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Bovine microvascular endothelial cells of separate morphology differ in growth and response to the action of interferon-γ

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Abstract. Five cell types recently isolated from the bovine corpus luteum differed in their epithelioid morphology and their cytoskeleton, but shared common criteria of microvascular endothelial cells[1,2]. To give strong evidence for the separate entity, the growth rate of the 5 phenotypically different cells was studied. They were seeded at low density on day 0. Most of these cells were treated with 200 to 1000 U recombinant bovine interferon-γ (IFN-γ) for 3 days. The untreated remainder served as controls. Cell counts were made for all cultures on days 4, 7, 10 and 13. Morphology: 13 d after treatment with IFN-γ senescent cells as well as intact cells occurred in cultures of cell types 1 to 4. Cultures of cell type 5 were apparently unchanged and resembled their untreated counterparts. Desmin-positive cells in cultures of cell type 2 developed cell processes. Growth rate: In the absence of IFN-γ, the growth rate was high for cell types 3 and 4, moderate for cell type 1, and low for cell types 2 and 5. The presence of IFN-γ caused anti-proliferative effects. These were higher for cell types 3 and 4 than for cell types 1 and 2. IFN-γ could be cytotoxic on cell type 3. In contrast, the cytokine tended to support the cell growth of cell type 5. These findings substantiate the postulate that endothelial cells exhibiting separate morphology in culture also function differently.
Key words. Microvascular endothelial cells; cell culture; interferon-γ; cell growth.

Interferon-γ (IFN-γ) has a variety of biological effects on endothelial cells, such as elevating the levels of major histocompatibility antigens, altering cell morphology, and modulating components of the extracellular matrix[3,4]. Interferon-γ is considered to be an anti-proliferative agent for certain normal cell lines and for numerous tumor cell lines[5]. However, the effect of IFN-γ on the growth of endothelial cells is not clear. Reports in the literature vary. Most authors have found IFN-γ to have an antiproliferative influence[6–8]. Ager did not detect any effect in some of her experiments[9]. Yet another group describes a stimulating influence, with a maximum response between 10 to 100 U[10]. These in vitro studies have primarily been performed on endothelial cells derived from the human unbilical vein. They offer little or no information on the anti-proliferative influence of IFN-γ on endothelial cells from large and small blood vessels, respectively. In view of the metabolic, biochemical and immunological differences between these two major sections of the circulatory system[11–13], IFN-γ may exert different effects.

Bovine corpora lutea have been used in our laboratory to isolate 5 different cell types, each showing a stable and separate morphology in long-term culture[1,2,14]. Since at the morphological level, all of the phenotypes respond to commonly applied criteria for endothelial cells, the heterous morphology seems to be constitutively expressed in endothelial cell cultures. This concept of heterous morphology which is supported by others[15–17] needs to be verified for two reasons. 1) Although different in morphology in situ, endothelial cells are known to become morphologically uniform cells in culture due to the absence of the physiological, cell-specific environment[18]. 2) The morphology of endothelial cells can easily be changed by manipulating the cell density, the serum batches, the growth factors or the matrix components used for coating culture dishes[19–22]. In order to prove that transformation does not occur, each particular MVEC must be monitored under well-documented, carefully controlled conditions. The growth response without and with IFN-γ may be used as such control factor.

Material and methods

Cell culture. Microvascular endothelial cells (MVEC) were prepared from bovine corpora lutea in the secretory stage as previously described[1]. To summarize, mechanically dislodged cells were suspended in a 50% Percoll (Pharmacia, Freiburg, Germany) solution, and centrifuged (16,000 *g*, 21 °C, 20 min) to obtain a density gradient. The 4 ml fraction above banded erythrocytes contained MVEC. Washed cells were seeded on 24-well-culture plates (Nunc) which had been coated with 1% collagen type 1 solution (Vitrogen®, Collagen Corp., Palo Alto, USA). The culture medium consisted of F12 (HAM, Gibco) and Dulbecco's modified Eagle's medium (DMEM, Gibco) mixed 1 + 1, with the addition of 0.15 mM Hepes, 0.22 mM $NaHCO_3$, 5% fetal calf serum FCS (Myoclone, Gibco) and antibiotics. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Once the cells had become confluent, they were classified as cells of type 1

