

Theme Issue Article

Endothelial immunogenicity – A matter of matrix microarchitecture

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Summary

The endothelium is a highly specialized active interface between blood and the underlying tissues, maintaining vascular tone, thrombo-resistance and selective permeability to cells and proteins. It is also an important regulator of inflammatory diseases, and endothelial-leukocyte interactions often herald complex diseases with an inflammatory component. Yet, the exact mechanisms promoting immune activation of endothelial cells (EC) are incompletely understood. Knowledge is accumulating that the immediate environment defines the cellular phenotype, whereby matrix composition and spatial formation (three- versus two-dimensional) seem to act as pivotal mediators. Here we summarize current findings denoting a key role of matrix environment in regulating endothelial immunogenicity. The immune response to three-dimensional matrix-embedded EC stands in stark contrast to the response engendered by injection of these same cells in their free state. Matrix-embedding confers

a quiescent endothelial state with reduced expression levels of chemokines, adhesion, costimulatory, and major histocompatibility complex II molecules. Compared to EC grown on two-dimensional tissue culture plates, cytokine-stimulation of matrix-embedded EC results in significantly reduced adhesion of natural killer cells and proliferation of co-cultivated allogeneic T cells. On the contrary, matrix-embedded EC induce an immune-inhibitory phenotype of dendritic cells and T regulatory cells to a greater extent than non-embedded EC. As vascular diseases are associated with profound changes in basement membrane composition and overall tissue architecture, our results indicate that the immediate environment of EC might play a pivotal role in initiating and regulating of different vascular diseases. Cell-matrix interconnections appear to govern endothelial immunogenicity and interactions between EC and immune cells.

Keywords

Endothelial cells, immunity, cell-matrix interactions

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Atherosclerosis as an inflammatory disease

Joseph Hodgson (1788–1869) and Rudolf Virchow (1821–1902) were among the first to describe atherosclerosis as an inflammatory disease. Research over the last decades indeed defined that immune cells from the innate and adaptive system are pivotally involved in coronary artery disease and other forms of atherosclerosis. Activated immune cells have been demonstrated in all stages of atherosclerotic disease and blood levels of inflammatory cytokines and other acute-phase reactants reflect the inflammatory process in the atherosclerotic arteries (1).

The prodromal stages of atherosclerotic lesions are already formed during fetal development (2–4). In children and young adults, fatty streaks become increasingly prevalent and may well progress to more advanced stages of atherosclerotic lesions (5, 6). Once initiated, progression of atherosclerotic disease is influenced by classical risk factors that promote vascular inflammation and plaque rupture (7). Hyperlipidaemia with increased concentrations of oxidized low density lipoproteins, hyperglycaemia and other metabolic disorders have been identified as risk factors for initiation of atherosclerosis (8). Atherosclerosis predominantly affects large and mid-size elastic and muscular arteries in hearts, brain and extremities. Predilection locations at branch sites and

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curvatures are also indicative for a pivotal role of hemodynamic influences such as turbulent flow and low shear forces. Metabolic diseases result in an early influx of pathologic metabolites into the subendothelial matrix space (9). Hemodynamic forces are sensed directly by the extracellular matrix and transmitted to cells within cardiovascular tissue, including endothelial cells, fibroblasts, cardiomyocytes, and smooth muscle cells, via integrin signaling (10, 11). In turn, cells within the cardiovascular tissues respond to mechanical stimuli by modulating the synthesis of almost all major components of the extracellular matrix, including collagen, elastin, proteoglycans, glycosaminoglycans, glycoproteins, and various soluble proteins. All of these mechanisms predispose to the development of endothelial dysfunction that has been identified as an early clinical hallmark of atherosclerotic disease. The imbalance of endothelial autocrine-paracrine factors that maintain normal vascular function is a main feature of endothelial dysfunction. Loss of acetylcholine-induced endothelium-dependent coronary vasodilatation as a critical indicator of endothelial dysfunction is independently associated with serum cholesterol levels, family history, age, and the total number of risk factors (12, 13).

