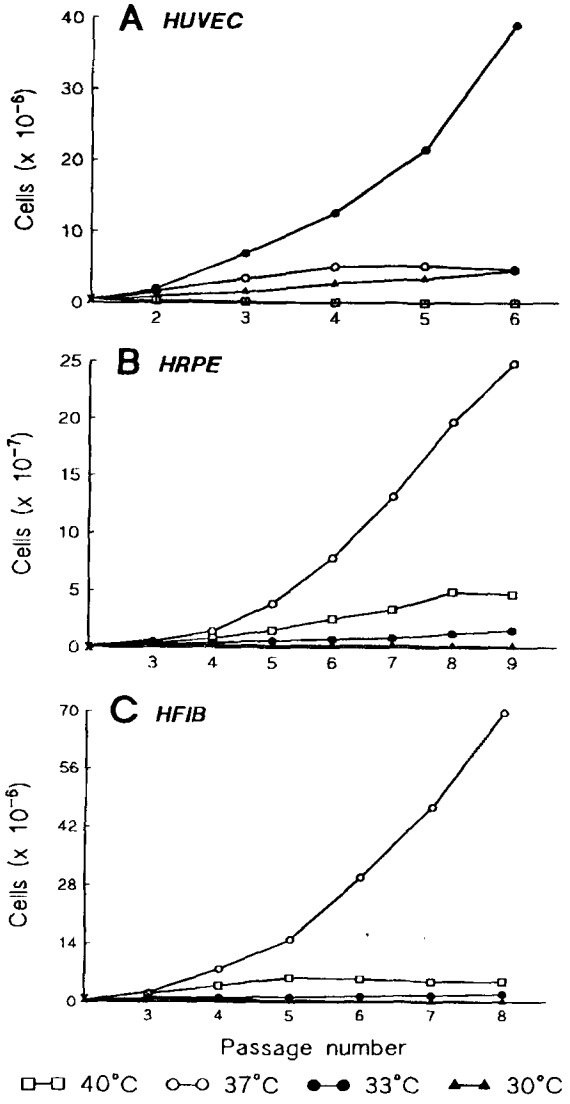

Figure 2. Effect of temperature on the proliferative activities of A) HUVEC, B) HRPE and C) HFIB. The cells were plated into T-25 flasks at a seed density of 2 × 10^4 cells/cm^2 in M199 supplemented with 20% HAS for HUVEC or 15% HAS for HRPE and HFIB. Four incubation temperatures were tested: 40 °C (□–□), 37 °C (○–○), 33 °C (●–●) and 30 °C (▲–▲). Trypsinization was carried out when the cells at an optimal incubation temperature reached confluence. The cultures were further passaged. The experiment was terminated after 30 days. During that time the cell growth was determined and recorded by cell count upon each passage. The results shown are the means (± SD < 10%).

though at a much lower rate. The proliferative tendency of both cells lines was more or less suppressed at 33 °C and completely suppressed at 30 °C.

In contrast, as shown in figure 2A, a marked proliferative activity was observed when HUVEC were incubated at 33 °C. HUVEC cultured at 37 °C and 30 °C sustained the ability to replicate, but to a smaller extent. At 40 °C the cells died off within 2 to 4 consecutive passages. The possible influence of different temperatures on cell attachment was minimized by the incubation of all newly-plated cultures at 37 °C for 2 hours before transfer to other temperatures.

Effect of temperature on the life span and the cell density at subculture of HUVEC. HUVEC subcultured at 37 °C, with an inoculum of 2 × 10^4 cells/cm^2 in M199 containing 20% HAS, with refeeding twice a week, ceased to proliferate at CPDLs ranging from 6.9 to 10.6. When the same cells were subcultured at 33 °C, they were able to reach from 29.7 to 51.2 CPDLs in serial subcultivations. The results are shown in the table.

In both 37 °C and 33 °C cultures, the cell density at harvest decreased with increasing CPDL (fig. 3). However, in 37 °C cultures the decrease was much more rapid, as greater numbers of large nondividing cells appeared and the cultures had a decreased tendency to form a confluent monolayer. The seeding efficiency was in the range 65%–90%. There was no significant difference between the cells incubated at 37 °C and 33 °C, 16 h after being seeded.

Additionally, some 33 °C subcultures at different passages, i.e. passages 7 to 15, were removed to 37 °C. These cultures soon showed the signs of senescence and the cells ceased to replicate within one to two subsequent passages (data not shown).