to 5 according to morphological criteria. Cells were judged to be endothelial cells due to contact-inhibited cell growth, tubule formation, immunoresponse of factor VIII-related antigen, and of angiotensin converting enzyme. Findings were obtained with immunolocalization as described below and recently[1,2]. Uptake of acetylated low density lipoprotein (Dil-ac-LDL, Biomedical Technologies, Stroughton, MA, USA) was verified for cells grown to confluency on 16-mm-glass cover slips. 10 µg Dil-ac-LDL was added to 0.5 ml culture medium (per 16-mm-well) at 37 °C for 4 h. Paraformaldehyde fixed cells were mounted in Glycergel® (Dako, Zürich, Switzerland) and viewed with a light microscope equipped with epifluorescence illumination. The uptake of Dil-ac-LDL was high for cell types 3 and 4, moderate for cell types 1 and 2, and low for cell type 5. Well-developed colonies were further purified by 'weeding' unwanted cells with a bent Eppendorf tip as well as by removing the selected cells after brief enzyme treatment with either 0.02% trypsin solution (cell types 1, 3 to 5) or 0.02% trypsin-0.02% EDTA solution (cell type 2). The subcultivations were performed at a 1:2 or 1:4 split ratio. After the 2nd or the 3rd passage, cells were dislodged at a postconfluent stage and stored at −196 °C. Cells from four wells were collected per ampoule. Cell cycle stages were partially synchronized in two steps: 1) Each cell strain was deep-frozen. 2) Thawed cells from one ampoule were grown to subconfluency in a Petri dish 10 cm in diameter. A timespan of 3–5 d was allowed. These cells of still subconfluent cultures were used for the assays.

Selection of cell strains. As a rule, 5 strains per cell type were thawed. Strains which mainly showed isolated cells 5 d after thawing were discarded. The final evaluation of a single cell type was based on 2 or 3 strains which when left untreated had become confluent cultures within 14 d, i.e., by the end of the experiment.

Attachment. Dislodged cells of subconfluent monolayers still differed in their rate of cell attachment. Considerable improvement was obtained by using a self-composed culture medium. It consisted of fresh DMEM-F12 supplemented with 20% FCS, and a conditioned culture medium, mixed 1 + 1. The conditioned culture medium was specific for each cell type being harvested from corresponding postconfluent cultures 3 d after a change of culture medium. The conditioned culture medium was filtered and stored at −20 °C until required.

Experiment. Recombinant bovine IFN-γ used in the following experiments was a generous gift from Dr. R. F. Steiger, Ciba-Geigy Industry Co. Ltd., Basel, Switzerland. Its specific activity was 2.5×10^6 U/mg protein. On day zero, 5×10^3 cells/0.5 ml 'attachment' medium were seeded per well of replicate 24-well-culture plates which had been coated with 1% Vitrogen solution. On day 1 the cultures were washed twice with culture medium. While 4 wells of each phenotype were maintained as controls and treated with normal culture medium, the rest, divided into 2 groups, were subjected to different doses of IFN-γ. Either 200 or 1000 U IFN-γ/0.5 ml culture medium was added. The application was made on day 1 for 3 d. On days 4, 7, 10 and 13 after cell seeding the culture medium was changed in all wells. To promote cell dislodgment, cultures were washed once with phosphate-buffered saline (PBS, 37 °C) and then, under gentle warming, treated with 0.02%-trypsin-0.02%-NaEDTA solution for 15 min. The number of cells was determined by counting with an electronic particle counter (Coulter Counter ZBJ, Coulter Electronics, Ltd). This instrument was set to count all particles with a diameter above 9.5 µm. Mean values were assessed for each cell strain of the phenotypes on days 4, 7, 10 and 13. For this, the cells from 4 corresponding wells were pooled into 2 samples. Each sample was measured and the average taken. Absolute cell numbers were calculated using the equation supplied by Coulter Electronics.

Morphology. At the end of the experiment (i.e., 14 d in culture), strains of each type whether untreated or IFN-γ-treated were photographed with an inverted phase contrast microscope (Axiovert 10, Zeiss, Zürich, Switzerland). Cultures of cell type 2 were studied further using indirect immunocytochemistry to localize desmin filaments because they were detected in some cells of cultures of cell type 2 only (unpubl. data). It has been speculated that the coexistence of desmin-positive cells and endothelial cells is an inevitable result of the collagenase-free technique for MVEC isolation used here. Connections of endothelial cells and myocytes may not be mechanically disrupted in areas where both cells types interdigitate heavily. This is the case with arterioles[23,24]. Since monolayers of cell types 2 never developed so-called hills and valleys, i.e., the characteristic structure of prevailing myocytes, the coexistence of endothelial cells and desmin-positive cells may represent a functional unit. The localization of desmin filaments allowed visula assessment of the harm caused by IFN-γ to desmin-positive and non-desmin positive cells, respectively.

