ENDOTHELIAL PROGENITOR CELLS IN CEREBRAL ENDOTHELIUM DEVELOPMENT AND REPAIR (REVIEW)

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The review covers the current concepts on the role of endothelial cells in the blood-brain barrier development and repair. The authors have characterized cerebral endothelial cells within a neurovascular unit, and endothelial progenitor cells, their development and migration to the brain, as well as molecular mechanisms of barriergenesis with a special focus on the modulation of intercellular communications. The review contains the analysis of application of endothelial progenitor cells of different origin in *in vitro* blood-brain barrier models. HIF-1-controlled regulation mechanisms of the regulation of functional activity of endothelial cells, angiogenesis, astrocyte-endothelial interactions, and cell metabolism within a neurovascular unit has been discussed. The authors have analyzed possible contribution mechanisms of these processes in endothelial dysfunction development as one of the pathogenetic components of neurodevelopmental disorders, ischemic brain injury, or neurodegeneration associated with alterations in key metabolic and transport processes, secretion of regulatory molecules, cell-to-cell communication in cerebral endothelial cells, as well as with endothelial cell death and the loss of structural and functional integrity of the blood-brain barrier. Deciphering the cellular and molecular mechanisms of barriergenesis in brain development, and its recovery after damage will provide new opportunities for pharmacotherapy of central nervous system disorders due to endothelial dysfunction.

Key words: endothelial progenitor cells; blood-brain barrier; brain development.

Heterogenicity of endothelium cell pool in the human body determines specifics of vessel functioning in various tissues and organs. Acquisition of endothelial phenotype specific for various tissues occurs at the stages of endothelial progenitor cells development through complex processes determining their differentiation and migration, besides these processes are very plastic and can change under external factors [1]. Endotheliocytes in the neurovascular unit (NVU) of the brain, being the basis of blood-brain barrier (BBB) structure, obtained in the evolution process the features allowing efficient regulation of interaction between the blood flow and central nervous system (CNS) [2]. Interestingly, this barrier function was not always associated with endothelial cells: initially, BBB was formed of glial cells [3]. The remarkable fact is that in the ontogenesis the chain of events is absolutely different — BBB is formed in the process of embryogenesis with endothelium cells and pericytes, and these events precede inclusion of astrocytes into the neurovascular unit [4].

Properties determining advantages of endothelial cells over glia cells in the context of regulation of BBB selective permeability are related to the option of forming a compact non-fenestrated monolayer with tight junctions, expression of high number of highly specialized transporter molecules and cell adhesion proteins, low level of transcytosis [5, 6]. Absence of fenestration determines low level of expression of glycoprotein MECA-32 (PVLAP) in the endothelium of brain microvessels. Besides, endothelium in the brain

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neurovascular unit is an object of regulation by other NVU cells — pericytes, astrocytes and neurons, as well as extracellular matrix molecules [7, 8].

Endothelial dysfunction is one of important mechanisms of pathogenesis of brain development disturbances, its ischemic damage or neurodegeneration, which is related to disturbance of key metabolic and transportation processes, release of regulatory molecules, with disturbance of intercellular coupling, BBB structural and functional integrity and death of endothelial cells. It is known that BBB integrity depends on many mechanisms, including Wnt-signalling, effects of retinoic acid, controlled expression of matrix metalloproteinases and aquaporins [9, 10]. Disturbances of BBB structural and functional integrity and pathologic permeability develop practically with all types of CNS pathologies, which determines not only the course of disease, but also efficiency of drug treatment. Most commonly mechanisms of BBB permeability disturbance are associated with dysfunction or damage of mature endothelial cells, however contribution of endothelial progenitor cells in these events are no less interesting.

Endothelial progenitor cells: development and migration to the brain

BBB formation, or barriergenesis, is a process realized in the embryonic and early postnatal stage of development, as well as after brain injury [11, 12]. An important component of barriergenesis is angiogenesis, which in many ways depends on activity, proliferation and differentiation of endothelial progenitor cells, in particular, microcirculation vessels in the brain are formed of meningeal vessels and spread to parenchyma of developing brain by angiogenic mechanism [13]. Besides, signs of angiogenesis activation in the brain are registered in case of neuroplasticity and memory consolidation [14].

In laboratory animals (mice, rats), BBB formation starts on day 10–17 of embryonic development, and by day 12 transporter and tight junction molecules are expressed (in a human embryo, expression of BBB markers is registered in week 8 of development, and angiogenesis itself is registered in week 2–3 of postnatal life) [10]. By day 21 of rodents' prenatal life transendothelial resistance is formed, it progressively increases during early postnatal life, thus corresponding to formation of tight junctions [12]. This fact versus data on dynamics of embryonic neurogenesis allows supposing that barriergenesis is initiated after formation of precommitted neuronal progenitor cells [15].