Immunogenicity of endothelial cells in endothelial dysfunction

Whereas active participation of innate and adaptive immune cells in all stages of atherogenesis has been well established (1), the driving mechanism of endothelial immunogenicity is less well characterized. As the active interface between circulating blood and underlying tissue the endothelium serves various biological roles, maintaining vascular tone and structure, regulating intravascular homeostasis and permeability, protecting against oxidative stress, and inhibiting the primary inflammatory response of leukocyte adhesion and migration. Endothelial dysfunction is characterized by a reduction of the bioavailability of endothelium-derived relaxing factor, nitric oxide, and an increase in endothelium-derived contracting factor (14). Moreover, endothelial dysfunction, aside from representing impaired endothelium-dependent vasodilatation, also comprises a specific immune state of endothelial activation, which is characterized by a pro-inflammatory, proliferative, and pro-coagulatory milieu that favors all stages of atherogenesis (15). As one consequence, atherosclerotic disease states are not only mirrored by activity of immune cells, frequency of circulating cytokines and other inflammatory markers, but also by circulating markers released by activated – and damaged – endothelial cells, e.g. intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor, plasminogen activator inhibitor-1 or thrombomodulin (16). Recent research has also demonstrated increased levels of circulating endothelial cells and CD31⁺/CD42⁻ microparticles in atherosclerosis (17, 18). Furthermore, atherosclerosis as well as vasculitis and allograft vasculopathy are associated with a profound immune reaction against diseased endothelium (19–21).

Immune phenotype of endothelial cells is matrix-dependent

Current research in our laboratory has employed tissue-engineering techniques to embed endothelial cells within three-dimensional collagen-based matrices. Three-dimensional cell culture systems offer a milieu to study endothelial biosecretory, migratory, and proliferative functionality that is perhaps more physiological and robust than conventional two-dimensional state of plated cultures (22–26). The three-dimensional state offers the opportunity to consider external mechanical and cell adhesion stimuli, which dramatically affect integrin ligation, cell contraction and associated intracellular signaling (27, 28). Others and we have demonstrated that embedding of endothelial cells in three-dimensional collagen-based matrices allows these cells to grow to confluence in a controlled environment (29, 30). Such constructs allow endothelial cells to retain endothelial quiescence, secretion of essential regulatory factors, and the associated potential for vasoregulatory control (Table 1), within vehicles that can be stored, manipulated, functionally validated and implanted at will at sites protected from environmental forces (30–33). Interestingly, our results furthermore indicate that embedding of endothelial cells within a physiologic three-dimensional environment affects their immunogenicity: whereas cytokine-stimulation of endothelial cells grown two-dimensionally results in a pro-inflammatory phenotype with increased expression and secretion of chemokines, adhesion, costimulatory molecules and major histocompatibility complex (MHC) class II molecules, cytokine-stimulated matrix-embedded endothelial cells retain a quiescent phenotype as analyzed by real-time PCR, ELISA, and flow cytometry (Table 2) (34, 35). Chemokine and adhesion molecule expression are essential for attraction of immune cells and subsequent diapedesis through the endothelial layer, expression of MHC class II and co-stimulatory molecules and costimulatory allows endothelial cells to act as antigen presenting cells, especially important in transplant rejection.

Muted expression of surface molecules on matrix-embedded endothelial cells was associated with reduced induction of allo- and xenogenic T-cell proliferation ($5,367.8 \pm 1,976.3$ vs.

Table 1: Compared to porcine aortic endothelial cells grown on two-dimensional tissue culture plates (PAETCPS), matrix-embedded PAE (matrix PAE) remain viable with full retention of biochemical secretory ability (30, 34).

	PAETCPS	Matrix PAE	P-value
Cell viability [%]	96 ± 2	95 ± 3	0.19
Glycosaminoglycan [µg/10 ⁶ cells/day]	2.77 ± 0.13	2.76 ± 0.19	0.18
Chondroitin/ dermatan-sulfate [µg/10 ⁶ cells/day]	1.22 ± 0.06	1.22 ± 0.13	0.21
Heparan-sulfate [µg/10 ⁶ cells/day]	1.56 ± 0.08	1.53 ± 0.02	0.17
Prostacyclin [pg/10 ⁶ cells/day]	76.8 ± 1.6	72.3 ± 3.8	0.11

Table 2: Cytokine-induced expression of costimulatory, MHC II, adhesion molecules and chemokines is significantly reduced when endothelial cells are matrix-embedded. In contrast, expression of MHC I, PD-L1, and PD-L2 is not affected by matrix-embedding (ELISA (MCP-I and IL-8) and flow cytometry analysis) (34).

