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EXPRESSION AND FUNCTION OF INFLAMMATORY MARKERS ASSOCIATES WITH CARDIOVASCULAR PATHOLOGY

ABSTRACT THESIS

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Keywords: Cardiovascular disease, atherosclerosis, inflammation, inflammatory markers, cytokine, chemokines, diabetes, resistin, fractalkine, MCP-1, human vascular smooth muscle cells, monocytes, cell-cell cross talk, MAPK-kinase, inflammatory transcription factors, promoter.

Cardiovascular disease with metabolic dysfunction, is the main socio-medical problem in developed countries. The number of patients with cardiovascular diseases is increasing, which requires improved prognostic algorithm by linked molecular risk factors with disease status and elucidating cellular and molecular mechanisms associated with them, as well as identifying new therapeutic strategies. Traditional risk factors involved in cardiovascular pathologies are diabetes, hyperlipidemia, obesity, hypertension and recently inflammatory processes have also been associated.

Pathological process leading of cardiovascular disease is **atherosclerosis**. Atherosclerosis is a multifactorial disease whose pathogenesis is still not fully understood, but complex cellular inflammation and its processes play an important role in the progression of the lesion. Atherosclerosis involves complex interactions between inflammatory cells (neutrophils, lymphocytes, monocytes/macrophages) and vascular cells (endothelial cells, smooth muscle cells). Inflammation is an independent risk factor for atherosclerosis, but the mechanisms involved are still unknown.

Immuno-inflammatory mechanisms are directly involved in the initiation and progression of cardiovascular disease (CV) from early asimtomatic stage *vascular injury* to clinical manifestations of *dysfunction* and *vascular remodeling*. Because vascular injury is in part reversible identification of inflammatory phenotype is essential to suppress inflammatory processes and development new therapeutic strategies. Inflammatory process of atherosclerotic lesion involves the participation of *molecular effectors* (pattern recognition receptors, inflammatory bioactive molecules) and *cellular effectors* (monocytes/macrophages, lymphocytes, antigen presenting cells and dendritic cells). Moreover, mediators involved in modulating inflammatory processes may be ***markers of vascular inflammatory processes***, and potential therapeutic targets. Through advanced technologies of proteomics, metabolomics and genetics has been: i) identified putative biomarkers or risk factors; ii) developed therapeutic targets; iii) enriched previously developed effective therapies, so that some biomarkers are experimental standard, closely correlated with disease status. Highlighting specific molecular signaling mechanisms involved in the expression and action of these markers in vascular cells have not yet been elucidated.

Trying to provide new data contribute to the development the mechanisms associated inflammatory atherosclerosis, this paper aims to identify the molecular mechanisms involved in the modulation of the inflammatory response of vascular cells wall. Identification of inflammatory

molecular mechanisms is required for early screening of patients with cardiovascular disease and monitoring anti-inflammatory therapy by quantification of inflammatory biomarkers but also may use in the development of targeted therapies.

In the first part of the thesis "The current state of knowledge", are presented through four chapters various features of the inflammatory process associated cardiovascular pathology.

Chapter I describes inflammatory mechanisms associated atherogenesis and the role of innate immunity effectors and adaptive immune system in inflammatory process. Innate immune mechanisms are pivotal role in initiating atherosclerosis by activating "pattern-recognition receptors" (PRR), that promotes the inflammatory response. In response to the binding and activation PRR are released immune *effectors of innate immunity* as well as a *peptides* (CRP, complement system), *nitric oxide* (NO), *eicosanoides* (prostaglandins and leukotrienes) and *inflammatory molecules* (cytokines, chemokines). Also, multiple studies indicate the important role of ***antigen-specific*** response in atherosclerosis modulated by effectors of adaptive immunity. Thus, the adaptive immune system can influence atherogenic processes in three different pathways: i) *cell-cell interactions* between antigen-presenting cells, macrophages, lymph B or T and CD; ii) *cytokine release* by activated lymph T, that mediates activation of macrophages and other cells of the plaque; iii) *antibody secretion* by lymph B dependent / independent of lymph T.

Chapter-II presented the role of cytokines in vascular inflammation and signaling mechanisms induced by pro-inflammatory cytokines. Increased levels of inflammatory cytokines keep inflammatory status associated vascular dysfunction in diseases such as atherosclerosis, hypertension, abdominal aortic aneurysm and varicose veins. Biological effects of pro-inflammatory cytokines TNF-α, IL-1 and IL-6 is reflected in the inflammatory mechanisms associated with atherosclerosis.

