Pathomorphism of Limb Major Vessels in Experimental Atherogenic Inflammation. The Role of Adventitial Intimal Relations (Review)

DOI: 10.17691/stm2017.9.3.20
Received November 28, 2016

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The review considers the problems of pathological destruction of a vascular wall at early signs of atherogenic inflammation of major arteries in hyperlipidemia in relation to modern technologies of local angioplasty. It has shown the role of molecular markers in atherogenic inflammation development and progression in intima and subintimal space. The emphasis is laid on modern genetically engineered and biopolymer technologies for vascular wall repair, the significance of adventitial and para-adventitial arterial layers in atherogenic inflammation, the formation of a therapeutic angiogenesis effect when using modern methods of adventitia bioengineering.

Key words: atherogenic inflammation; atherogenesis markers; vascular wall; therapeutic angiogenesis; morphological angioplasty; genetically engineered drugs; biodegradable polysaccharide structures; vascular adventitia engineering.

The success in combating such diseases as infarction, stroke, lower limb chronic ischemia depends mainly on solving the problems of atherosclerosis diagnosis, management and prevention. It is significant that effective treatment of patients with lower limb chronic ischemia and inflammatory process regression in arteries is possible subject to an early detection and treatment of the disease [1–3]. Lower limb peripheral arterial occlusive disease is an important and very frequent manifestation of systemic atherosclerosis resulting in a significant decrease in patients' mobility, deterioration in life quality, and associated with a high risk of cardiovascular incidence and mortality rate [4, 5], its incidence growing steadily [6, 7]. Moreover, the development of lower limb critical ischemia indicating nearly complete circulatory decompensation occurs as frequently as 400–1,000 per a million of population a year, or in 15–20% patients with lower limb peripheral arterial occlusive disease [8, 9]. The expected mortality of patients with critical ischemia is increasing from 25% within the first year of the syndrome development to 60–70% during the next 3–5 years. The indications for high, i.e. above the knee joint, amputation reach 52–95% within 3 years, total mortality rate during the period increasing from 10–40 up to 71% [10]. Experimental practice shows [11] that a chronic cholesterol diet in experimental rats and rabbits is an adequate model to form the early signs of atherogenic inflammation in limb major vessel walls. The use of pure cholesterol as a part of a diet for outbred rats for 60 days results in high hyperlipidemia increasing the level of total lipid fractions by 2.5 times, triglycerides — twice as high, unesterified fatty acids — by 26 times. The use of a cholesterol diet in chinchilla rabbits within 80–110 days provides the increase of triglycerides by 1.5–2.5 times, cholesterol level — by 30–90 times, low-density lipoprotein (LDL) fractions — by 29–56 times and higher; the fraction of very low-density lipoproteins (VLDL) — by 2.5–5.0 times in blood plasma forming a very high atherogenicity coefficient.

High hyperlipidemia in animals corresponds to a high level of lipid fractions in vascular wall tissues of major arteries and exceeds the level of total lipids in femoral arteries thrice, and triglycerides — by 2.5 times forming the accumulation of xanthoma cells in the intima. Atherogenic inflammation in experimental animals causes a marked intimal edema in a form of disintegrated fiber component of internal elastic membrane, the

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occurrence of xanthoma cells, proliferation of smooth muscle cells subintimally and in media, with its irregular thickening and the formation of a typical lipid ("soft") plaque covered by a fibrous membrane resulting in a reduced vascular lumen diameter [12, 13].

Cholesterol feeding of animals, rabbits in particular, regularly leads, in addition to hypercholesteremia, to cholesterol deposits in tissues, and to the utmost — in the internal membrane of major arteries. Vascular lipidosis develops, it being observed usually in a 3–4-month experimental period. Marked atherogenesis has revealed on average by a feeding day 90–100, though it can become evident within a significantly shorter period of time. Vascular pathomorphism in atherogenic inflammation involves whitish or slightly yellowish spots or stripes, usually a bit rising over the surface, formed on the intima of major vessels. When cerasin red stained, they are well red dyed, it presenting a bright picture of lipid inclusions against a white intima background. Lipid accumulations are primarily in the apical part of the ascending aorta above the valves, as well as in the aortal arch and in the orifice area of major vessels coming from the aorta [14–17].

