



Toxicity of Lps and Opa Exposure on Blood with Different Methods

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Introduction

BLOOD

Blood is considered a specialized form of connective tissue, given its origin in the bones and the presence of potential molecular fibers in the form of fibrinogen. Blood is a tissue composed of formed elements suspended in a liquid portion called plasma. Red blood cells (erythrocytes), white blood cells (leucocytes), and platelets are the three types of formed elements. Wright's stain a combination of methylene blue and eosin is commonly used to stain blood cells. Red blood cells, which contain no nucleus, are far more numerous than white blood cells, which have a nucleus (4.5 million / mm³ compared to 9 000 / mm³). It is composed of a liquid called blood plasma and blood cells suspended within the plasma. The blood cells present in blood are red blood cells (also called RBCs or erythrocytes), white blood cells (including both leukocytes and lymphocytes) and platelets (also called thrombocytes). Plasma is predominantly water containing dissolved proteins, salts and many other substances; and makes up about 55% of blood by volume. Mammals have red blood, which is bright red when oxygenated, due to hemoglobin. The most abundant cells in blood are red blood cells. These contain hemoglobin, an iron-containing protein, which facilitates transportation of oxygen by reversibly binding to this respiratory gas and greatly increasing its solubility in blood. White blood cells help to resist infections and parasites, and platelets are important in the clotting of blood. In mammals, mature red blood cells lack a nucleus and organelles. The red blood cells (together with endothelial vessel cells and other cells) are also marked by glycoproteins that define the different blood types. The proportion of blood occupied by red blood cells is much greater than that occupied by white blood cells. White blood cells are part of the immune system; they destroy and remove old or aberrant cells and cellular debris, as well as attack infectious agents (pathogens) and foreign substances. Platelets are responsible for blood clotting (coagulation).

There are certain membrane receptors for bacterial toxins in blood cells which act as binding sites .In plasma membrane of eukaryotic cells these are found to be Gangliosides which are sialic acid containing glycosphingolipids. Specific gangliosides have been

found to bind IL-2, while others are implicated as macrophage receptors of interferon. LPS can activate macrophages and cause diverse pathophysiologic effects. Studies have shown the ability of gangliosides to alter LPS induced macrophage function, including Glucose metabolism and Fc mediated phagocytosis. (Charles S. Berenson, Herbert C. Yohe, and John L. Ryan).

A red blood cell (rbc)

b white blood cell (lymphocyte)

c white blood cell (neutrophil)

d white blood cell (eosinophil)

e plasma (matrix)

LPS recognition by mammalian cells occurs through a multiprotein interaction. First, a plasma protein, LPS-binding protein (LBP), binds LPS and transfers LPS monomers to CD14. CD14 is a high-affinity receptor for LPS present both as a soluble form in blood or as a glycosphosphoinositol (GPI)-anchored protein on the surfaces of myeloid lineage cells. LPS signaling in cells can only occur if the transmembrane molecule TLR4 is activated. TLR4 belongs to the family of Toll-like receptors that are type 1 transmembrane proteins characterized by an extracellular domain containing multiple leucine-rich repeats, a single transmembrane domain, and an intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain. Stimulation of TLR4 by LPS activates a signaling cascade that is characterized by the production of proinflammatory cytokines and subsequent immune response. The importance of TLR4 in LPS-induced signaling is emphasized by the fact that TLR4def mice (C57BL/10ScCr), TLR4 knockout mice, and mice with a single point mutation in the TLR4 gene (C3H/HeJ) are resistant to the immunostimulatory and pathophysiologic effects of LPS (Kubes et.al, 2005). TLR4 is present in many different cell types, including dendritic cells, neutrophils, macrophages. It has also been demonstrated that ganglioside composition of LPS responsive C3H/HeN murine peritoneal macrophages is profoundly altered (Andonegui et.al, 2005).

LEUCOCYTE:

Leucocytes are also known as the White blood cells in the body. Human leukocytes, when incubated with bacterial endotoxins (lipopolysaccharides, LPS) are stimulated to generate a procoagulant-tissue factor activity (TFa) (Niemetz and Morrison, 1977). This

activity is associated with the lipid A region of the LPS molecule. LPS by treatment with mild alkali abrogated its capacity to stimulate TFA generation. In addition, such altered preparations of LPS partially inhibit the stimulatory effect of native LPS. Similarly, treatment of LPS (or lipid A) with the antibiotic polymyxin B substantially inhibited the stimulatory effect of LPS.

They are produced and derived from a multipotent cell in the bone marrow, called hematopoietic stem cell. They are called white blood cells because when whole blood is centrifuged, these cells separate into a thin layer that is typically white in color.

There are two types of leucocytes according to the presence of differently staining granules in their cytoplasm. The granulocytes (also known as polymorphonuclear leucocytes) are leucocytes with very distinctive cytoplasmic granules, e.g. neutrophils, basophils and eosinophils. The second is agranulocytes (mononuclear leucocytes), which are characterized by the lack of apparent granules, e.g. lymphocytes, monocytes and macrophages.

