ORIGINAL ARTICLE

Asclepius

Beta Tubulin in Extracellular Traps and Mitochondrial Dynamics in Autologous Cultures of Human Leukocytes Stimulated With Lipopolysaccharide

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ABSTRACT

Introduction: Extracellular traps (ETs) are structures of chromatin and intracellular proteins which are extruded in leukocytes under inflammatory conditions. Some protein components of the cytoskeleton have been described in the traps, but the presence of beta-tubulin has not been reported. Not all ETs are created equal; this depends on the source of stimulation. On the other hand, "mitochondrial dynamic" is the set of characteristics of shape, position, and size of mitochondria. These organelles are currently considered as regulating functions of innate and adaptive immunity and are determinant in the phenotypes adopted by immune cells in their responses. **Objectives:** The objectives of this study were to generate ETs in cultures of leukocytes challenged with lipopolysaccharide (LPS) and to carry out the beta-tubulin labeling and, on the other hand, to observe the morphological characteristics of the mitochondria in lymphocytes in the LPS assay. **Materials and Methods:** Autologous cultures from healthy human blood samples (n = 10) with ethical consent (Hospital Nacional de Clinicas, Facultad de Ciencias Medicas), anticoagulated with heparin, were stimulated with 25 ng/ml LPS, 30 min. Immunofluorescence technique performed with anti-beta tubulin antibodies, DNA staining with 4,6'-diamidino-2-phenylindole. Paired blood samples provided the controls. Samples of cell cultures were studied with transmission electron microscopy. **Results:** Beta-tubulin molecules were localized in the ETs. Alterations of mitochondrial morphology of lymphocytes were observed in the samples with LPS with an increase in size and complexity of the ridges with electrolyte images (*t*-test for paired samples, $P < 0.0001$). **Conclusions:** The expression of beta-tubulin contributes to the better understanding of the composition of the ETs generated by LPS and may have significance as a therapeutic target. Is this similar in ETs triggered by different stimuli? The ETs would affect the DM of the surrounding cells, influencing cellular function. Changes in the morphology of mitochondria lead to the improvement or deterioration of lymphocyte functions. Further experiments are necessary to elucidate this. The endotoxemia produced by the effects of endotoxins such as LPS in the blood circulation leads to inflammation in multiple organs.

Key words: Beta tubulin, extracellular traps, human leukocytes, lymphocytes, mitochondrial dynamics

INTRODUCTION

The endotoxemia produced by the effects of endotoxins such as lipopolysaccharide (LPS) in the blood circulation leads to inflammation in multiple organs. Extracellular traps (ETs) are structures of chromatin and intracellular proteins which are extruded in leukocytes under inflammatory conditions. The ETs are structures composed of chromatin, histones, and granular proteins and were first described in neutrophil polymorphonuclear leukocytes (PMNs) by Brinkmann *et*

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al.[1] calling them "neutrophil extracellular traps" (NETs). The NETs, in addition to their implication in mechanisms of defense against pathogenic microorganisms, have been implicated in tissue damage, thrombosis,[2] autoimmunity, and even in the immunoedition of cancer.[3] In patients with gout, the formation of NETs with sterile stimuli such as monosodium urate crystals has been observed,[4] and it has been described that the interaction between PMN and activated platelets can generate NET formation.[5] The formation of NETs in human PMNs has even been reported from interaction with *in vitro* herbicides.[6] It is currently known that other leukocytes in addition to PMNs are capable of generating ETs.[7,8] ETs constitute a new defense mechanism of the immune system in response to various microorganisms and other stimuli.[1] In stimulated cells, the development of these traps begins with the decondensation of the chromatin simultaneously with the loss of the nuclear structure. The nuclear membrane and the granule membrane disintegrate, resulting in the mixture of contents of the nuclear, cytoplasmic, and granular compartments, which is finally extruded into the extracellular space generating the ETs.[9] Cell motions and vesicle transport involve polymerization and depolymerization of microtubules.[10]

The importance of ETs in innate immunity has been well established, but the molecular mechanisms responsible for their formation still remain to be fully elucidated.[8] Only about 30 proteins have been described as part of the NETs despite being composed of cytoplasmic, granular, and nuclear components.[11,12] Some protein components of the cytoskeleton have been described in the traps, but the presence of beta-tubulin has not been reported. Not all ETs are created equal; this depends on the source of stimulation. In previous work in this laboratory, colocalization of B7 costimulatory molecules in NETs was observed in autologous cultures of total leukocytes from blood samples from healthy humans.[13] This has implications in the possibility of rupture of the immunological tolerance. The importance of this finding lies in the functions of neutrophils, the possibility of acquiring competence to be an APC (Antigen Presenting Cell), and playing a role in immunomodulation.