Endothelial dysfunction manifests itself as increased permeability and adhesion, as well as enhanced secretion of pro-coagulants and vasoconstrictors. The most significant damaging factor is the increase in LDL level [18–20]. The first stages of pathomorphism development of atherogenic inflammation are characterized by gradual lipid accumulation: first, inside, and then extracellularly with the formation of a lipid spot/stripes followed by a new atherosclerotic plaque [21, 22]. Such microscopic picture in vessels is accompanied by signal molecular transduction of markers starting with the oxidation of LDLs and VLDLs, therefore, the occurrence of weak angiogenic structures able to determine a weak immune response. Electrostatic relations of oxidized LDL as a part of apoprotein B form antigenic determinants to interact with immune cells.

An important trigger mechanism in atherogenic inflammation development is the initiation of presentation of antigenic epitopes to immune cells (A and T cells) in the major vessel intima, the circulation of oxidized LDL in vessels [23, 24]. A level of specific antibodies to oxidized LDL indicates primarily the activity of atherogenic inflammation development [25]. However, it may happen that the marker also signals the atherosclerotic damage degree [26]. Thus, oxidized LDLs fulfil the role of neoantigens initiating inflammatory reactions, which lead to atherosclerosis progression, and the antibodies to such LDL can be considered an independent predictor of atherosclerotic disease progression and a marker of LDL oxidation [27].

Oxidant system activation in the presence of hyperlipidemia forms the accumulation of monomycyclic endoperoxides with metabolism into aldehyde compounds and Schiff's bases. It is of importance that the process activates in the subintimal space of an arterial wall, where LDLs are oxidized. Oxygen metabolites of activated macrophages captured a large mass of LDL, T lymphocytes concentrate in an intima, trigger lipid oxidation inside and outside the cells, active absorption of oxidized forms forming a great number of foam (vacuolated) cells [28, 29]. The overload of cells by lipid oxidized forms an apoptotic cascade with macrophage damage. A large number of cholesterol crystals in the subintimal space outside the cells clearly reveal the picture of atherogenic inflammation known to morphologists.

Proliferation of smooth muscle cells in the middle layer of a vessel with destabilization of their orientation, trafficking to intima space from a middle layer gives to the inflammatory process the stability of a future atherogenic construction in a form of connective tissue proteins (collagen, proteoglycans, elastins) secreted to the extracellular space. This is a future fibrous frame of an atherogenic product. It will separate a lipid nucleus from a blood flow lumen. The stimulation of vascular endothelial growth factor (VEGF) by intimal cells will provide the proliferation of vascular endothelium, its migration to atheromatous plaque location. New formation of microvessels in intima will increase the volume of an atherogenic product, enhance the inflow of foam cells and their destruction. The accumulation of oxidized LDL in the subendothelial space enhances vascular endothelial dysfunction increasing the adhesion of endothelial cells, and forming lipid and cellular leakages.

Active escape of blood stream by monocytes, and the activation of receptor phenotype under endothelium many times increase the capture of a lipid mass [30]. Impaired expression balance of pro-atherogenic (pro-inflammatory) and anti-atherogenic (anti-inflammatory) cell markers if hyperlipidemia conditions are preserved stabilize destruction in subendothelial space. The expression of blood residual cell receptors under vascular endothelium and of vascular endothelium itself, namely: endothelin-1 [31–35], caveolins-1, -2, and -3 [36, 37], selectins P (CD62P), E (CD62E), L (CD62L), and antibodies to them on vascular endothelium, lymphocytes and platelets [38–40], intercellular adhesion molecule-1 (ICAM-1) marker [41–43], vascular cell adhesion molecule-1 (VCAM-1) marker [44–47], monocyte chemotactic protein (MCP-1) [48–50], macrophage colony-stimulating factor (MCSF) [51–57], pleiotropic cytokine (TNF-α) [58–64], C-reactive protein (CRP) [65–70], platelet derived growth factor (PDGF) [71–77], interleukin family in atherosclerosis [78–81] create a picture of multicellular destruction involving all layers of a major vessel including adventitial space [82].