Chapter III describes **chemokines** as mediators in vascular cell-leukocyte interaction.

Chemokines are chemotactic mediators for different subpopulations of leukocytes but also nonhematopoietic cells cells (CMN) in response to inducers stimuli. Chemokines and chemokine receptors comprise a complex system that attract specific leukocyte subsets in each step of the atherogenic process. Recruitment of leukocyte subsets site involves various inflammatory chemokines, involved in heterophilic interactions with proteoglycan. Recent studies have shown that circulating levels of chemokines may be associated with various inflammatory diseases, as well as **MCP-1** is independent prognostic factor in acute coronary syndrome. Vascular inflammation can be modulated by axis chemokine/receptor ***MCP-1***(CCL2)/***CCR2*** and strategies such as gene therapy (MCP-1 gene deletion) or pharmacological targeting reduces the inflammatory response thus, MCP-1 can be both **prognostic marker** and **therapeutic target**. CX3CL1/CX3CR1 axis may also be involved in the dialogue between vascular cells-leukocytes, immunohistochemical studies confirmed the presence of CX3L1/CX3CR1 axis in the coronary atherosclerotic plaque

(monocytes/macrophages, CE, CMN). Smooth muscle cells (CMN) of atherosclerotic plaque neointima express CX3CR1 which demonstrates that CMN migrate from the media to intima by CX3CL1-dependent gradient. CX3L1/CX3CR1 axis mediates anchoring of monocytes/macrophages to CMN in the plaque, which supports therapeutic targeting of this pro-inflammatory pathways in cardiovascular disease. In conclusion, the *Fk/CX3CR1 axis* can be considered the most promising target for therapeutic intervention to pro-inflammatory atherosclerosis process but intracellular signaling mechanisms must be fully understood to develop a new class of anti-inflammatory agents.

Chapter-IV presented diabetes as a major cardiovascular risk factor independently associated complications micro/macrovascular by accelerating atherosclerotic process. Both diabetes and atherosclerosis are multifactorial conditions that seem to have a common inflammatory basis because both diseases are released *acute phase inflammatory markers* (CRP, IL-6 sialic acid, amyloid A, cortisone) specific innate immunity. Both diabetes and atherosclerosis are multifactorial conditions that seem to have a common inflammatory basis because both diseases are released acute phase inflammatory markers (CRP, IL-6 sialic acid, amyloid A, cortisone) specific to innate immunity.

In the attempt to find cellular and molecular mechanisms of inflammation associated with atherosclerotic processes, and to develop a therapeutic strategy to reduce this process with an important role in initiating subsequent cardiovascular disease, this paper focused in second part II of the thesis **"original contributions"** on the following main objectives:

I. The role of high glucose concentration in smooth muscle cells in exacerbate inflammation and modulating effect of fibrates (PPARα activators) in the expression and function of chemokines (MCP-1 and fractalkine).

II. Cellular signaling pathways involved in smooth muscle cells activation by high glucose and regulates expression of chemokines (MCP-1 and fractalkine).

III. Cross talk between smooth muscle cells and monocytes via CX3CL1/CX3CR1 axis augments expression of pro-atherogenic molecules

IV. Signaling pathways involved in cross talk CMN-monocyte dependent by CX3CL1/CX3CR1axis.

V. The role of resistin in modulating smooth muscle cell phenotype; molecular mechanisms involved in the action of resistin on vascular smooth muscle cells.

VI. Characterization of functional promoter of human fractalkine.

In **Chapter I** included studies on the effects of high glucose concentration in modulating MCP-1, fractalkine expression in smooth muscle cells and functional role of these chemokines, monocyte adhesion and cells chemotaxis to CMN activated by high glucose. In addition, we investigated effect of PPAR-α activators (fenofibrate and clofibrate) in fractalkine and MCP-1

expression in human smooth muscle cells exposed to high glucose concentration but also signaling mechanisms involved.

Our results shown high glucose concentration increased fractalkine and MCP-1 expression in human smooth muscle cells dependent to the concentration of glucose. The results support previous studies that have identified fractalkine in the intima. Furthermore, activators of PPAR-α , **fenofibrate** and **clofibrat** also reduced gene/protein expression of MCP-1 and fractalkine in CMN activated by high glucose. High glucose effect is reflected by the increased number of monocytes adhered to CMN by functional chemokines MCP-1 and fractalkine. The adhesion of monocytes to CMN activated by high glucose is dependent by chemokines, inhibition of signaling with *pertussis toxin* reduces the number of monocytes adhered in continuous laminar flow conditions. In particular, the role of MCP-1 and fractlkine is underlined by experiments in which of these chemokine receptors were blocked (by incubation with specific antibodies), which led to a decrease in the number of monocytes adhered.