Effect of temperature on the proliferative activity of HUVEC at low seeding densities. At 37 °C, although the cells inoculated at density of 2 × 10^3/cm^2 proliferated (fig. 4A), the culture failed to achieve confluence during 28 days. Instead, the pre-confluent cells migrated into clusters and formed a weblike network (fig. 4B). Under the same conditions, the cells were not able to survive at clonal seed densities of 200 and 20 cells/cm^2 (fig. 4C,D). At 33 °C, the cells grew actively in the culture with an inoculum of 2 × 10^3 cells/cm^2, and reached 95% confluence by day 14 (fig. 4E). Furthermore, at 33 °C the cells in a confluent monolayer were still responsive to the growth mitogens and hormones present in the serum, and proliferated continuously until a dense monolayer was formed (fig. 4F). Moreover, the same cells did survive and grow at 33 °C at clonal seed densities of 200 and 20 cells/cm^2, as distinct colonies of various sizes could be observed (fig. 4G,H). However, our experience with HUVEC cultures older than the third passage was not as good. The variation of the cloning efficiency became so big that the results were statistically not reliable (data not shown).

Discussion

Previously HUVEC have been grown in vitro on a plastic substrate at 37 °C in medium containing 20 to 30% serum[4,11,19]. Using this system initially, we failed to bring up the HUVEC cultures beyond the seventh passage. To date, serial and/or clonal cultivation of normal HUVEC has been possible only with the assistance of the addition of one or more growth factors and attachment substances[6–9,20].

Table. Effect of incubation temperature on the life span of HUVEC.

HUVEC strain	37 °C		33 °C	
	Number of CPDL achieved	Number of passages achieved	Number of CPDL achieved	Number of passages achieved
1	10.6 ± 2.1	8	51.4 ± 4.7	27
2	8.0 ± 1.4	6	38.8 ± 3.3	20
3	7.6 ± 1.5	5	33.5 ± 4.1	18
4	6.9 ± 2.0	5	29.7 ± 3.6	16
5	8.5 ± 1.5	7	35.3 ± 3.8	19
6	9.4 ± 1.7	7	46.5 ± 2.7	24

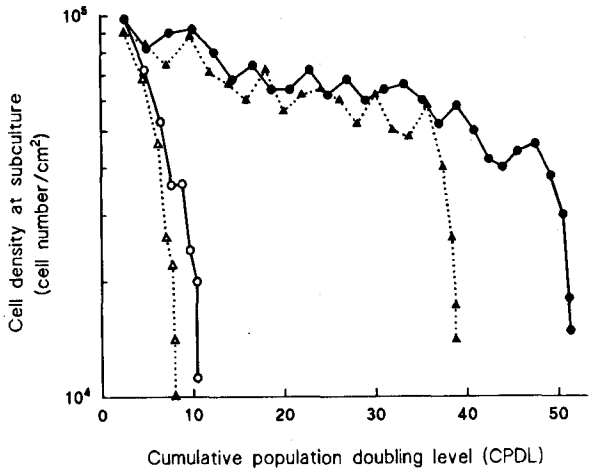

Figure 3. Effect of temperature on the cell density at subculture versus CPDL for HUVEC strain A cultivated at 37 °C (○–○) or 33 °C (●–●); and strain B cultivated at 37 °C (△–△) or 33 °C (▲–▲). HUVEC cultures, both at 37 °C and 33 °C, were seeded at 2×10^4 cells/cm^2 and grew in M199 containing 20% HAS with refeeding every 3 to 4 days. The cultures were passaged weekly and the cells were counted. The CPDL at each subculture was calculated as described in 'Materials and methods'. The results shown are the means (± SD < 10%).

In the present study, however, we demonstrate that the serum-supplemented medium can be effective for long-term propagation of HUVEC in culture, provided the cells are incubated at 33 °C. Although the alteration was merely a matter of incubation temperature, the increase in life-span of the cultures was striking. 33 °C HUVEC cultures achieved a CPDL at least 4 times higher than that of the same cells incubated at 37 °C. Moreover, at 33 °C the cells were able to proliferate at seeding densities as low as 200 and 20 cells/cm^2, while at 37 °C similar sparsely-seeded cultures failed to survive. It is interesting to note that the values achieved by 33 °C cultures are comparable to the ones that were achieved by adding growth substances to the HUVEC cultures in serum-supplemented medium[7,8].