A level of pro-inflammatory cytokines circulating in blood plasma correlates with a cytokine level in an atherogenic plaque [83]. In addition, multifunctional interferon activating macrophages (IFN-γ), as well as IL-6, IL-12, and IL-15, tumor necrosis factor (TNF-α) are expressed highly by NK and T cells in a soft
plaque compared to the intact artery exceeding the activity levels of anti-inflammatory cytokines such as IL-4, IL-10 [84–86]. Among cytokines, IFN-γ is a key factor in atherosclerosis pathogenesis, since the marker plays a multifunctional role in the development of both early (factors VCAM-1, ICAM-1, cytokines, chemokines, class I and II antigens of expression induction of major histocompatibility complex (MHCs) on macrophages, T lymphocytes, NK cells) and late stages of atherogenic inflammation including the pool formation of macrophages saturated with oxidized LDL forms, cholesterol accumulation in an atheromatous plaque with cytokine activation, NO-synthase of monocyte chemotactic protein (MCP-1), increased synthesis of proteins of metalloproteininas [87, 88].

The deficiency of a gene controlling IFN-γ synthesis in experimental atherogenesis results in the reduction of pathomorphological disturbances in a vessel wall, and restenosis [89]. Mass capture of oxidized LDL by macrophages is provided mainly by the expression of category A (SR-A) and B (SR-B1, CD36) scavenger-receptors [90–92]. Any artificial external interference in the pathological molecular cellular cascade for the purpose of therapy is oriented on a break in interrelated processes of progressive atherogenic inflammation. Such external interferences are known as treatment methods aimed primarily at the consequences of the process: surgical reconstructive vascular operations [93–99], physiotherapeutic interventions [1, 100, 101]. The introduction of genetic engineering constructions [102] in ischemic tissues, implantation of polymer polysaccharide constructions in perivascular cases for [102] in ischemic tissues, implantation of polymer [93–99], physiotherapeutic interventions [1, 100, 101]. process: surgical reconstructive vascular operations methods aimed primarily at the consequences of the disease [102–104] in ischemic tissues, implantation of polymer polysaccharide constructions in perivascular cases for major arteries [103–107] aims at local reconstruction of a vascular wall. It should be mentioned that the use of only genetic engineering constructions administered intravenously or directly in limb ischemic tissues — synthetic analogues of prostaglandins: Iloprost [108–110], prostanoids; registered in Russia Alprostan, Vazaprostan (PGE-1-alprostadil) [102, 111], VEGF165 gene [112, 113], Neovasculgen [114–122] — are meant for rearrangement of subendothelial, middle and adventitial layers of major and small limb arterial vessels. Such rearrangement concerns the activity of vascular endothelial proliferation, its translocation in interstitial tissue with the formation of a great number of new additional microvessels (therapeutic angiogenesis) that by itself is the objective for researchers.

A modern trend in atherogenic inflammation regulation includes the methods of controlling the progressive atherosclerosis degree, the methods aiming at applying the technologies counterbalancing the effect of pro-atherogenic molecules both at the level of forming antigen-antibody complexes, and also at the level of genetic engineering constructions blocking the operation of genes responsible for the synthesis of pro-atherogenic proteins such as VCAM-1, ICAM-1, TNF-α, GM-CSF, M-CSF, G-CSF, or the administration of antagonists of cytokines or their receptors, e.g., to IL-1 or to IL-1R (IL-1Ra) [78, 123]. The control of atherogenic inflammation degree by regulating the expression of pro-atherogenic markers results in the development of new preventive and treatment modalities for atherosclerosis [124].

Constructions based on liquid polysaccharides (sulfated forms of chitosan, chitosan ascorbate, chitosan hydrochloride, alginate) when introduced in fascial compartments of neurovascular bundles [12, 13] create an effect of therapeutic angiogenesis. Local administration of chitosan copolymer containing endothelial cells of the umbilical vein to the mice with experimental lower limb ischemia within 2 weeks clearly formed a new vascular net in the arterial trunk [125]. Endothelial cells reprogrammed from embryonic stem cells encapsulated in chitosan hydrogel in combination with VEGF, when administered locally in animals with lower limb ischemia model, resulted in neovascularization through the mechanisms of vasculogenesis and angiogenesis, with effective blood flow recovery in ischemic tissues [126]. In addition to therapeutic angiogenesis effect, chitosan copolymers create a direct contact with an atherogenic construction in the subendothelial space of major arteries due to electrostatic, concentration and affine relations and, more precisely, with cholesterol-containing molecules of both: a lipid nucleus of soft plaques, and also foam cells of an intimal layer.