Signaling mechanism involved in MCP-1 and fractalkine overexpression induced by high glucose-induced promote phosphorylation ERK1/2, p38MAPK protein kinases and translocation NF-kB/AP-1 transcription factors dependent by reactive oxygen species (ROS) released. Inhibition of reactive oxygen species with PDTC and DPI reduced activation of ERK1 /2 and p38 MAPK, suggesting the involvement of oxidative stress in MAPK activation. Also, inhibit p38MAPK and ERK1/2 kinases with specific inhibitor (SB203580 or PD98095) reduces phosphorylation of ***IkBα*** and ***c-jun*** suggesting role p42/44MAPK and p38MAPK in activating NF-kB and AP-1 respectively. Furthermore, we studied the role of NF-Kb/AP-1 transcription factor in MCP-1 and fractalkine expression by transient transfection of CMN with oligodeoxinucleotide for p65 and c-Jun. Blocking binding sites of NF-kB and AP-1 reduces protein expression of MCP-1 and fractalkinei, suggesting the involvement of transcription factors in modulating both chemokines. Clofibratul and fenofibrate reduces phosphorylation/ activation of p38MAPK and ERK1/2 kinases, that is prevented translocation of transcription factors NF-kB and AP-1 redcuced MCP-1 and fractalkine release.

In conclusion, the studies in this chapter demonstrate that activation of vascular CMN induced overexpression of chemokines, induces increased adhesive interactions between monocytes and CMN,; study may explain one of the mechanisms induced accelerated atherosclerosis in diabetic conditions.

In **Chapter II** we investigated the role ***CX3CL1/CX3CR1*** axis in cross talk between smooth muscle cells and monocytes and involvement this pathway in excessive expression of proinflammatory molecules, based on previous studies providing expression of fractalkine and CX3CR1 receptor in the coronary atherosclerotic plaque. Thus we assumed that the interaction between CMN and monocytes can be achieved through *fractalkine-CX3CR1* which may influence

each cell type by modulating inflammatory molecules with important role in atherosclerotic plaque progression.

The results demonstrates the cross talk between CMN/monocytes or CMN/activated monocytes with LPS (lipopolysaccharide) increased expression of TNF-α, IL-1β IL-6, CX3CR1 and MMP-2, -9 in both cell types and additional activate of monocytes with LPS exacerbates TNF-α, CX3CR1 and MMP-9 expression. Modulation of TNF-α CX3CR1 and MMP-9 expression is dependent by *fractalkine-CX3CR1 axis* while the expression of IL-1β, IL-6 and MMP-2 is independent of this receptor-ligand pair. Cross talk CMN/monocyte-activating p38MAPK kinase which successive transduction NF-kB and AP-1 transcription factors which induced the production of pro-atherogenic molecules (cytokines and MMP). Communication between CMN-monocytes by binding fractalkine recepors is dependent by transduction ***AP-1*** transcription factor involved in regulating the expression of pro-atherogenic molecules TNF-α, MMP9 and CX3CR1.

The data of this study extend the known role of fractalkine and its receptor and suggest in atherosclerotic plaque smooth muscle cells interact direct with monocytes, amplify the inflammatory response by *fractalkine-CX3CR1 axis* induce progression of atheroma. Thus, ***CX3CL1-CX3CR1 pair*** may be a new therapeutic target to modulate inflammatory process associated with atherosclerosis.

In **Chapter III** we involved inflammatory effect of resistin in smooth muscle cells by modulating fractalkine and CX3CR1 expression dependent by TLR4 receptor.

The aim of this study was to investigate the role of resistin as a modulator of CMN phenotype and pro-inflammatory effects respectively. Since resistin and fractalkine are present in atherosclerotic lesions and CX3CR1 receptor is predominantly associated with CMN we hypothesized that resistin may be inducer of fractalkine (CX3CL1) expression and contribute both to CMN phenotype modulation (pro-inflammatory) and increased monocyte transmigration.