During prenatal development, endothelial cells develop from the common progenitor and take on phenotypical properties characteristic to a specialized tissue. In embryogenesis, endothelial cells populate brain regions enriched with neuroepithelial cells, radial glia, neuroblasts and neurons [16]. Immunophenotyping of endothelial progenitor cells demonstrates their expression of certain key antigens (e.g., CD34, CD31, CD133, CD45, VEGFR2, CD144, etc.) [17], which identification on resident cells of vessel wall or progenitor cells of bone marrow origin allows considering mechanisms of development and trafficking of endothelial cells to tissues.

Embryonic stem cells precommitted to endothelial development, early start expressing intercellular junction molecules (adherence molecules, tight and gap junction proteins) [18]. At the same time, according to experimental findings [19], endothelial progenitor cells circulating in the peripheral blood are capable of acquiring a phenotype typical of BBB under special culture conditions *in vitro*, which should be considered when developing BBB models.

Mechanisms of endothelial progenitor cell trafficking to the brain tissue are studied insufficiently. Most probably, mobilization and recruitment of endothelial progenitor cells in a developing and mature brain occur due to paracrine and endocrine signals produced by NVU cells under (patho)physiological conditions.

Radial glia cells and neuroepithelial cells interact with endothelial cells in the brain during embryogenesis. Radial glia cells express glutamate-aspartate transporter GLAST, which indicates their role in regulation of metabolic events at early stages of brain development, as well as connexin 43 (Cx43), ensuring efficient paracrine and autocrine signaling in the regions of intensive cell proliferation [20]. Radial glia cells produces retinoic acid that is one of the most efficient BBB formation regulators [9]. In the context of neurogenesis, it is believed that in mature brain a portion of cells having the phenotype of radial glia, which is most active in the period of embryonic neurogenesis, is retained in neurogenic niches, not turning into astrocytes, which ensures reparative neurogenesis [21]. YKL-immunopositive cells related to astroglia progenitor cells are found in the brain in the early development period exactly in the areas of intensive angiogenesis and the BBB being established [22]. Interestingly, the astrocytes expressing YKL-40 mark neuroinflammation or neurodegeneration zones in the mature brain [23, 24], thus confirming the role of astroglia in reparative angiogenesis regulation. In the period of embryogenesis and in the mature brain, endothelial cells retain their important function of neurogenesis regulators, which is supported by their stimulating effect on maintaining population of stem and progenitor cells of the brain, as well as effects of growth factors (e.g., VEGF, BDNF, LIF, PDGF, IGF) and other regulatory molecules of endothelial origin affecting neuronal and astroglial progenitors [25, 26].

In the postnatal stage, neovasculogenesis, e.g., in case of the brain damage, is ensured by endothelial progenitor cells of bone marrow origin [27] and, most likely, exactly this mechanism is important for BBB repair, thus explaining the observed dependence of clinical effect after brain injury on the levels of endothelial progenitor

cells mobilization [28]. Endothelial cell proliferation can be induced, e.g., by hypoxia, resulting in alterations in the expression of cell adherence proteins [29].

Disturbance of various stages of barriergenesis and BBB functioning is induced by many factors: 1) vasculogenesis and neurogenesis suppression during prenatal stage; 2) impact of pregnancy failure and perinatal stress on regulation of BBB development and functioning, regulation of transport function of endothelial cells by humoral factors (stress hormones, neuropeptides, interleukins); 3) neuronal damage, activation of astrocytes and microglia, neuroinflammation development in case of neuroinfections and ischemia, which causes higher BBB permeability, especially evident in premature infants; 4) systemic inflammation and hyperproduction of pro-inflammatory cytokines in the prenatal and early postnatal stage causing early BBB damage and vasculogenesis disturbance with subsequent BBB pathological permeability in adults [30, 31].