It has been well recognized that the incubation temperature, being one of the important physical environmental factors, can greatly influence the growth of cultured cells. It is commonly assumed that the proper incubation temperature for cells derived from humans and other homeothermic animals is close to 37 °C[21–23]. In agreement with the findings of others[24,25], we observed the HFIB and HRPE cells did indeed grow best at 37 °C, while temperatures lower than normal body temperature, 33 °C, and 30 °C, apparently suppressed the proliferative activity of the cells. However, the proliferative response of HUVEC to different temperatures differed markedly from that of HFIB or HRPE cells. HUVEC proliferated most vigorously at 33 °C, while at 37 °C the cells not only grew to a lesser extent but also ceased to replicate after a small number of passages. The growth pattern of HUVEC at 37 °C is similar to that of HFIB or HRPE cells cultivated at 40 °C. Our study thus shows that 33 °C, not 37 °C, is the optimal incubation temperature for the propagation of cultured HUVEC.

The fact that a moderately low temperature, i.e. 33 °C, potentiates the proliferative capacity of cultured HUVEC appears to be paradoxical, since it would be expected that cellular metabolism would be slowed down at a temperature 4 degrees lower than the normal body temperature. The explanation for this is complex. It is possible that endogenous growth inhibitors are synthesized and secreted less actively when HUVEC are at 33 °C, so that the cells can better respond to and utilize the hormones and growth factors present in the serum. This suggestion is supported by the observation that at 37 °C, a HUVEC culture from an early passage stopped replicating at the moment when confluence was just reached, as was also found by Haudenschild et al.[5], whereas the same cells at 33 °C continued to proliferate until a dense monolayer was formed. We have also observed that conditioned medium (CM) from HUVEC cultured at 37 °C seemed to inhibit the proliferation of HUVEC, while no inhibition effect was detected when the HUVEC-CM was obtained from cells cultivated at 33 °C (unpubl. observ.).

One of the distinct characteristics of cultured vascular endothelial cells is the ability to align and organize in vitro into two- or three-dimensional tubule-like structures. Furthermore, the cells seen in this stage of differentiation have been shown to have ceased proliferating[26–30]. In agreement with Maciag et al.[27], we have observed