The results show that resistin may induce significant expression of CX3CL1 and CX3CR1 in vascular CMN, which demonstrates resistin inducing pro-inflammatory status and contribute to the development of atherosclerosis. Since resistin receptor in vascular cells is not yet known, experimental results of this study demonstrate that both anti-TLR4 and PTX inhibits CX3CL1 and CX3CR1 expression induced by resistin CMN vascular, suggesting that resistin may use both TLR4 receptors and Gi-proteins receptors in modulating these molecules.

In addition, the results of this study show that resistin activates p38MAPK and STAT3 but not ERK1/2. Pharmacological inhibitors of p38MAPK reduced significantly CX3CL1 and CX3CR1 expression in CMN activated with resistin. P38MAPK activation by resistin can induce translocation p65 and c-Jun subunits of NF-kB and AP-1 transcription factors. Moreover, *SP100030* inhibitor (dual inhibitor of NF-kB and AP-1) reduces fractalkine and CX3CR1, demonstrating the role of NF-kB and AP-1 in their regulation. Silencing p65 and c-Jun prior to resistin activation

reduces significantly expression of two molecules. Together these results indicate NF-kB and AP-1 are involved in resistin action to fractalkine and its receptor expression in the vascular CMN. The results demonstrate also that blocking JAK/ STAT pathway with specific inhibitor *WP-1066* or silencing STAT1/STAT3 with specific ODN, reduced CX3CL1 and CX3CR1 expression, demonstrates STAT transcription factors are involved in this process additionally to NF-kB and AP-1 action.

Chemotaxis experiments performed xCELLigence system demonstrates fractalkine induced by resistin is functional in transmigration of monocytes. Conditioned medium from CMN activated with resistin induced chemoatractant signal for monocytes. Addition of anti-CX3CL1 in conditioned medium reduces significantly the number of transmigrated monocytes. All these results show the chemotactic effect of soluble fractalkine released into the conditioning is dependent by CX3CR1 expression/interactions with monocytes.

In **Chapter IV** we designed experiments to reveal whether promoter activity and CX3CL1 expression is regulated by direct/indirect mechanisms of NF-kB, AP-1 and STAT1/STAT3 transcription factors.

In silico analysis of ***human CX3CL1 promoter*** indicate the existence of specific promoter elements *CCAC* and *TATA box*. Additionally, we identified binding sites relevant to vascular physiology and pathology including ***NF-kB***, ***AP-1***, ***GAS*** (STAT1/STAT3), ***C/EBP***, ***GATA*** and ***GAGA***. Analysis of deletion mutants of CX3CL1 revealed NF-kB, AP-1 and STAT1/STAT3 are not essential to the promoter activity in CMN but have role in modulating CX3CL1 expression in response to inflammatory stimuli. To investigate the function elements NF-kB, AP-1 and GAS we analyzed the activity of nuclear transcription factors by ChIP technique in CMN treated with resistin. The results indicated binding complex c-Jun, p65 and STAT1/STAT3 corresponding AP-1, NF-kB and GAS provided the many *non-canonical* interactions between proteins p65/NF-kB, c-Jun/AP-1 and STAT1/STAT3.

In conclusion, these results shows for the first time CX3CL1 human promoter characterization. The results demonstrate that complex interactions between NF-kB, AP-1 or STAT1/3 and associated pathways regulate directly/indirectly CX3CL1, mechanism may be responsible for CX3CL1 overexpression in human atherosclerotic plaque. These transcription factors are essential for many transducer cardiovascular risk factors and modulating regulators "up-stream" of the promoter CX3CL1 may represent an effective pharmacological strategy to reduce pro-inflammatory action of CX3CL1.

Together these results obtained in the "original contributions" underscores the importance of complex interactions of transcription factors, coactivators, and/or corepressors of fractalkine overexpression. Inhibition of "up-stream" kinase pathways (p38MAPK, JNK and JAK2) and suppression of transcription factors NF-kB, AP-1 and STAT1 / 3 by interference RNA technology

reduced fractalkine expression, depndent induced by resistin and high glucose. All these results show the importance of **NF-kB**, **AP-1** and **STAT1/3** signaling pathways in regulating expression of CX3CL1, which explains its presence in human atherosclerotic lesions dependent - resistin secreted by macrophages or hyperglycaemic conditions specific to diabetes.

Since this thesis is a predominantly fundamentally, goal of these studies is the application nature. The experiments can help elucidate the mechanisms of inflammatory atherosclerotic plaque, optimization of therapies to reduce inflammatory dynamic processes respectively.

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