Immunocytochemistry. Cultures of cell type 2 grown on 24-well-culture-plates were rinsed with PBS and fixed for 20 min at room temperature with 2% paraformaldehyde buffered with 1 mM $MgSO_4$, 1 mM EGTA, 100 mM PIPES, pH 6.8. They rinsed with PBS buffer containing 1 mg bovine serum albumin/ml PBS and 0.1% Triton x-100 (Sigma) between each of the following steps.

Step 1) the primary mouse monoclonal antibody against muscle cell desmin (DAKO-Desmin, D33, Code No. M 760, Dako) diluted 1:100 with PBS containing 10 mg BSA/ml, was applied for 20 min.

Step 2) the secondary antibody, a biotinylated goat-anti-mouse IgG (No. B-7264, Sigma) was diluted 1:50 and applied for 20 min.

Step 3) the binding of biotinylated molecules was localized with streptavidin-biotinylated peroxidase complex (No. RPN 1051, Amersham) diluted 1:50, and incubated for 20 min.
Step 4) the peroxidasae complex was detected by using 0.05% 3,3'-Diaminobenzidine (No 26,189–0, Aldrich) in the presence of 0.03% H_2O_2.
Statistics. The percentage of viable cells present at the end of the experiment after IFN-γ treatment and the corresponding percentage in the untreated controls was calculated and compared for all strains of phenotypes 1 to 5. The Mann-Whitney U test was performed to test for differences between these 2 data sets.

Results
Morphology. Untreated cultures (fig. 1a-f) of cell types 1, 3 and 4 had become confluent 13 d after plating. Monolayers of cell types 2 and 5 still exhibited subconfluent areas. The morphology of each control monolayer maintained cell-specific criteria. However, IFN-γ caused striking changes which varied for each cell type (fig. 1g-l). Monolayers of treated cell type 1 reached confluency, yet showed an inhomogeneous aspect (fig. 1g). There were regions of large-sized, flattened cells sometimes containing 2–3 nuclei. These cells were considered to be senescent cells. On the other hand, areas were apparent which consisted of normal-sized cells of cobble-stone appearance. Interferon-γ treated monolayers of cell type 2 (fig. 1h) seemed to consist of one large-sized cell population. Contact-inhibited cell growth was lost because the desmin-positive cells present extended long cell processes either to contact each other or to attach to endothelial cells. Compared to the controls, the proportion of desmin-positive cells seemed to have increased. Interferon-γ treated monolayers of cell type 3 exhibited either intact cell islands or groups of heavily damaged cells which were of huge size and contained striking vacuoles (fig. 1i). This pattern developing in treated cultures whose untreated counterparts formed tubules within 13 d was termed reaction pattern 1 of subline A. Reaction pattern 2 of subline B demonstrated only groups of injured, large-sized cells (fig. 1j). Pattern 2 occurred in those treated cultures of cell type 3 whose untreated monolayers lacked tubule formation, but exhibited normal-sized cells with conspicuous vacuoles. As reaction pattern 1 was detected in two independent strains of cell type 3, and reaction pattern 2 in three independent strains, monolayers of cultures of cell type 3 apparently represented a population of two different cell sublines. Treated monolayers of cell type 4 displayed islands of normal-sized cells (fig. 1k). In treated monolayers of cell type 5, confluency was more advanced and mitotic figures became more noticeable (not shown) than in controls (fig. 1f, l).
Cell growth. A particular growth pattern was observed for each single MVEC type (fig. 2). The growth rate was high for cell types 3 and 4, moderate for cell type 1, and low for cell types 2 and 5. Each cell type showed a continuous, almost uniform increase in growth during the period of study (exception: cell type 3 of subline B). The behaviour of the two sublines of cell type 3 was compared. Subline A which finally formed tubules proliferated well on day 4 after seeding, while subline B without tubules grew slowly. Interferon-γ had an antiproliferative effect on cell types 1–4. The number of cells which were viable after a single treatment with IFN-γ at the end of the experiment was statistically higher in cultures of cell types 1 and 2 (20–30% vs untreated monolayers) than in treated cultures of cell types 3 and 4 (less than 10% vs the untreated monolayer). The statistical difference between cultures 1 and 2 compared to cultures 3 and 4 was $p < 0.05$. The number of viable cells appeared to be independent of whether the treatment was with either 200 or 1000 U IFN-γ. In contrast to these clear-cut antiproliferative effects, IFN-γ was non-inhibitory on the cell growth of cell type 5. The average values obtained on days 7 and 10 after cell seeding were higher than those of the untreated cultures. Due to high variation in cell number between these cell lines the quantitive data lack statistical significance.