Few studies have evaluated the potential effects of NETs on APCs, and some observed pro-inflammatory and other effects, anti-inflammatory.[14] There are works that report to NETs as negative regulators of LPS-induced activation in dendritic cells derived from monocytes[14] and other authors propose instead as activators of macrophages.[15]

Mitochondria are currently considered as regulating functions of innate and adaptive immunity and are determinant in the phenotypes adopted by immune cells in their responses. Mitochondria are double-membrane organelles that provide the cell with metabolic functions that include the production of energy through oxidative phosphorylation. Mitochondrial morphologies vary widely from one cell type to another.[16] It is defined as "mitochondrial dynamics" (MD) to the set of characteristics of shape, position, and size of mitochondria.[17] It is considered to encompass three processes: (a) The remodeling of the mitochondrial reticulum by fusion/fission, closely linked to the cellular metabolic state; (b) subcellular mitochondrial motility, particularly relevant in polarized cells and what ensures the local supply of adenosine triphosphate (ATP), and (c) the remodeling of the mitochondrial ultrastructure and the condensation of its matrix.[18] Recent evidence indicates that such MD is important in both metabolic and immune functions in immune cells.[17] Mitochondria play a key role not only in the generation of ATP but also in the production of reactive oxygen species and the induction of apoptosis.[19]

The lymphocytes undergo metabolic changes during their activation, and they must adapt the energy and biosynthetic requirements to the demand for cell proliferation in the clonal expansion and for their effector functions.[20] Memory cells act more quickly and immune responses are stronger. In the case of CD4 T cells, modifications are described in MD in the memory cells with respect to the virgin cells. Mitochondria are more complex, elongated, branched, and more abundant in effector memory cells.[21] In CD8 T cells, it was reported that effector cells increased their mitochondrial mass with respect to virgin lymphocytes and this was proposed as a factor that influences the speed of secondary responses.[22] Activation of T cells involves mitochondrial biogenesis that leads to the growth and division of pre-existing mitochondria.[23]

The position of mitochondria controls the influx of calcium into the IS (Immunological Synapse) in T cells.[24,25] Activation of T cells requires mitochondrial translocation toward IS.[26]

It is known that the endotoxemia produced by the effects of endotoxins such as LPS in the blood circulation leads to inflammation in multiple organs. T lymphocytes respond innately to LPS with signaling through the toll-like receptor 4. LPS does not affect the proliferation or cytokine secretion of T lymphocytes but increases adherence and inhibits their migration.[27] It has been described in rat hepatocytes stimulated with LPS mitochondrial degeneration and extrusion of its content through the system of autophagolysosomes.[28] However, mitochondrial biogenesis leading to ischemic tolerance was reported in cultures of neurons with sublethal doses of LPS.[29] Studies have been conducted on alterations in MD associated with cellular responses to lesions in ischemic neurons. One of these responses includes mitochondrial biogenesis. Sublethal stimulus of LPS (1 μg/mL) can induce mitochondrial biogenesis. This was the first demonstration that preconditioning of LPS initiates multiple signaling pathways that lead to mitochondrial biogenesis in neurons and that these dynamic changes contribute to ischemic tolerance as neuroprotective effects of LPS.[29] Citrate of mitochondrial origin has been proposed with an important role in LPS signaling.[30] Thereby, for all the above, the study of MD is proposed here.

On the other hand, to provide new knowledge about the constitution of the ETs, we propose their ultrastructural analysis and the study of the expression of β-tubulin in ETs generated by human leukocytes in autologous cultures obtained from blood stimulated with LPS.

Objectives

The objectives of this study were to generate ETs in cultures of leukocytes challenged with LPS and to carry out the beta-tubulin labeling and, on the other hand, to observe the morphological characteristics of the mitochondria in lymphocytes in the LPS assay.

MATERIALS AND METHODS

Autologous cultures from samples of healthy human blood (n = 10) with ethical consent (Hospital Nacional de Clinicas [HNC] Facultad de Ciencias Medicas [FCM]), anticoagulated with heparin, were stimulated with 25 ng/ml LPS, 30 min. Immunofluorescence technique performed with cell signaling technology anti-beta tubulin antibodies (phycoerythrin [PE]), DNA staining with SIGMA 4,6'-diamidino-2-phenylindole (DAPI). Paired blood samples provided the controls. Cell cultures of the cultures were studied with transmission electron microscopy. DM analysis was performed using FIJI software.