Special attention should be paid to the adventitia in tissue engineering of major vessels [127, 128]. It has been clearly shown [129] that hypercholesterolemia remodels an intimal vascular layer and induces endothelial dysfunction. Hyperoxidation in an adventitial layer of a vessel using NADPH-oxidase induces an inflammatory response of a vessel with the expression of pro-atherogenic markers (MCP-1, IL-6) in the middle and endothelial layers of a major artery, stimulates the translation of myofibroblasts into an intimal layer of the artery [130, 131]. The use of apocynin, an inhibitor of NADPH oxidase on myofibroblasts, and probably, on macrophages results in blocking neointimal growth and endothelial dysfunction [132]. Thus, adventitia is no less important layer of a vascular wall actively involved in atherogenic inflammation associated with the middle and intimal layers of major vessels.

Similar changes resemble a morphological picture in nonspecific aorta-arteritis or Takayasu disease. However, it is characteristic that in Takayasu disease a granulomatous inflammatory process starts initially in adventitia and internal media layers; microscopic study of granulomas in adventitia reveals the accumulations of lymphocytes, plasma, and reticular cells. Later on, there is granuloma fibrosis, hardening and rupture of media, endothelial proliferation. Immunogenetic basis of such inflammation is presented by markers-antigens HLA-A1, HLA-Bp, HLA-Bw52, HLA-DR2. The lumen narrowing or the occlusion of aorta, its large branches in proximal regions of upper and lower limbs is not related to atherosclerosis and the picture of hypercholesterolemia.
The concept of morphological rearrangement of qualitative and quantitative characteristics of internal layers of the vessel affected by atherogenic inflammation suggested by the authors consists in an artificial arrangement of such ingredients in the perivascular space, which are able to have an effect on intimal and middle layers leading to the normalization of both cellular and intercellular structure, lipid spectrum of a vascular wall, but also macroscopic characteristics of a vessel, such as the thickness of intima, media, the diameter of major arteries. It is important [82] that adventitial and para-adventitial layers take their primary place in treatment intervention and drug delivery in order to solve the problems of controlling an inflammatory process including atherogenesis. Therefore, the attempts to control cell-tissue compartment in an adventitial layer in atherogenesis are sensible, if the purpose is to “develop” the main events of atherogenic inflammation, and its reduction in adventitial and para-adventitial areas, rather than in the zone of internal layers of a major vessel, especially in an early inflammatory process. It has been proved demonstratively [133] that about 20% cells of adventitial population of the mouse aortic root are composed of the precursors of smooth muscle cells capable of forming atherosclerotic impairment of the middle layer of a vessel under hyperlipidemia. The precursor cells applied on a decellularized venous wall result in their migration via a vascular wall with partial formation of a neointimal layer. In recent years, vascular adventitia is considered as a key component of vascular response to damage, the engineering of which is able to regulate the proliferation of smooth muscle cells of the middle and endothelial vascular layers that are of importance in atherogenic inflammation development [134]. Experimental data indicate the presence of the relationship between vasa vasorum and neointima formation after experimental damage [129]. The relationship is proved by a number of studies on vascular impairment modeling and minimally invasive arrangement of biopolymer particles containing allogenic endothelial cells near to a major vessel [135]. Tissue engineering in the adventitia area in open or closed surgeries in experimental models inhibit intima thickening, restenosis formation, negative remodeling [136–138].

The technologies to obtain a therapeutic angiogenesis effect are one of the promising methods used to treat chronic (critical) lower limb ischemia in patients, who are not candidates for surgical or endovascular revascularization. A concept of therapeutic angiogenesis implies creating in ischemic tissues such concentration of angiogenic growth factors, which will enable to achieve an effective stimulation of natural angiogenesis. For this purpose, in clinical practice there can be used preparations of recombinant angiogenic growth factors or their genes, as well as stem and progenitor cells [139, 140]. Angiogenesis is a complex process including a series of events occurring in strict sequence. These processes involve endothelial cells and pericapillary cells [141], which are located in arterioles, capillaries, and postcapillary venules. Under the influence of angiogenic stimuli, which include hypoxia, ischemia, mechanical strain and inflammation, endothelial cells and pericapillary cells activate and start producing proteases (collagenases and plasminogen activators) promoting the destruction of basal membrane, the detachment of these cells.