	TCPS	Matrix-embedded	P-value
CD58 [%]	16.2 ± 5.3	3.8 ± 4.5	<0.002
VCAM-1 [%]	31.2 ± 7.8	18.5 ± 4.3	<0.05
ICAM-1 [%]	22.1 ± 7.6	10.2 ± 4.9	<0.01
CD62E [%]	62.1 ± 22.2	17.5 ± 9.9	<0.005
CD62P [%]	23.1 ± 4.5	3.9 ± 2.2	<0.001
MHC I [%]	97.9 ± 2.3	96.5 ± 4.1	n.s.
MHC II [%]	27.2 ± 9.1	5.9 ± 6.6	<0.001
CD80 [%]	36.3 ± 4.7	14.6 ± 1.3	<0.0002
CD86 [%]	81.2 ± 4.4	26.9 ± 1.2	<0.0005
CD40 [%]	34.4 ± 11.2	15.9 ± 4.3	<0.001
PD-L1 [%]	92.4 ± 8.8	89.5 ± 7.6	n.s.
PD-L2 [%]	62.0 ± 23.9	62.6 ± 8.1	n.s.
CX ₃ CL ₁ [%]	76.5 ± 8.6	22.8 ± 5.7	<0.0001
MCP-I [ng]	148 ± 21	49 ± 16	<0.002
IL-8 [ng]	131 ± 38	42 ± 8	<0.002

17,087.2 ± 3,749.75 cpm, $p < 0.01$), activation and differentiation of naïve T cells into cytokine-producing and effector T cells (22 ± 4 vs. 43 ± 3% CD4⁺CD62L^{low}CD44^{high} T cells, $p < 0.001$), and adhesion of natural killer cells *in vitro* (1,735 ± 135 vs. 6,335 ± 420 cpm; $p < 0.0002$) when compared with endothelial cells grown two-dimensionally (34–36). In-vivo analysis revealed nearly complete absence of infiltration with innate and adaptive immune cells when matrix-embedded instead of two-dimensional grown endothelial cells were implanted subcutaneously in mice and rats (34, 36).

Matrix embedding promotes an immune regulative endothelial phenotype

Matrix embedding is far more than passively protective; it enables active regulation of endothelial immune phenotype. Matrix-embedded endothelial cells secrete 6.2-fold more of the immune-regulative cytokine transforming growth factor (TGF)- β than two-dimensional grown endothelial cells ($p < 0.002$). Though not because they are simply shielded from forces and chemical stimuli, embedded endothelial cells do sense their local environment and express the same amount of negative costimulatory molecules (e.g. programmed cell death ligand (PD-L)1 and PD-L2; Table 2) as endothelial cells grown on tissue culture polystyrene plates (34). The increase in TGF- β and retention of negative costimulatory molecule expression contributes to an overall shift towards immune regulation, rather than immune activation, by matrix-embedded endothelial cells. Hence, induction of CD4⁺CD25⁺ Foxp3 expressing T regulatory cells was en-

hanced when endothelial cells were presented matrix embedded in a three-dimensional environment (37). Furthermore, whereas two-dimensional grown endothelial cells induced full dendritic cell maturation, characterized by loss of phagocytosis, expression of CD83, CD80, CD86, and MHCII, matrix-embedded endothelial cells induced a different dendritic cell phenotype (unpublished results). This dendritic cell phenotype has recently been characterized as "alternatively activated" (lacking maturation markers, increased secretion of interleukin-10 and TGF- β) (38). Indeed, induction of T-cell proliferation was significantly hampered when dendritic cells had been matured with matrix-embedded endothelial cells instead of two-dimensional grown endothelial cells.