Human blood samples

Human blood samples. Samples of human blood, anticoagulated with heparin, of healthy people (n = 10) with informed consent donated by the Blood Bank, Institute of Hematology and Hemotherapy (IHH) of the UNC, were used, in anonymity, with serology data. Ethical approved by the Ethics Committee of the HNC, FCM, UNC. RePIS 3381 y RePIS 3412.

Autologous cultures of total leukocytes

Autologous cultures of total leukocytes. Cultures of total leukocytes of human blood were made. The blood samples were subjected to the following tests at the IHH, UNC: Hudleson (Wiener), VDRL (Wiener), Chagas HAI (Wiener), Chagas EIE (BioMerieux), HBs EIE (BioMerieux), HBc (BioMerieux), HCV EIE (Murex), HIV Ac EIE (BioMerieux), HIV Ag EIE (BioMerieux), and HTLV EIE (Murex). Cells were cultured in suspension, in 24-well sterile culture dishes (some samples with sterile coverslips in the bottom) in stove gassed at 37°C in TC199 medium (with Earle and L-glutamine salts) (SIGMA, St. Louis, MO) added with serum from the same donor. The classic 0.5 Tripan Blue exclusion test were used for cell viability. All cell cultures were prepared under sterile conditions under hood equipped with ultraviolet light and laminar flow. Samples were taken at 30 min.

Stimulation with LPS

Stimulation with LPS Total leukocytes in autologous cultures were stimulated 30 min with LPS (from *Escherichia coli* Sigma-Aldrich) 25 ng/ml from time zero at 37°C.

Generation of ETs

Cells grown in medium with serum from the same donor were stimulated: a) with formylated peptides (fMLP) N-formyl (Sigma-Aldrich) and (b) with LPS (LPS from *E. coli,* SigmaAldrich) 25 ng/ml, to form ETs at 37°C in a gassed stove. Culture samples were taken at 30 min. The ETs were visualized with fluorescence microscopy using DAPI (Sigma, St. Louis, MO).

MD analysis with FIJI software, Nat Methods.; 9.(7). doi:10.1038/nmeth.2019.

Cytopreparations for electron microscopy

Cytopreparations for electron microscopy. Cell sediments were prepared with aliquots of samples of the cultures carried out. They were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer for 1 h and post-fixed in 1% OsO_4 in the same buffer, for 1 h. Then, the materials were dehydrated in ketones of increasing graduation and included in epoxy resin (Araldite) at 60°C, for 24–48 h. Subsequently, ultrafine cuts of 60–80 nm thickness (silver/gold interference color) were made, whiches were collected in copper grids of 250 bars per inch, contrasted with uranyl acetate and lead citrate, and studied with MET Zeiss LEO-906E

Immunofluorescence

The pelleted cultured cells were washed briefly in phosphate-buffered saline (PBS), fixed in paraformaldehyde 4% 10 min and washed 3 times in PBS. They were incubated with "blocking serum" 5% albumin in PBS to prevent nonspecific staining for 20 min. They were washed in PBS. Samples

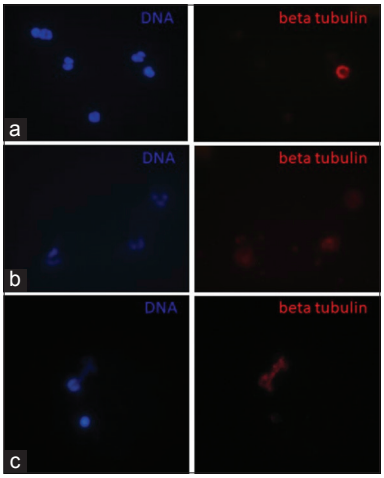

Figure 1: Representative images of immunofluorescence microscopy. 30 min of culture. Observe DNA (blue) and expression of beta-tubulin (red). (a) Control paired leukocyte culture samples without lipopolysaccharide (LPS) ×1000. (b) Total leukocyte autologous culture samples stimulated with 25 ng/ml LPS ×1000. (c) ET in total leukocyte autologous culture samples stimulated with 25 ng/ml LPS×1000. Antibodies: Primary antibody anti-beta tubulin (cell signaling technology), secondary anti-mouse antibody (m-IgG BP-PE, phycoerythrinconjugated; Santa Cruz Biotechnology). DNA staining with 4,6'-diamidino-2-phenylindole

were incubated with primary antibody anti-beta Tubulin (cell signaling technology) for 1 h at 37°C, then washed with PBS, and incubated with secondary anti-mouse antibody (m-IgG BP-PE, phycoerythrin-conjugated; Santa Cruz Biotechnology) at 4°C all night. Washed with PBS and make DNA staining with DAPI (Sigma, St. Louis, MO). In all experiments, the secondary antibody were only a negative control. It were mounted with mounting medium 90% glycerol in PBS. Observations were made in video microscope Axioscop 20, MC80, trinocular, Carl Zeiss. Paired blood samples provided the controls.