The interest of angiology is focused on therapeutic angiogenesis — a process aimed at managing impairments in insufficient tissue perfusion by stimulating the growth and arborization of blood vessels or endothelial function modulation. The formation and growth of new vessels in a postnatal period include the appearance of new capillaries and arterioles. Neo-angiogenesis is the activation of endothelial cells, the synthesis of protease enzymes in them, degradation of intercellular matrix, proliferation and migration of endotheliocytes, and the formation of primary highly-permeable vascular structures followed by the stabilization and “growing-up” of primary vascular structures due to the attraction of pericapillary cells and smooth muscle cells resulting in organizing a three-dimensional vascular network.

A key stimulus to angiogenesis in pathological conditions is hypoxia or ischemia, which induce the formation of many angiogenic factors and, primarily, the main angiogenesis regulator in both an embryonic and also postnatal period of body development: VEGF and its receptors. VEGF selectively stimulates the proliferation and migration of endothelial cells, their precursors and monocytes synthesizing the receptors to it. It enhances vascular permeability promoting plasma protein transudation to perivascular space, which should be prepared for migration of endothelial cells. VEGF causes the synthesis of endothelial NO-synthase and NO formation that contributes to vasodilation and stimulates the formation of proteases enzymes destroying the relations between endothelial cells and extracellular matrix that is necessary for cell trafficking. Prolonged local presence of VEGF is of importance during the stabilization and “growing-up” of a newly formed immature vascular network. Genetic constructions function in a target tissue from one to several weeks, and provide the longer presence of angiogenic factors.

A great number of neo-angiogenesis stimulators have been distinguished: VEGF, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), angiogenin, angiopoietin. In addition, there have been found nonspecific vascular growth stimulators, such as insulin-like growth factor 1 (IGF-1), transforming growth factor, TNF-α, nitric oxide, IL-8, matrix metalloproteinases. However, the extensive use of angiogenic factors, e.g., FGF, eventually can result in angiogenesis enhancement and active formation of vascularized atheromatous foci [142]. Induced proliferation of vascular endothelium, its translation in the intimal...
space with the formation of microvessels under angiogenesis stimulation is likely to be troublesome. The displacement of inflammation and angiogenesis center from the sub-intimal to para-adventitial space requires close attention of researchers.

VEGF is a significant and the most investigated factor. Fibroblast growth factor that can independently stimulate neo-angiogenesis has a synergistic effect with VEGF. VEGF is an angiogenic glycoprotein increasing vascular permeability. It is produced by different cell types [143], and endothelial cells have the receptors for the factor actively producing it under hypoxia or anoxia [144–146]. Experimental and clinical works on therapeutic angiogenesis have used the modulation strategy of both endo-angiogenous growth factors (FGF-1, FGF-2, VEGF165) and their genes (VEGF165, VEGF121, VEGF189, FGF-5), and also the administration of recombinant exogenous agents. Their effect has been studied in patients with lower limb vascular ischemia, and on hind limb ischemic models in small and middle-sized rodents, and acute and chronic myocardial ischemic models in dogs and mini pigs [147–151]. Scientific researches offer the challenges confirming or denying an actual mitogenic effect of VEGF on vascular endothelium [152]. Without dwelling on the results of these numerous studies in detail, it is necessary to mention the main thing: nearly all the cases, when growth factors or their genes were administered, were found to have stimulated the development of collaterals and new capillaries, which did not regress after discontinuation of growth factors administration; numerous introduction of the gene substituted numerous injections or infusions of recombinant growth factors. It is of importance that the administration of genes was accompanied by none or slight side effects, e.g., hypotension, which was observed when growth factors were administered. And, finally, the intramuscular administration of plasmid DNA without adenoviral vector managed to stimulate effectively angiogenesis and arteriogenesis in ischemic lower limbs.