Discussion
The present results strongly support our postulate that MVEC maintain a separate morphology in culture because the growth rates were clearly different for each MVEC type. Furthermore, these details on cell growth add to the catalogue of functional differences of our MVEC, which already includes the differential adhesion of granulocytes, the particular expression of NCAM molecules as well as the separate structure of the cytoskeleton[2,25,26].
When MVEC cultures are started from a low cell density and treated with IFN-γ for 3 d, two distinct consistent responses are found within single cultures of cell types 1 to 4. Some cells which look like senescent cells do not resume cell division, while others which appear to be healthy are capable of cell mitosis. Both morphological responses are already described in the literature. An increased in vitro aging is observed for endothelial cells isolated from large blood vessels when the cells are repeatedly treated with tumor necrosis factor and IFN-γ[27,28]. Recovery in morphology and proliferative activity is seen for endothelial cells as soon as IFN-γ has been removed[7].
Our results show that cultured endothelial cells derived from small blood vessels of the corpus luteum are targets for the anti-proliferative, mainly cytostatic action of IFN-γ. This finding is not self-evident from previous studies because the anti-proliferative effect of IFN-γ has mainly been verified in endothelial cell cul-

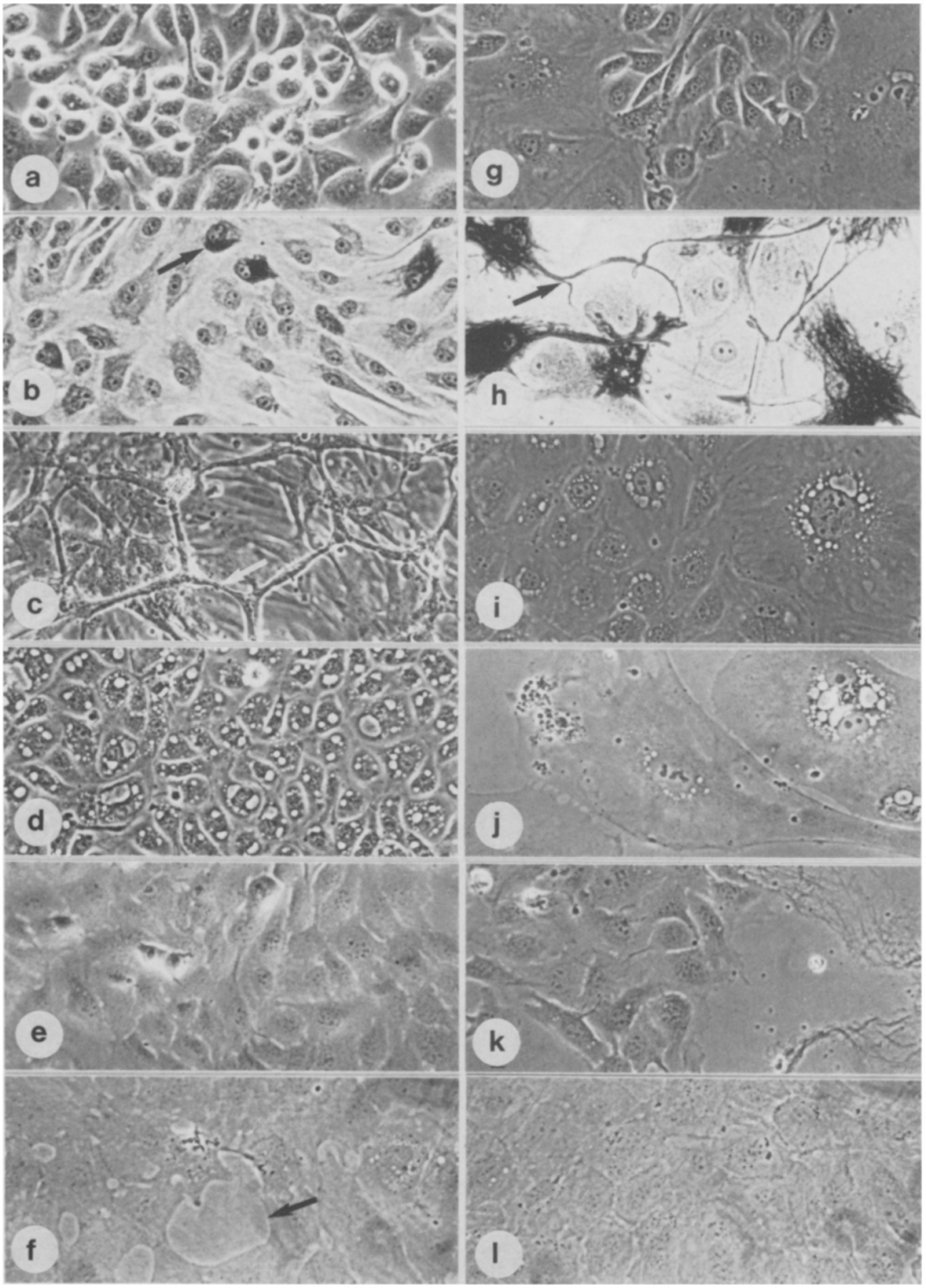

Figure 1. Cultures of cell types 1 to 5 without or with treatment of 200 U IFN-γ for 3 d. Phase contrast microscopy and immuno cytochemistry (*b*, *h*). × 240.
a–f: Untreated monolayers are displayed. *a* Cells of type 1 develop the cobble stone morphology. *b* Cells of type 2 exhibit desmin-positive cells. *c* Subline A of cells of type 3 has formed tubule-like structures (arrow). *d* Subline B exhibits polygonal cells of type 3 with striking vacuoles. *e* Cells of type 4 are of opaque appearance. *f* Cells of type 5 show subconfluent areas (arrow).

