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Glycated Collagen I Impairs Angiogenesis in Vitro - a Study Using an Innovative Chamber for Cell Research

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Abstract

Studies of cell-matrix, cell-cell interaction, or angiogenesis on different matrices require simultaneous comparison of read-out parameters from differently treated companion cells. The culture conditions (cell number, temperature, volume of culture medium) in different chambers are not completely equalized using conventional methods. It has been reported that cells growing in different environmental conditions may exhibit different proliferation patterns (2). Herein we describe an innovative chamber which could resolve this problem by significantly improving the standardization of experimental conditions. The chamber was manufactured from a standard cell culture well by its division with a septum into two sections. We utilized the chamber and recently developed topological analysis to examine the effects of glycated matrices on the capillary network by endothelial cells. Glycated Collagen I resulted in dose-dependent changes to all measured topological characteristics of the capillary-like network, such as the number of branching points, number of meshes and total capillary length. These differences were observed only in neighbored compartments coated with different matrices, but not in the compartments coated with the same matrix. The novel chamber brings an opportunity for better standardization of experimental conditions and simultaneous observation of different experimental groups, reducing the possible effect of any systematic error.

Keywords

cell culture; angiogenesis; glycated collagen; diabetes mellitus

Introduction

Studies of cell-matrix, cell-cell interaction, or angiogenesis on different matrices require simultaneous comparison of read-out parameters from differently treated companion cells (1). Using conventional methods sometimes is difficult to achieve equal culture conditions

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(cell number, temperature, medium volume) in different chambers because subtle differences in the environment of separate cell culture dishes introduce variables unrelated to the treatment conditions (2). Therefore, it is not easy to assess the degree of variability from experimental treatments versus extraneous conditions. Hence, a new experimental chamber for cell research was designed to resolve this problem by significantly improving the standardization and uniformity of experimental conditions. This novel chamber was applied to simultaneous study of angiogenesis on different matrices.

Using the Matrigel assay of angiogenesis and a topological analysis of capillary-like tube formation by human umbilical vein endothelial cells (HUVEC), we examined the effects of glycated matrices on the capillary network formation. We have previously demonstrated that glycated collagen I (GC), as a model of diabetes-like microenvironment, significantly affected the branching of capillary cords formed by HUVEC (3). Here we utilized the novel chamber and recently developed analyses of angiogenesis to extend these findings.

Materials and methods

Cell culture

HUVEC were obtained from Clonetics Corporation (Walkersville, MD) and cultured in EBM-2 medium supplemented with 2% fetal bovine serum (FBS) and growth factors (Clonetics) at 37°C in a 95% air + 5% CO₂ atmosphere. HUVEC passages 3-5 were used for the experiments. Glycated matrices were prepared as described early (3).

A novel cell culture chamber and its application for studying angiogenesis

A well of 24-well culture plate was divided into two compartments with a plastic septum (0.1 mm width, 1.5 mm high). (Figure 1). Matrigel (growth-factor reduced and phenol red-free, BD Bioscience, San Jose, CA), containing different concentrations of Glycated (GC) or Native (NC) collagen (3), was pipetted to the resulting compartments (total volume 140 µl into each compartment) and was allowed to solidify for 30 min in the incubator at 37°C. Mixture of Matrigel and Collagen I was used in order to facilitate the capillary-like network formation, since HUVEC do not form well the capillary network on pure glycated Collagen I (3). Thereafter, 200,000 HUVEC in 1.0 ml of endothelium basal medium (EBM-2, Clonetics) with 2% FBS were seeded into the wells (4) and were uniformly distributed between two compartments. Cells were grown in a cell culture incubator at 37°C and 5.0% CO₂ and 95% air. In order to monitor the same field at different time points, we randomly marked two-three spots on the bottom of each compartment of the culture well with a fine-point marker and used these marks as a guide to facilitate finding the same spots and further analysis (4). Cells were photographed at 6, 12, 24, and 48 hours using a Nikon TE2000-U microscope equipped with a CCD camera (Diagnostic Instruments). From 1 to 4 pictures from each reference point were obtained. The topology of the capillary-like networks was measured semi-automatically using a modified analysis of the capillary-like network. Photographs from each assay were analyzed using a MetaMorph software (Universal Imaging, USA). For each image, the number and average area of meshes as well as the number of branching points were calculated. The capillaries were traced and the average integrated length of the capillary-like network was measured (4). To minimize human errors, we double-checked the number of meshes and branching points of the vascular-like networks using automated morphological image analysis, adopting a method we derived from Guidolin *et al* (5) and previously reported in Mezentsev *et al.*(4) and in Merks *et al.*.(6).

Statistical analysis

All experiments were reproduced at least in triplicate. All calculations were completed by two independent investigators blinded to the data source, and the number of meshes and branching

points were double-checked with non-supervised, automatic image analysis. All data are presented as mean $\pm$ SEM, unless specified otherwise. The parameters of angiogenesis were analyzed between different experimental groups, as well as for the cells from the same experimental group growing in different compartments. The means of two groups were compared using the two-tailed Student *t*-test. For multiple comparisons, the ANOVA was used, followed by the Tukey post-hoc test. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

HUVEC's angiogenesis on Matrigel

HUVEC seeded on Matrigel were initially dispersed evenly between two compartments containing different concentrations of glycosylated matrices. Some cell culture wells were filled with the same matrix in the both compartment to study the equivalency of angiogenesis in different neighboring matrices (Figure 1, D). Within 6-12 hours cells spontaneously formed a network of capillary-like structures (Figure 1, C), the number of branching points and meshes initially dropped quickly (50% of the initial values at 12 h of culture) and then stabilized (Figure 2 A, C). Simultaneously, the total capillary length decreased during this time, but more gradually (Figure 2, D). The mesh area increased and reached a plateau phase at 24 h (Figure 2, B), representing the shrinkage of small meshes and dissolution of the borders between neighboring meshes, which was described early (4).