Statistical treatment of the data

Statistical treatment of the data The *t*-test of the Student for paired samples were used. The Infostat program of statistics were used for its analysis. Infostat software version 2010, developed by Grupo Infostat, FCA, UNC.

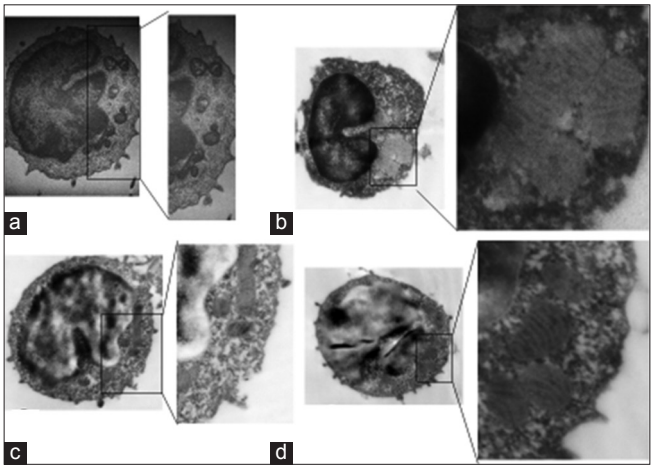

Figure 2: Representative microphotographs of transmission electron microscopy of leukocytes in autologous cultures of healthy humans. (a) Lymphocyte micrograph in total autologous leukocyte culture without stimulation (control paired sample), 30 min, ×6000. (b and d) Lymphocyte micrograph in the culture of total autologous leukocytes, stimulation with 25 ng/ml lipopolysaccharide, 30 min ×6000

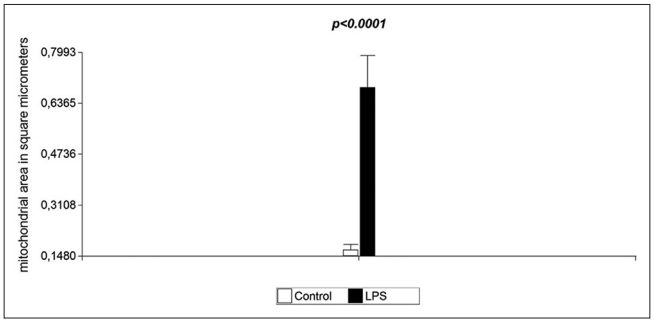

Figure 3: Graph of mitochondrial area in μm² of lymphocytes from total autologous cultures, 30 min, controls and stimulated with 25 ng/ml LPS. The mean mitochondrial area in control lymphocytes yielded a value of 0.15 μm², while in those stimulated with LPS, the mean was 0.69 μm², showing a significant difference $P < 0.0001$ according to Student's *t*-test for paired samples

RESULTS

In LPS assay, beta-tubulin molecules were observed with fluorescence microscopy in ETs at 30 min of culture. Total leukocyte autologous culture samples were stimulated with 25 ng/ml LPS [Figure 1].

Ultrastructural observations

Altered mitochondrial morphology was observed in test samples with LPS with an increase in size and complexity of the ridges with Electrolucid images in the lymphocytes (*t*-test for paired samples, $P < 0.0001$) [Figures 2 and 3].

The mean mitochondrial area in lymphocytes of paired control samples showed a significant difference $P < 0.0001$, compared with lipopolysaccharide's stimulated samples.

DISCUSSION

Since the ETs were described for the 1st time, research work has focused on their microbicidal properties and recently on their implication in diseases; however, the functional consequences in the interaction with immune cells have not been deepened.

The expression of beta-tubulin contributes to the better understanding of the composition of the ETs generated by LPS. MDs are of great importance in metabolic and immune functions, and its study in relation to ETs is considered of interest. The ETs would affect the MD of the surrounding cells, influencing cellular function.

The potential importance of the findings lies in the fact that ETs such as NETs are involved in the pathogenesis of various diseases, among which are infectious diseases, autoimmune diseases, and cancer, so the contribution to new knowledge about their composition and influence on different cell types can contribute to the potential therapeutic targets of them.

CONCLUSIONS

The expression of beta-tubulin contributes to the better understanding of the composition of the ETs generated by LPS and may have significance as a therapeutic target. Is this similar in ETs triggered by different stimuli? Do changes in the morphology of mitochondria lead to the improvement or deterioration of lymphocyte functions? Further experiments are necessary to elucidate this.

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