Matrix – integrins – endothelial phenotype

Integrins mediate binding between cells and the surrounding/underlying matrix. Integrin expression largely varies with composition of the matrix proteins, microarchitecture, tension, mechanical stimuli, etc. It is well established that early stages of atherosclerosis are associated with changes in matrix composition (9). Hyperlipidaemia and hyperglycaemia induce changes in proteins, tension, increased deposition of fibronectin and fibrinogen that in turn provoke alterations in the integrin expression pattern (39, 40). Integrins not only act as simple binding structures but also actively affect intracellular signaling, e.g. via focal adhesion kinase, and signaling via different integrins modulates cellular (immune) phenotypes (41). Alterations in cellular immune behavior by matrix environments have been described previously: fibroblast MHC class II expression is limited in a signal transducers and activators of transcription (STAT)-1-dependent pathway (42), smooth muscle cell response is limited when cultured in a three-dimensional environment (43) and T-cell development in the thymus depends on the spatial tissue formation (44).

Whereas the physiologic endothelial phenotype has been defined quiescent, activated dysfunctional endothelial cells display a different integrin expression pattern (e.g. increased expression of integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$) (45). Atherosclerotic lesions are marked by integrin expression patterns that have been associated with an activated endothelial phenotype (39, 40, 46). We demonstrated earlier that the predominant endothelial phenotype in three-dimensional matrices is quiescent and marked by a physiologic integrin expression pattern (e.g. $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$). In contrast, endothelial cells grown on two-dimensional polystyrene tissue culture plates are easily activated via cytokines and display an integrin expression pattern previously being demonstrated for atherosclerotic lesion-derived endothelial cells (35). Our own results now indicate a profound change of intracellular signaling pathway activities in endothelial cells by the underlying matrix (35). We demonstrated muted phosphorylation of Janus kinase and STAT in interferon- γ -treated matrix embedded EC (35). As one explanation, baseline levels of the inhibitory mediators suppressors of cytokine signaling (SOCS)1 and SOCS 3 are increased in matrix-embedded endothelial cells. Furthermore, matrix embedding seems to interact with regulation of tumor necrosis factor- α signaling pathways (unpublished results). Yet, modifications of intracellular signaling path-

ways via outside-in signaling need to be further characterized to fully elucidate the mechanisms matrix environment imposes on cellular phenotype. Additionally, it will be interesting to see if modulation of the matrix environment can sustain modulation of endothelial immunogenicity even in the face of local and systemic circulating pathologic signals (e.g. disturbed shear stress, hyperlipidaemia, hyperglycaemia, uremia).

Furthermore, it will be important to understand if the effect of a physiologic matrix environment on endothelial cell immunogenicity sustains in a pathophysiologic milieu (e.g. hyperlipidaemic, hyperglycaemic, disturbed shear stress).

Our findings might be crucial for our understanding how endothelial cells might become immunogenic in arterial disease states in first place. Matrix architecture is critical for modulation of endothelial immunogenicity. As three-dimensional matrix embedding mimics basal anchorage of endothelial cells, these findings might offer novel insights to our understanding of various endothelial-mediated diseases, highlighting the importance of spatial formation and endothelial cell-matrix-interactions in maintaining endothelial cell (immune)phenotype and vascular

homeostasis. Earlier findings have revealed a well regulated multistep process consisting of signaling via integrins, intracellular signaling cascades, down- and up-regulation of specific genes and finally expression and secretion of a variety of pro-inflammatory mediators. Tissue engineering with three-dimensional matrices offers a tool to increase our understanding of determinants for endothelial health and also of the pathophysiology of diseased endothelium. Further work is needed to develop physiologic three-dimensional matrix structures that might also allow for examination of more realistic milieu (e.g. application of flow). In addition, specific effects of the different extracellular matrix components on endothelial immunogenicity need to be further elucidated. In this regard the chosen collagen-based matrix provided a good tool to study effects of collagen and three-dimensional structure on endothelial immunogenicity but it lacks the full diversity of a physiologic endothelial extracellular matrix. In the future, three-dimensional culture systems may become more widely used to characterize cellular responses and phenotypes to a variety of stimuli.

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