Technical Bulletin #417
Optimization of Cell Culture Systems that Support Growth or Differentiation of Endothelial Cells

CORNING

Introduction

Extracellular Matrices (ECM), growth factors, and cells all interact to define the cellular microenvironment. These interactions play a crucial role in regulating cellular physiology both *in vivo* and *in vitro*. In an effort to construct *in vitro* environments providing the optimal conditions for endothelial cells (EC), various combinations of ECM and growth factors were examined for their abilities to induce either cell growth or differentiation. EC from several sources (species and vessel types) were seeded into standard tissue culture flasks, or onto collagen I, fibronectin, or laminin, and were grown in a low-serum medium containing various growth factors including bFGF, EGF, or ECGS/ECGF. Cells grown on collagen I for five to seven days demonstrated a two- to four-fold increase in cell number compared to those seeded onto plastic, laminin, or fibronectin. The mitogenic effect of collagen I was maximal when EGF, ECGS, and heparin were included in the optimized low-serum medium. For induction of differentiation, however, the optimal ECM was either fibronectin or Corning® Matrigel® Matrix, a reconstituted basement membrane. Optimization of key components of the microenvironment *in vitro*, such as the ECM, growth factors, and other soluble factors thus help to increase the rate of cellular growth and enhance the level of differentiation.

Results

Construction of a Cell System for Rapid Growth of EC

Optimization of Culture Supplements for Rapid Growth

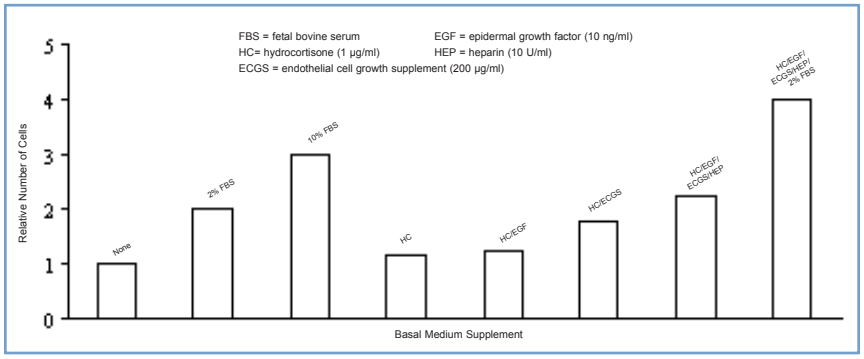

Figure 1. *Various combinations of growth factors and medium supplements were examined for their effects on proliferation of HUVEC. Cells (5 x 10⁵) were seeded onto Falcon® 75 cm² tissue culture flasks containing the indicated medium formulations (all using MCDB 131 as base), grown for three days with medium changed on day two, and then harvested and counted. Results are compared and normalized to basal medium with supplements; they represent means of three separate experiments.*

These results indicate that the combination of hydrocortisone, EGF, ECGS, heparin (heparin sulfate proteoglycan gave similar results), and 2% FBS out-performed all other media tested. The medium formation (Corning Endothelial Culture Medium) thus provides an optimized environment to promote rapid growth of EC under low-serum conditions.

Optimization of ECM for Rapid Growth

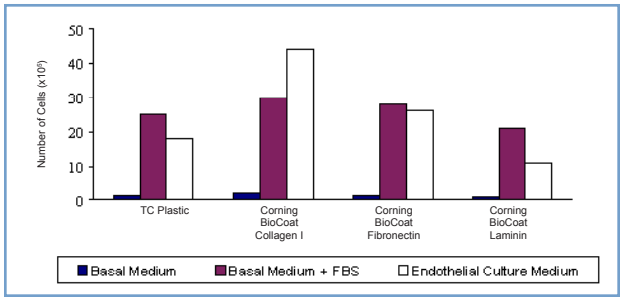

Figure 2. *In addition to an optimization of the culture medium, various ECM components were examined to determine their effects on the proliferation of HUVEC. Cells (5 x 10⁵) were seeded onto Falcon 75 cm² Tissue Culture Flasks or onto Corning BioCoat 75 cm² Flasks containing type I collagen, fibronectin, or laminin. Cells were grown for five days in basal medium (**Figure 1**) with or without 10% FBS, or in Corning Endothelial Culture Medium, and then harvested and counted. Results represent means of four separate experiments.*