Molecular mechanisms of barriergenesis: role of intercellular interactions

Many interesting literature reviews are devoted to barriergenesis mechanisms [10, 12, 32, etc.]. According to up-to-date views, complicated process of barriergenesis is regulated by a huge number of factors, among which the leading roles are played by Wnt/catenin, sirtuins, Notch, HIF-1, NF-kB, FOX, Rho GTPases, GSK-3, as well as humoral regulators (vascular endothelial growth factor (VEGF), matrix metalloproteinases, neurotransmitters, neuropeptides, neurosteroids, TNF-α, IL-1) [12, 32, 33]. At all the barriergenesis stages, development of local microenvironment supported by regulatory molecules is of great importance, ensuring various intercellular interactions, controlling cell proliferation and differentiation [34]. An important role in the formation of an adequate local microenvironment belongs to interaction of neighbor (within a neurovascular unit) cells — endothelial cells, astrocytes, pericytes, and neurons. Interestingly, these interactions are actual for all ontogenesis stages, and it is evident that they undergo specific changes along BBB development even under conditions of its damage or repair. Thus, it is shown that *in vitro* co-culture of embyonic neuronal progenitor cells with endothelial cells of cerebral capillaries induce in the latter the barrier properties typical for BBB endothelium [35], later the possibility to induce neurogenic or angiogenic program from the stem cells *in vivo* was demonstrated by M. Ii et al. [36].

Induction of phenotype characteristic of BBB cells in endothelial cells during prenatal development stage of the brain is ensured by impact of neuronal cells on endothelium [4].

Pericytes play an important role in angiogenesis in a developing and mature brain. These cells participating in BBB formation are an object of regulatory influence by a high number of humoral factors of endothelial origin, in particular VEGF and angiopoetines [37–39]. Interestingly, various signal pathways are involved in mechanisms of pericytes participation in forming vessels and in barriergenesis [40].

As a whole, the barriergenesis process demonstrates three main lines of progenitor cells specialization: 1) neuronalization (Wnt/Notch-regulated and other associated mechanisms); 2) astroglialization (STAT3/RAR/Zac1-regulated and other associated mechanisms); 3) endotheliolization (HIF-1/SIRT1-regulated and other associated mechanisms).

Although mature astrocytes do not participate in the early stages of BBB formation (mainly radial glia cells are found in the close vicinity to endothelium cells migrating to the brain), there is an opinion that astrocytes ensure formation of a matrix for angiogenesis in brain development by producing VEGF, being in close interaction with progenitor cells forming vessels [41]. Besides, there is data confirming that metabolic dysfunction of astrocytes determines the character of BBB damage [42]. Retinoic acid — an important regulator of barriergenesis [9] — is a product of astrocytes activity [43]. Reactive astrocytes regulate interaction of cerebral endothelial cells and endothelial progenitor cells, releasing HMGB1 group proteins to extracellular environment (e.g., as a result of cell damage and activation of inflammasomes), interacting later with RAGE-receptors in cells of endothelial origin [44].

HIF-1-coupled mechanisms in astrocytes and endothelial cells control processes of angiogenesis and barriergenesis, BBB permeability [45]; action of neurotoxic factors, in particular, amyloid, is associated with damage of astrocytes, and causes intensive angiogenesis, disturbs BBB permeability [46]. Peptides of astroglial origin, like VEGF, stimulate angiogenesis, while thrombospondin has angiostatic effect, but both of them are important for reparative barriergenesis and neurogenesis [47, 48]. Interestingly, hippocampal mechanisms of plasticity depend on specifics of thrombospondin production by astrocytes [49], and angiogenic potential of endothelial progenitor cells is suppressed by thrombospondin-1 [50]. Factors influencing biological effects of these molecules (matrix metalloproteinases (MMP) and their inhibitors TIMPs, thrombospondin receptors CD47 and CD36) may be considered as molecules participating in the pathogenesis of a wide range of diseases [51].

One of the most powerful factors stimulating angiogenesis, in particular in pathological conditions, associated with the necessity of additional vascularization of the tissue and recovery of damaged vessels, is hypoxia. Interestingly, hypoxia increases PVLAP expression in BBB endothelial cells, thus suggesting elevation of fenestration rate and transcytosis level [4]. The role of Rho GTPases in hypoxic conditions is not clear yet but it might have an importance, e.g., for Rac1

that controls the following events in cells of various nature: 1) HIF-1 expression determining the character of cells response to hypoxia, insulin effects and glucose metabolism [52]; 2) changes in cytoskeletal proteins, in particular, in astrocyte stellation [53]; 3) production of free radicals, secretion of matrix metalloproteinases (MMP-2, MMP-9) that is important for remodeling the extracellular matrix [54]. It is evident that all the above mechanisms are actual for both BBB functioning and for barriergenesis. Indeed, maintaining structural and functional integrity of endothelial barrier needs in Rac1 activity in various organs and tissues [55–57]. Migration of endothelial cells to specialized tissues requires participation of Rac1 in realizing SDF-1 effects (stromal cell-derived factor-1), which is actual for angiogenesis [58], e.g., in case of tissue damage [59].