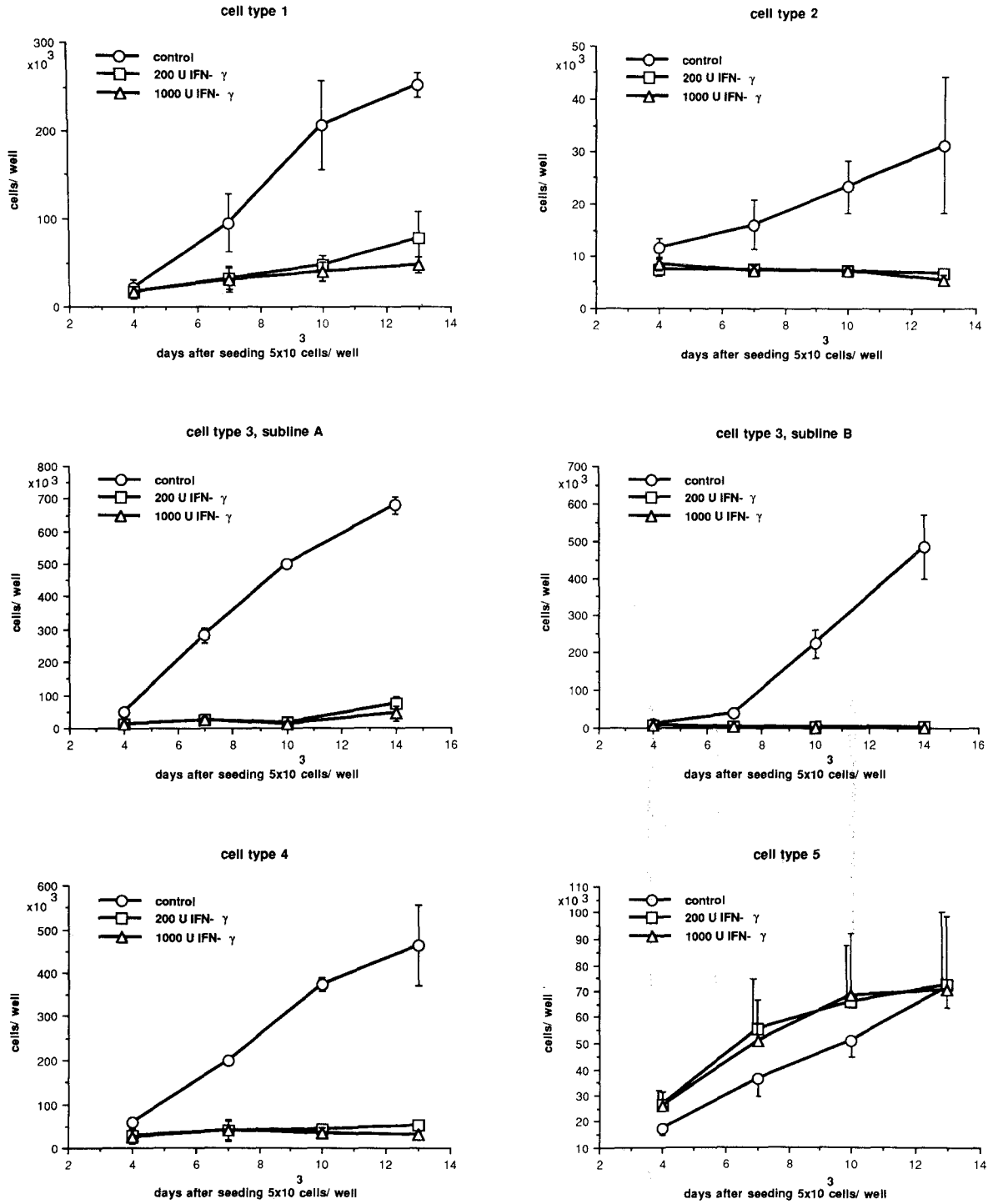

Figure 2. The growth response curve of untreated and interferon-γ (IFN-γ) treated cells varied between the different cell types as indicated in 'Results'. Each value shows the mean $\pm$ SD of 2–3 independent assays.

tures stemming from large blood vessels[6–8, 10, 27, 28]. In addition, the anti-proliferative effect is reported to be dose-dependent. The absence of such relationship as observed in our experiments may be due to a maximum effect with 200 U IFN-γ. The degree of the anti-proliferative influence depended on the particular MVEC type.

Figure 1 (continued). *g–l*: IFN-γ treated monolayers are shown. *g* Cells of type 1 become large-sized apart from areas with the 'cobble stone' aspect. *h* Note cell processes in desmin-positive cells (arrow). *i* Subline A of cells of type 3 develop huge cells and normal-sized cells. *j* Subline B of cells of type 3 is senescent. *k* Surviving cells of type 4 look healthy. *l* Cells of type 5 are confluent.

For example, it was lower in cell types 1 and 2 than in cell types 3 and 4. In cultures of cell type 3, subline B even appeared to react in a cytotoxic manner because cells were less numberous at the end of the experiment than at the beginning. Up to now, IFN-γ when in combination with tumor necrosis factor has only been reported to exert cytotoxicity on subconfluent endothelial cell cultures[6,10,29]. We speculate that cell type 3, subline B, is of older age than subline A. The onset of cell senescence in subline B is assumed because of the presence of conspicuous vacuoles. If so, it would explain the pronounced anti-proliferative effect observed for IFN-γ in subline B in accordance with the finding that IFN-γ is more operative in endothelial cell cultures of older than of younger age[27,28].