These results indicate that collagen I is the optimal ECM promoting rapid proliferation of EC, supporting previous findings suggesting a mitogenic effect of collagen I for EC.[1] Furthermore, the results indicate that the combination of type I collagen and the optimized Corning Endothelial Culture Medium (forming the Corning BioCoat™ Endothelial Cell Growth Environment) supports rapid growth of EC better than all other ECM and medium combinations tested.

Proliferation Rates of Several Types of EC

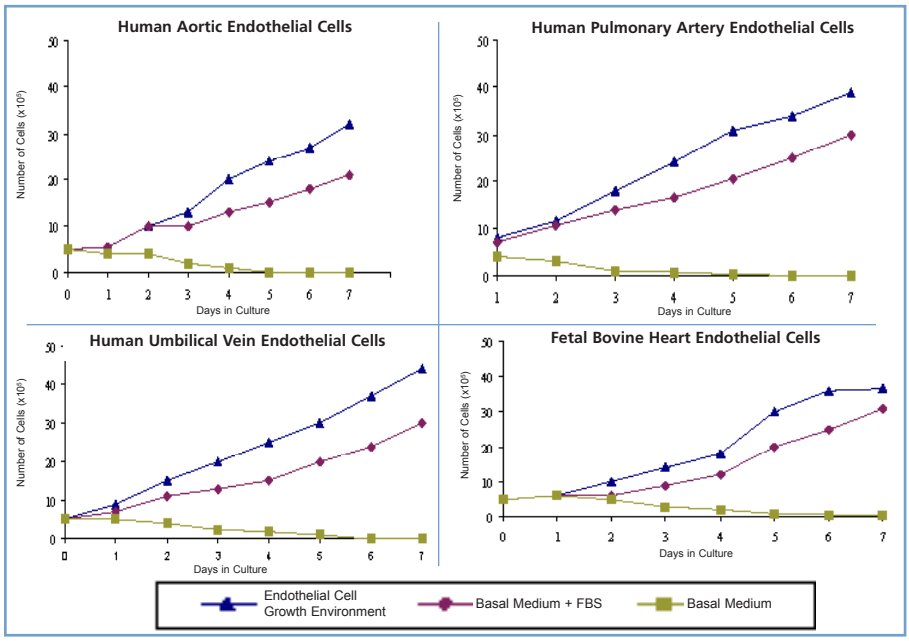

Figure 3. *To characterize the optimized culture system further, EC from a variety of sources (vessel type and species) were cultured for seven days in the Corning® BioCoat™ Endothelial Cell Growth Environment, and growth rates compared to those obtained in standard culture systems. EC were seeded onto Falcon® Tissue Culture Flasks (5 x 10⁵ cells/per 75 cm²) in medium with or without 10-20% serum, or onto the Corning BioCoat Endothelial Cell Growth Environment (containing Corning Endothelial Culture Medium and Corning BioCoat Collagen I Flasks). Cells were harvested daily for counting. Results are representative of three separate experiments.*

Light Micrographs of EC Monolayer

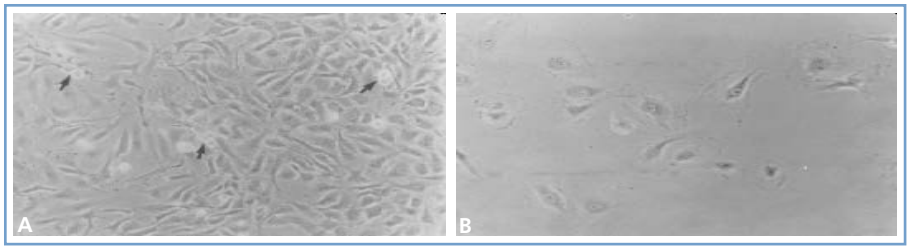

Figure 4. *At day five, HUVECs grown in the Corning BioCoat Endothelial Cell Growth Environment had formed a nearly confluent monolayer and showed numerous mitotic cells (**A**), while those grown in standard flasks in medium containing 10% FBS grew much more sparsely (**B**). Similar results were obtained for FBHEC, HAEC, and HPAEC (data not shown).*

These results indicate the Corning BioCoat Endothelial Cell Growth Environment provides all types of EC tested with culture conditions that optimally support their growth. In addition, this culture system allows for more rapid growth of EC than do standard culture conditions.