GC resulted in dose-dependent changes to all measured topological characteristics of the capillary-like network. Specifically, addition of 200 $\mu\text{g/ml}$ of GC to the Matrigel resulted in a significant decrease in the number of meshes and branching points within the course of experiment (Figure 2, A, C). Thus, the number of meshes was significantly lower already after 6 hours of culture even though the HUVEC were equally distributed between the compartments of the chamber. This was due to effects of GC on angiogenesis, since cells growing in two different compartments filled with the same matrix did not show any differences in any of the parameters calculated (Figure 1, D). The most significant differences were observed in the number of branching points, which was about 50% lower in a network formed on GC after 6 hours of culture and these significant differences persisted at 12, 24 and 48 h of culture (Figure 2, C), thus confirming and expanding our previous observations (3). At the same time, the total capillary length and the number of meshes were also lower in the network formed on GC compared to the native collagen at early stages of angiogenesis (Figure 2, A, D), suggesting that GC changed the adhesive properties of the matrix, which play a significant role in the capillary-like network formation (7-9). The data obtained using automated (non-supervised) image analysis were systematically lower than the semi-automatic counts (Figure 2, inserts A and C). The automated analysis introduces spurious branch points at rounded cell aggregates, while it misses other branching points at faint areas of the image. The automated analysis also misses many meshes detected by human observers: the algorithm cannot pick up meshes that are not yet entirely closed, or closed only by a thin endothelial extension. Despite these differences, the automated analysis confirmed the trends observed with semi-automatic counts. Also, different approaches in the mesh counting - touching the borders or not - resulted in the same trends in the mesh number dynamics (Figure 2, A and A-insert), although the total mesh numbers were different. The mesh area dynamics was not statistically different across experimental groups through the entire experiment. (Figure 2, B). We have also looked at topological and positional orders of the vascular networks (4,6), but did not detect significant differences, suggesting that matrix glycation affects the network scale, not its topology and distribution of nodes.

Angiogenesis plays a significant role in many physiologic and pathologic processes (10;11). The formation of a network-like pattern, the primary capillary plexus is one of the initial stages

of angiogenesis (10). There are several methods for studying the capillary like network formation *in vitro* and *in vivo*. Of the many *in vitro* assays of angiogenesis, the Matrigel assay has been widely used to study the capillary-like network formation for many years (9). However, there are several pitfalls associated with this assay, one of them is unequal distribution of cells between different experimental chambers (reviewed in 1). Several solutions were offered to eliminate this problem, including a robotization and miniaturization of the angiogenesis assay (12). This requires a fully automated high-throughput screening system and cannot be widely used due to significant costs of the equipment.

The novel chamber for cell research was successfully applied to study angiogenesis on matrices with different properties. It allowed us to significantly improve standardization of the experiment. First, cells were equally distributed between two compartments of the chamber. Second, all experimental conditions (cell/medium ratio, temperature etc.) were equalized for two compartments of the chamber, allowing the precise comparison of the experimental groups. The network formed in different compartments of the chamber was similar when the neighboring compartments were coated with the same matrix. However, the parameters of angiogenesis were significantly different in the compartments coated with different matrices, suggesting an important role of glycosylated matrices in an impaired angiogenesis, as it is seen in diabetes mellitus.

Another possible application for the novel chamber is to study cell-cell interaction. Different cell types may be grown in different compartments of the chamber in the same culture medium, allowing free exchange in different secretory substances. Using this chamber, it will be possible to monitor cells under a microscope simultaneously (Figure 1), studying the dynamics of the cell-cell interaction in different cell types, which is impossible with conventional inserts. Another applicable area for the chamber is pharmacological research. Using this method, it will be possible to monitor simultaneously effects of drugs on different cell types (13)

In conclusion, our novel chamber facilitates cell research, bringing an opportunity for better standardization of experimental conditions and simultaneous observation of different experimental groups, reducing the possible effect of any systematic error.

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Figure 1.

A novel chamber. A, B - A schematic view of a modified cell culture chamber. A - view from the top, B - sagittal view; C - representative photograph of two compartments of the chamber filled with different matrices after 12 hours of culture. 1 - a mixture of matrices containing Native Collagen I only and 2 - a mixture of matrices containing 100 mcg/ml of Glycated Collagen I. D - the numbers of branching points as a parameter of angiogenesis were analyzed after 6 hours of culture between two compartments of the chamber filled with the same matrices. NC/NC - both compartments were filled with a mixture of matrices containing Native Collagen I only, GC/GC - both compartment were filled with a mixture of matrices contained 100 mcg/ml of Glycated Collagen I. HUVEC were seeded into the chambers (2×10^5 /ml) and equally distributed between two compartments. Cells were photographed at 12 hours of culturing. Magnification 90x.

Figure 2.

Effects of Glycated Collagen I on topological parameters of angiogenesis *in vitro*. HUVEC (2×10^5 /ml) were seeded on different matrices in the novel chamber and photographed at different time points. In each photograph the number of branching points, meshes, mesh area, and total capillary length were calculated. Different topological parameters of angiogenesis: A - the number of meshes per field; B - the area of the meshes; C - the number of branching points; and D - the total capillary length were analyzed off-line and presented as mean $\pm$ SER (n=16 per group). Insets A and C - the results of the automated image analysis (mean $\pm$ SD). * - $p < 0.05$ compare with control.