The world laboratories engaged in gene therapeutic preparations are being currently at different stages. So, in China, there are two genetic therapeutic agents on the market — Gendicine and H101 designed to treat advanced cases of head and neck skin cancer. In Europe and USA, some genetic therapeutic pharmaceuticals are at the 3rd phase of clinical trials. Collategene, a pharmaceutical engineered in Japan to treat the conditions associated with critical lower limb ischemia, is at the extended 3rd phase of clinical trials in the United States, Europe, Japan, and other countries. Preliminary research protocols have been approved by FDA. FDA is known to have provided fast track designation program for Collategene. The program implies an accelerated prosecution for the preparations, which have demonstrated a high potential for solving the problem with unfulfilled medical needs in serious diseases.

In the early 2000s in Russia, there were carried out the first studies and pilot clinical trials of genetic therapeutic constructions designed on the basis of a gene encoding VEGF. In 2012 Neovascugen, a new Russian pharmaceutical with VEGF165 gene, designed for managing chronic lower limb ischemia came to the market. Neovascugen is an innovation and unique product in the world market. Its mode of action is based on a principle of a controlled therapeutic angiogenesis. Neovascugen is a circular DNA molecule, which contains a region responsible for VEGF synthesis. Local multifocal administration of the agent into lower limb muscles provides controlled angiogenesis, stimulating growth and development of new vessels [153–157].

Protonated gel forms of hydrochloride and chitosan ascorbate, as well as chitosan sulfated form implanted in the perivascular fascial layer in rats result in significant local extraction of total lipid fractions, LDL and triglycerides from the femoral artery wall, by reducing their level, respectively, by 2.2, 2.5, and 1.9 times [12, 13]. The implantation of liquid polymer forms demonstrates local recovery of the middle layer thickness by 29–38% reducing the relation of the media thickness to the vascular lumen diameter by 37–44%. The effect was also observed in the overlying segment of a major vessel. Dislocation of sulfated chitosan form in the perivascular space within 20 days provides the decrease of a sub-intimal myocyte coefficient in the middle arterial segment by 27% enhancing a reconstruction effect in the upper vascular segment by 68%. An implantation effect concerns the new formation of microvascular bed at the site of polymer resorption increasing the number of new vessels in rats by 22–49%, in rabbits — by 56–66%. The more profound effect of therapeutic angiogenesis is also clearly observed when chitosan constructions are implanted in animal intact tissues, on day 30 of a post-implantation period the increase being 85–96%. The rearrangement of intimal and sub-intimal space of a major artery at early stages of atherogenic inflammation provides the local perfusion increase of limb soft tissues 20 days after implantation, by 68–86% on average. On day 30 of post-implantation period perfusion decreases, though being 34–36% higher compared to a control area [12, 13].

The mentioned findings of a local angiogenic effect of chitosan hydrogels are confirmed by fundamental researches, when using in experiment noncovalent and covalent copolymers containing chitosan and sulfated ingredient, heparin serving as an example. An angiogenic effect develops due to binding of proangiogenic growth factors by heparin fixed on copolymer [158–160]. However, the role of heparin is not obligatory. In fact, any biodegradable copolymers containing chitosan are able to bind and release angiogenic growth factors resulting, in their biodegradability, in the formation and growth of vascular tissue [161, 162]. When using chitosan biopolymers, VEGFs and those of the vessels proper, as well as multipotent cells being
a part of a hydrogel, are safely protected by a chitosan polymer against temperature factors and the effect of hydrolyzing enzymes [126]. Thus, the diagnostics of separate markers in atherogenic inflammation analysis is confined by nature to form a strategy for controlling atherogenesis [38]. Current strategy in atherogenic inflammation study with a view to advanced technologies of making drug and medical products aimed at indirect and direct effective control of molecule-cell-tissue structures of vascular walls with early atheromatosis requires revealing fine reconstruction mechanisms. The analysis of molecular markers indicating atherogenic inflammation and considering anti-atherogenic direction of modern treatment technologies for chronic lower limb ischemia will enable to develop a control algorithm in the disease development and progression. Perivascular space should become one of the main application points for anti-atherogenic control mechanisms.

**Study Funding.** The study was supported by Russian Foundation for Basic Research, Krasnoyarsk Territory government, Krasnoyarsk Territory Foundation for Scientific and Applied Research Activity Support within the framework of a scientific project No.16-44-240506.

**Conflicts of Interest.** The authors have no conflicts of interest related to the present study.

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