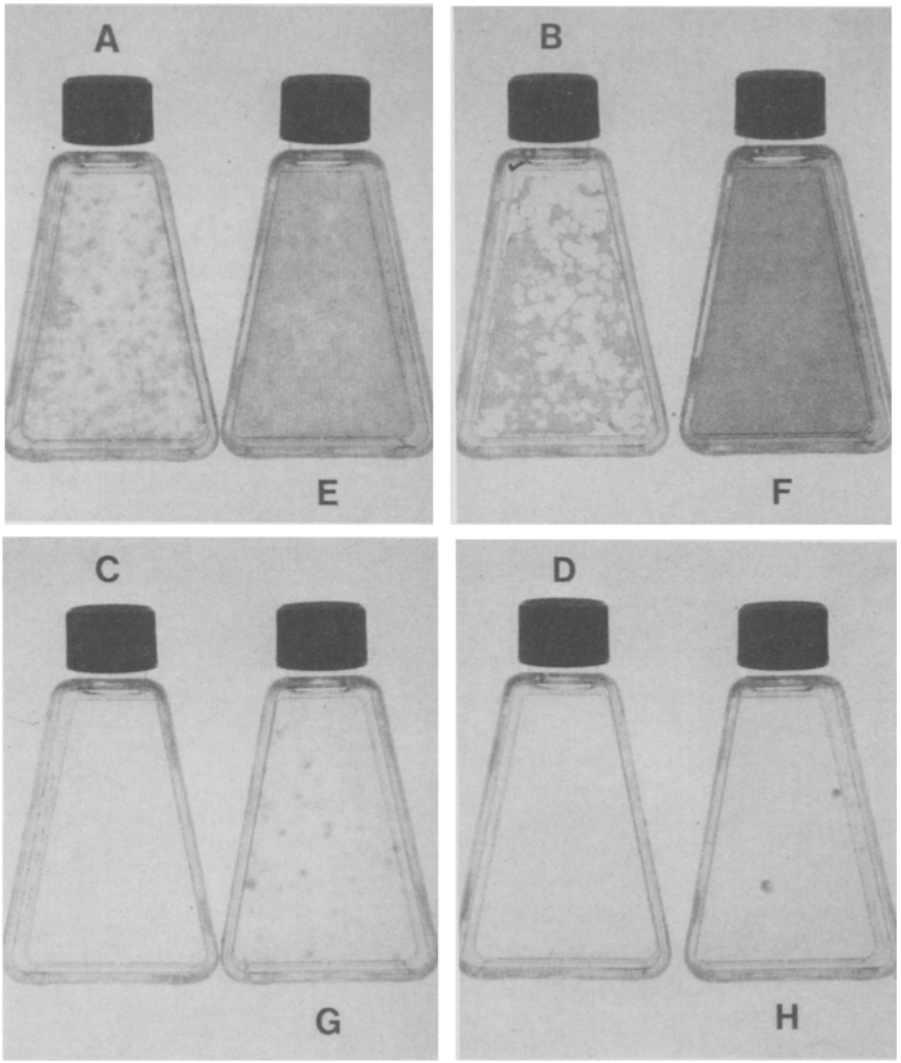

Figure 4. Growth of HUVEC at low cell seed densities. HUVEC (passage 2) were plated into T-25 flasks at seed densities of 2×10^3, 200 and 20 cells/cm^2 in growth medium. Half of the cultures with three different seed densities were incubated at 37 °C and the other half were incubated at 33 °C. All cultures were fed every 4 days with fresh growth medium. After 14 days incubation at 37 °C or 33 °C, half of the cultures with an initial inoculum of 2×10^3 cells/cm^2 were teminated by fixation in methanol and then stained with Giemsa. The rest were further cultured for another 14 more days. Then all cultures were fixed and stained. *A*) 2×10^3 cells/cm^2, incubated at 37 °C, 14 days in culture; *B*) 2×10^3 cells/cm^2, incubated at 37 °C, 28 days in culture; *C*) 200 cells/cm^2, incubated at 37 °C, 28 days culture; *D*) 20 cells/cm^2, incubated at 37 °C, 28 days in culture; *E*) 2×10^3 cells/cm^2, incubated at 33 °C, 14 days in culture; *F*) 2×10^3 cells/cm^2, incubated at 33 °C, 28 days in culture; *G*) 200 cells/cm^2, incubated at 33 °C, 28 days in culture; *H*) 20 cells/cm^2, incubated at 33 °C, 28 days in culture.

that cultivation of a HUVEC subculture at 37 °C for a period longer than 3 weeks resulted in the appearance of organized structures. This was particular striking in cultures seeded at low density and incubated at 37 °C. Interestingly, the organizational behaviour was not apparent when the same cells were cultured at 33 °C and 30 °C. Thus, our results indicate that the incubation temperature also affects the organizing behaviour of cultured HUVEC. It is tempting to assume that a moderately low temperature might have a negative effect on extracellular matrix metabolism, which is essential to the in vitro differentiation of HUVEC[29]. The failure to form organized structures in a 33 °C culture suggests that the majority of the cells are still capable of proliferating, whereas a rapid formation of tubule-like network in a culture at 37 °C means a higher rate of cells entering non-terminal differentiation rather than cell division.

Normal diploid cells in vitro have a finite life span, which has been most thoroughly studied in human diploid fibroblasts (HDF)[31]. HDF life history has been divided into three phases[32,33]: 1) a primary outgrowth phase, 2) the vigorous growth of the cell population during subsequent passages, and 3) a phase of senescence with declining proliferation, increasing cell size, the emergence of a long-lived postmitotic population,

and the eventual death of the culture. Our study shows that HUVEC cultured at both 37 °C and 33 °C undergo all three phases, like HDF. However, in 37 °C cultures, the phase of rapid cell proliferation was very short and early senescence of the cells occurred. In contrast, the cells cultured at 33 °C showed a relatively normal length of phase 2. In other words, cultivation of HUVEC at 33 °C significantly delayed the premature senescence of the cells. In addition, it is important to note that cultivation of HUVEC at 33 °C early in their life span gave the greatest extension. The effect was very much diminished when a 33 °C culture was removed to 37 °C, or when the change of temperature to 33 °C was only made after the cells had already become senescent at 37 °C.

The advantage of culturing HUVEC at 33 °C is highlighted by the fact that long-term growth of cultured HUVEC was achieved without adding growth and attachment substances to serum-supplemented medium. Furthermore, the cells serially subcultured at 33 °C retained morphological features and specific immunological markers of EC. Most importantly, because no added angiogenic stimuli are involved in the cultivation of HUVEC, these cell strains will provide an adequate model for the study of the biological effects of various mitogens on the growth and functioning of vascular endothelium.

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