Many events in intercellular interactions depend on activity of HIF-1 transcription factor mediating the cells response to hypoxia. It is well known that HIF-1-controlled reactions of energy metabolism are reflected in changes of processes of glycolysis, lactate accumulation and changes in the character of neuron-astroglia metabolic coupling. HIF-1 activity regulates VEGF expression [60] and cerebral angiogenesis. Besides, among all the HIF-1-controlled genes, genes encoding for SDF-1, glucose and lactate transporters, glycolytic enzymes, are required to support cell functioning under the conditions of acute or chronic hypoxia. Interestingly, lactate as a product of anaerobic glycolysis (key regulator of gliovascular control ensuring adequate local blood flow in functionally active brain regions) facilitates realization of angiogenesis program acting at endothelial cells [61], stimulates circulating vasculogenic stem cells and increase their expression of HIF-1-controlled angiogenic growth factors [62]. Lactate proangiogenic activity is evident under conditions when adding lactate to the matrix for development of vessel cells significantly improves angiogenesis parameters in the implant placement zone [63].

HIF-1 activation in cells can be induced both by hypoxia and non-hypoxic stimuli, e.g. production of reactive oxygen species by mitochondria [64]. It is important to note that lactate production is closely related to cell redox-condition [65], in particular to NAD+/NADH ratio in mitochondria, therefore non-hypoxic mechanisms of HIF-1 activation can occur under physiological conditions of BBB functioning. It is hard to tell how important mitochondrial events in these cells are in this context, especially taking into the consideration specific features of mitochondrial content in endothelial cells versus other cells [66].

Within blood-brain barrier, the major lactate producers are endothelial cells. Thus, it is shown that NO production by endothelial cells drives HIF-1α-dependent effects (specifically, anaerobic glycolysis activation) in contacting astrocytes [67]. On the other part, endothelial cell proliferation is inherently controlled by glucose metabolism, and the tissue-produced lactate is transported to endothelium cells thus inducing HIF-1α-controlled events resulting in angiogenesis stimulation [68] as it was demonstrated in tumor tissue. Truthfulness of a similar mechanism for astrocyte-endothelial interactions in cerebral vessels needs in experimental approval, however, taking into account high glycolytic activity in astrocytes vs. other neurovascular unit cells and their control functions over metabolic cell coupling processes, this mechanism is rather possible.

It is of interest that response of astrocytes to hypoxia associated with HIF-1 activation is important for pathological angiogenesis associated with central nervous system cell damage but has no significant effect on angiogenesis in the course of neurodevelopment [69], however, according to other research group data, astrocytes take a key role in the generation of angiogenic microenvironment but HIF-1-α-mediated processes are evident mostly in neurons contacting with astrocytes [41].

In the central nervous system, HIF-1 affects the processes of glucose transport and uptake by cells, memory acquisition [70], lactate acts as a glio-transmitter controlling neuronal excitability [71], as well as a key regulator of local blood flow in active brain regions. At the same time, HIF-1 is the subject of epigenetic regulations in neurovascular unit cells being deacetylated by sirtuin 1 [72] which can bear on both epigenetic regulation of cognitive functions in developing brain [73] and NAD^+ (nicotinamide adenine dinucleotide) homeostasis which determines activity of NAD^+-converting enzymes (ADP-ribosyl) polymerase, ADP-ribosyl cyclase/CD38/CD157, sirtuins) [74]. Interestingly, lactate as a product of glycolysis suppresses activity of deacylases (sirtuins), thereby causing long-term changes in gene expression [75]. There is an interrelation between glycolysis and synaptogenesis, and expression of genes specific for early brain development stages [76]; between expression of genes encoding for proteins involved in neuron-astroglia metabolic coupling, and conditions related to alterations in metabolite clearance through the blood-brain barrier [77].

Development of up-to-date pharmacotherapeutic strategies focused on regulation of HIF-1-regulated angiogenesis mechanisms is a promising direction of treatment efficiency improvement in ischemic injury and tissue degeneration, neoplastic proliferation control [78], however, by now, there are only few evidences that such strategies prove its efficacy for correction of barriergenesis alterations or BBB damage [79–81].