Construction of a Cell System for EC Monolayers that Exhibit Barrier Function

Optimization of ECM for EC Monolayer Formation – Light Micrographs

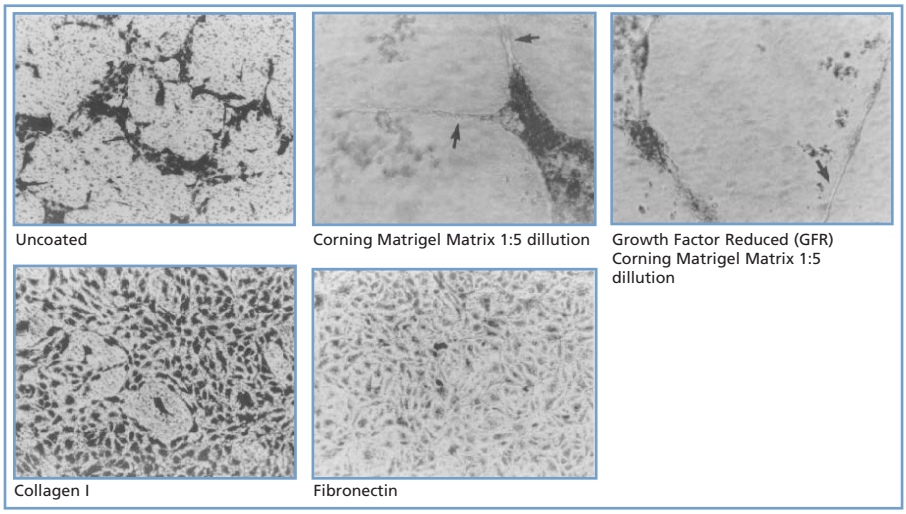

Figure 5. *HUVECs (3 x 10⁵) were seeded onto Falcon Cell Culture Permeable Supports (0.31 cm², PET track-etched membrane, 3 µm pores) without ECM, or into Corning BioCoat Cell Culture Permeable Supports containing Corning Matrigel Matrix (a reconstituted basement membrane), collagen I, or fibronectin. Cells were grown for 48 hours and were then formalin-fixed, stained with crystal violet, and examined microscopically for the formation of intact monolayers.*

EC grown in the Corning BioCoat Endothelial Cell Growth Environment may be used in a variety of applications. Some of these applications, however, may require intact EC monolayers exhibiting barrier function (e.g., studies of transendothelial leukocyte traffic or drug transport). Corning, therefore, sought to construct and optimize an *in vitro* environment promoting EC differentiation and the formation of a physiological barrier.

EC grown on permeable membranes with fibronectin, but not on those with collagen I, formed confluent monolayers demonstrating barrier function (determined by measurement of transendothelial electrical resistance [TEER] and dye diffusion), and displayed markers of EC differentiation (vWF expression; AcLDL binding). EC seeded onto Corning Matrigel® Matrix, however, underwent tubulogenesis and generated capillary-like networks, simulating the process of angiogenesis. Monolayers of differentiated EC formed on microporous membranes with fibronectin proved useful in a variety of applications, including the study of transendothelial leukocyte traffic.