Two positive effects of IFN-γ were noted in our experiments design.

1) While in cultures of cell types 2 the desmin-negative, actual endothelial cells appeared to decline in number, desmin-positive cells were stimulated to extend cell processes. Interestingly, in co-cultures with microvessel endothelial cells, IFN-γ stimulated myofibroblasts to secrete a growth factor which leads to tube formation[30].

2) When cultures of cell type 5 were treated with IFN-γ mitotic figures seemed to become more apparent. Quantitative proof of this proliferative effect is still required since a statistically significant increase in cell numbers has not been obtained to date. Additionally, the growth data with cell type 5 may be misleading since very little growth was observed in all populations relative to the other strains. Thus, we are unable to confirm in full the observation made by others that low concentrations of IFN-γ stimulate the growth of endothelial cells from the human umbilical vein[10].

The application of tumor necrosis factor either alone or together with IFN-γ has been found to reduce the growth of endothelial cells originating from large veins rather than from large arteries[31]. Such a selective influence on different segments of the circulatory system has also been observed for interleukin-6. This has no anti-proliferative effects on MVEC from the bovine corpus luteum, but on endothelial cells from the human umbilical vein[32]. The particular responses of our separate MVEC cells to the action of IFN-γ may thus indicate that they have different origins within the microvascular tree. However, this conjecture should be viewed with caution until our MVEC can be definitely assigned to their place of origin.

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