Use of endothelial progenitor cells *in vitro* blood-brain barrier models

One of the goals in the establishment of BBB *in vitro* models is production of system cellular components

which to the utmost match the phenotypical properties of BBB cells *in vivo* (intercellular junctions, metabolism, transport protein activity, endo- and exocytosis), and also a precise reproduction of natural cell-to-cell interactions which are basic for the barrier properties [82]. The most sticking point is the use of the most relevant endothelial cells with respect to *in vivo* situation (in the context of endothelial cells barrier function). Another critical aspect is reproduction of adequate BBB cell-component secretory activity ensuring intercellular communications and maintaining barrier integrity. Thus, a considerable number of endothelial humoral factors and BBB basement membrane proteins affect the status of astrocytes within the neurovascular unit [83].

Several approaches have been proposed to solve these problems (See the Table). The most frequently used method is obtaining the mature neurons, astrocytes, endothelial cells derived from cerebral blood capillary vessels, and pericytes (pericapillary cells). At the same time, over the last few years several attempts have been done to get BBB cellular components from progenitor cells with their compete characterization with metabolomics and proteomics approaches [84]. This approach is of interest because it provides for both reproduction of natural so-called barriergenesis mechanisms *in vitro* which by definition represents a combination of neurogenesis and angiogenesis and identification of key cellular events determining BBB functional competence. Specifically, analysis of endothelial progenitor cell secretion made it possible to identify molecular mechanisms of their impact on angiogenic activity of cerebral endothelium [27].

At present, the source of neurons and astrocytes in such protocols are neuronal progenitor cells (NPCs) [85], and in order to get endothelial cells differentiating human pluripotent stem cells (hPSCs) are used. Interestingly that about 6% of neuronal stem cells cultured in the presence of endothelium may be converted into cells of endothelial nature [86] which offers opportunities for target-specific differentiation of neuronal stem cells *in vitro* into BBB cellular components (neurons, astrocyte, endothelial cells). However, there is a serious methodological problem relating to high heterogeneity of neuronal stem cell population, therefore selection of adequate culture conditions is rather difficult.

Production of endothelial cells with cerebral endothelial characteristics from hPSCs will get a real breakthrough in BBB modeling *in vitro* to solve problems relating to patient-specific screening of neuropharmacological substances, since all current BBB models utilize either endothelial cells derived from blood capillary vessels (and it makes practically impossible to create an absolutely adequate patient-specific BBB model), or endothelial cells lines of non-cerebral origin, i.e. HUVEC (which cast doubt on feasibility of the full data extrapolation to central nervous system barrier structures).

Application of soluble factors affecting barriergenesis *in vivo*, i.e. retinoic acid added to the culture medium [87] would significantly improve the results of endothelial cells development with the phenotype close to cerebral endothelial cells.

Another methodological approach is the production of BBB endothelial cell components from umbilical blood haemopoietic stem cells which in appropriate conditions start to express tight junctions proteins and transporters specific for cerebral endothelial cells within the BBB [88].

Use of hPSCs as a source for production of so-called primitive endothelium (endothelium which is specific for early ontogenesis stages) sets a further task to get optimal conditions for co-culture of neurons, astrocytes (derived from pluripotent or progenitor cells) and endothelial cells so that the latter can achieve the mature phenotype (which is specific for mature brain) [16].

Development of protocols for production of iPSCs (induced pluripotent stem cells) introduce a new era in experimental neurology and neuropharmacology [89–91]. Application of iPSCs for *in vitro* modeling of cerebral structures, including BBB, opens new opportunities for development of patient-specific screening of drugs, and for the assessment of individual features in the pathogenesis of central nervous system disorders.

BBB cellular and molecular mechanisms of barriergenesis and maintenance of its integrity in pre- and postnatal stages of brain development differ from each other, and the reason for this not only specific properties

Endothelial cell sources in BBB models *in vitro*

Name	Source	Advantages	Disadvantages
Mature endothelial cells	Mature cerebral blood capillaries	Identity to BBB cells *in vivo*	Complexity of isolation and culture (limited proliferative activity), assessment of barriergenesis is impossible
Embryonic endothelial progenitor cells	Embryonic brain	Assessment of barriergenesis, cell migration	Difficulties in target-specific cell differentiation
Pluripotential stem cells (hPSCs, iPSCs)	Somatic genetically reprogrammed progenitor cells	Assessment of barriergenesis, cell migration, establishment of patient-specific models	Difficulties in hPSCs and iPSCs production, target-specific cell differentiation

of endothelial progenitor cells but rather the complexity of their interactions with other BBB cells. Progress in our understanding of these mechanisms will give us new opportunities to pharmacotherapy of brain disorders associated with alterations in BBB permeability.

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