Optimization of ECM for EC Monolayer Formation – Scanning Electron Micrographs (SEM)

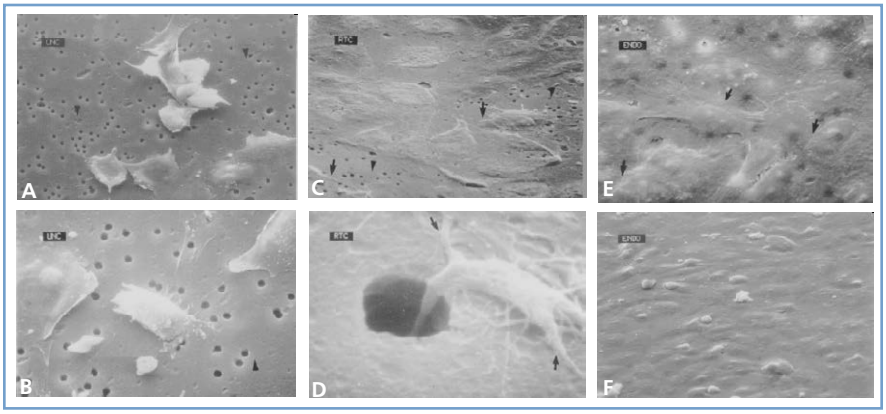

***Figure 6**. To examine the influence of the ECM on EC monolayer formation at the ultrastructural level, HUVECs were seeded (as described in **Figure 5**) onto Falcon® Cell Culture Permeable Supports without matrix or Corning® BioCoat™ Cell Culture Permeable Supports with collagen I or fibronectin. After 48 hours, monolayers were fixed with glutaraldehyde and OsO_4, and processed for examination by SEM.*

SEM examination of these cultures yielded several observations:

1. After two days, HUVECs cultured on microporous membranes without ECM (**A, B**) demonstrated both flattened and rounder morphologies, indicating suboptimal culture conditions. In addition, large open areas were seen on the permeable support membrane (**arrowheads**), indicating lack of confluency.

2. After two days, HUVECs cultured on microporous membranes with type I collagen (**C, D**) were flatter than on membranes without ECM (**C**), but a number of cells were observed to be forming sprouts (**C, D, and arrowheads**) and to be migrating through the pores of the membranes (**D**). These monolayers were not confluent either, as indicated by the open areas of membrane visible between cells (**C and arrowheads**).

3. On microporous membranes with fibronectin (**E, F**), HUVECs were found to be completely flattened and demonstrated close intercellular junctions (**E and arrowheads**); when viewed more obliquely (**F**), the cells appear to form a confluent monolayer with a typical cobblestone morphology, and no open areas on the membrane.

Together (***Figures 5 and 6***), these results indicate that fibronectin is the optimal matrix for the rapid induction of intact monolayers of EC, while cells grown on membranes without ECM or with type I collagen do not form intact monolayers over this time course.

Optimization of ECM for EC Monolayer Formation – TEER Measurements

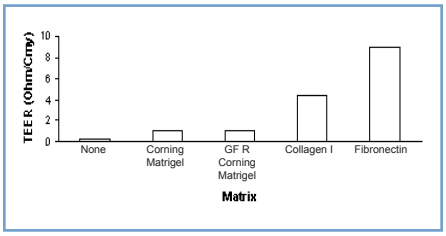

***Figure 7**. To examine the formation of a physiological barrier in EC monolayers, TEER across HUVEC monolayers (see **Figure 5**) was measured using the Endo-Ohm 12 resistance meter (World Precision Instruments). HUVECs were cultured for 48 hours on microporous membranes containing Corning Matrigel® Matrix, collagen I, fibronectin, or without ECM (**Figure 5**). Results represent means of three experiments, and are corrected for background by subtracting electrical resistance of like-coated microporous membranes containing no cells.*

These results indicate that HUVEC monolayers grown on fibronectin microporous membranes form an intact barrier, as determined by TEER measurements, in agreement with those previously reported using standard culture conditions and much longer culture periods.[2] In agreement with microscopy analysis, TEER values for EC cultured on other ECMs or without ECM after 48 hours were lower, indicating a less intact (and thus more permeable) monolayer (***Figures 5 and 6***).

Having constructed an *in vitro* environment resulting in the rapid formation of intact EC monolayers demonstrating barrier function, the utility of this system in studies of transendothelial leukocyte trafficking was examined. Human neutrophils, lymphocytes, and monocytes were isolated from peripheral blood and seeded at 1.5 x 10^6 leukocytes/permeable support onto intact EC monolayers on Corning BioCoat Fibronectin Cell Culture Permeable Supports (3.0 µm), and were incubated for 30 minutes to allow leukocytes to attach to and migrate through EC in response to various inducers. EC monolayers were then processed for SEM analysis by glutaraldehyde and OsO_4 fixation, or were rinsed to remove nonmigrating cells and the number of migrating cells expressed as a percentage of the number of leukocytes seeded. Results represent means of three experiments.

Transendothelial Leukocyte Trafficking – Scanning Electron Micrographs (SEM)

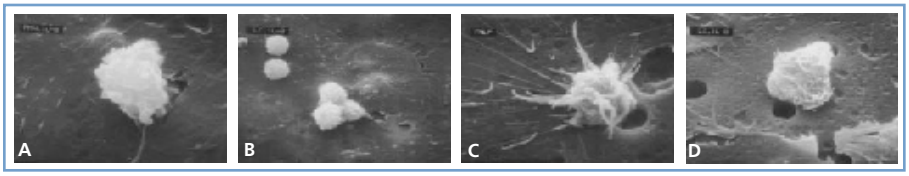

***Figure 8**. Activated lymphocytes adhering to an endothelial monolayer prior to transmigration (**A, B**). Activated neutrophil (**C**) and lymphocyte (**D**) emerging from underside of a 3 µm membrane following transendothelial migration.*

Migration of Leukocytes in Response to Various Inducers

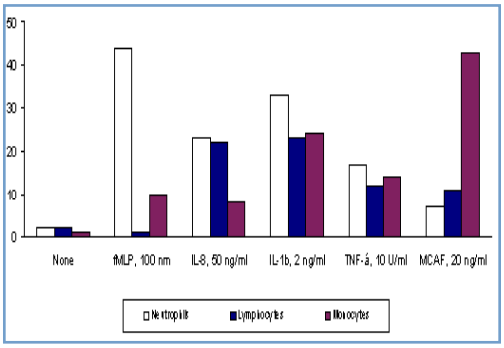

***Figure 9**. SEM observation (**Figure 8**) demonstrated that in response to an appropriate inducer of migration present in the lower compartment of the cell culture permeable support (e.g., IL-8, fMLP), neutrophils will attach to and migrate through the endothelium, and emerge from pores on the underside of the membrane. Similar results are observed with lymphocytes which penetrate the endothelial monolayer and migrate through to the underside of the microporous membrane. This system has been used successfully to examine the transendo-thelial migration of neutrophils, lymphocytes, and monocytes in response to a variety of inducers and is thus a model of the* in vivo *process of leukocyte migration through endothelium and into tissues in response to inflammatory mediators.*

Together, these results indicate that the culture system optimized to promote formation of intact EC monolayers can be used successfully in studies of transendothelial leukocyte trafficking. In addition, the monolayers obtained with two-day EC cultures are similar to those reported for standard culture systems that require longer culture periods for the establishment of intact EC monolayers.[2-4]

Conclusions

1. Rapid growth of EC from a variety of sources can be achieved using an optimized combination of growth medium and a type I collagen substrate (the Corning® BioCoat™ Endothelial Cell Growth Environment).
2. Use of this growth environment results in more rapid growth of EC than use of traditional culture conditions (e.g., standard culture flasks, high serum-containing media).
3. While collagen I is the optimal ECM for promotion of EC growth, fibronectin is the matrix of choice for growth of intact monolayers of differentiated EC demonstrating barrier function.
4. Rapid differentiated function of endothelial cell monolayers can be achieved using an optimized culture medium and microporous membranes (3 µm) with fibronectin; the EC monolayers may be used to examine transendothelial migration of various types of leukocytes, and may find use in other applications (e.g., transendothelial drug transport).
5. Use of this differentiation environment results in the formation of an intact EC monolayer more rapidly than standard culture systems.

References

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