LPS can interact with endothelial cells and it induces leucocytes sequestration to some tissues which is entirely dependent on both CD14 and TLR4 but there are also CD14-independent, TLR4-dependent endothelial cell responses. (Kubes et.al, 2005). LPS could activate peripheral blood leucocytes especially monocytes and express CD14 and CD16 and regarded as pro-inflammatory because upon stimulation produce TNF- α and also IL-10. CD14(high) CD16(+) monocytes exhibited an increased phagocytic activity and a decreased antigen presentation in comparison with CD14(dim), CD16(+). As expected, lipopolysaccharide (LPS)-stimulated CD14(dim) CD16(+) monocytes produced TNF- α but little IL-10. By contrast, LPS-stimulated CD14(high) CD16(+) subpopulation produced significantly more IL-10 than CD14(dim) CD16(+) and CD14(high) CD16(-) MO. Studies suggested that human peripheral blood CD16(+) monocytes are heterogeneous in function and consist of two subpopulations: CD14(dim) CD16(+) pro-inflammatory and CD14(high) CD16(+) with anti-inflammatory potential. In Gram-negative infections, lipopolysaccharide, can initiate a cascade of inflammatory mediators that could lead to systemic inflammation. Neutrophils play a key role in tissue injury during systemic inflammation. Recently, Toll like receptor-4 (TLR4) was found to mediate the intracellular signaling after LPS binding to CD14. (Leeuwen et.al, 2005). In contrast, other serum factors and membrane receptors have been identified which bind LPS and neutralize its toxic activity. Lipoproteins, apolipoprotein A-1, apolipoprotein B, lactoferrin, bactericidal/permeability increasing protein (BPI),

soluble CD14 (sCD14) and receptors on macrophages (scavenger receptors, CD11/CD18 receptors) have all been implicated in the clearance and detoxification of LPS. (Leeuwen et.al, 2005).

High-density lipoprotein (HDL) plays an important role in protecting against atherosclerosis as well as in innate immunity. Several lines of evidence showed that HDL could ameliorate the toxic effects of endotoxin or lipopolysaccharide (LPS). HDL could inhibit LPS-induced leukocyte adhesion on endothelial cells in rats. .

The release of two mediators, Leukocyte Inhibiting Factor (LIF) and Tissue Factor (TF) from human mononuclear cells are described. These factors may play either beneficial effects (LIF) or noxious activities (TF liberation in certain pathological conditions). This LPS effect can be performed directly or via a lymphokine release, Platelet Slowing Factor (PSF). Interaction between LPS and platelets has also been suggested.

Leukocyte adhesion is triggered by upregulation of cell surface adhesion molecules and counteracted by the shear forces of the flowing blood. Since both factors, inflammatory response and flow dynamics are severely altered during endotoxemia, the effects of endotoxin on leukocyte-endothelial interactions under different levels of shear stress in vitro.

MACROPHAGES

Macrophages (In Greek means: "big eaters", (makros "large" + phagein "eat") are cells within the tissues that originate from specific white blood cells called monocytes. Monocytes and macrophages are phagocytes, acting in both non-specific defense (or innate immunity) as well as specific defence (or cell-mediated immunity) of vertebrate animals. Their role is to phagocytose (engulf and then digest) cellular debris and pathogens either as stationary or mobile cells, and to stimulate lymphocytes and other immune cells to respond to the pathogen. When a monocyte enters damaged tissue through the endothelium of a blood vessel (a process known as the leukocyte adhesion cascade), it undergoes a series of changes to become a macrophage. Monocytes are attracted to a damaged site by chemical substances through chemotaxis, triggered by a range of stimuli including damaged cells, pathogens, histamine released by mast cells and basophils, and cytokines released by macrophages already at the site. One important main role of macrophage is the removal of necrotic debris and dust in the lungs and is done by fixed macrophages, which will stay at strategic locations such as the lungs, liver, neural tissue, bone, spleen and connective tissue, where microbial invasion is likely to occur, ingesting foreign materials such as dust

and pathogens, calling upon wandering macrophages if needed. They rid the body of worn-out cells and other debris and act as scavengers and act as secretory cells. The phenomenon of LPS - induced in vitro macrophage cytotoxicity has been reported by a number of investigators but has often been difficult to reproduce and to quantitate. It has been examined the effect of LPS on the ability of macrophages to ingest ⁵¹Cr-labeled, opsonized sheep erythrocytes as a method for examining the direct toxic effects of LPS on macrophages in vitro. The assay was more sensitive than LPS-induced cytotoxicity, since inhibition of phagocytosis was detectable in cultures of LPS-sensitive macrophages even when cytotoxicity, assessed by trypan blue exclusion, was not. Thus, this assay represents an extremely sensitive method for analyzing the direct effects of LPS on macrophages.

Macrophages play a dual role in host defence. They act as the first line of defence by mounting an inflammatory response to antigen exposure and also act as antigen presenting cells and initiate the adaptive immune response. They are also the primary infiltrating cells at the site of inflammation. Inhibition of macrophage activation is one of the possible approaches towards modulating inflammation. Both conventional and alternative approaches are being studied in this regard. Ginger, an herbal product with broad anti inflammatory actions, is used as an alternative medicine in a number of inflammatory conditions like rheumatic disorders. The effect of ginger extract on macrophage activation in the presence of LPS stimulation is studied.

Lipopolysaccharide (LPS) stimulates immune responses by interacting with the membrane receptor CD14 to induce the generation of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6. The mechanism by which the LPS signal is transduced from the extra cellular environment to the nuclear compartment is not well defined. Recently, an increasing amount of evidence suggests that protein tyrosine kinases especially the Src-family kinases Hck, Fgr, and Lyn, play important roles in LPS signaling.

DNA DAMAGE

Cell death can occur by either of two distinct mechanisms, necrosis or programmed cell death (apoptosis). Necrosis is a pathological process which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis is a physiological and controlled process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis can be detected in populations of cells or in individual cells.

Apoptosis (Ptosis tosis dropping off, Greek) or "programmed cell death" in the tissues of an organism

is not associated with inflammation or scarring, unlike necrosis (meaning dead, Greek). Apoptosis is a normal event that occurs both during and after development. It is an important and inevitable event in the remodeling of tissues during development and aging. This phenomenon occurs in cells injured by certain levels of toxic agents. It is also a crucial process for eliminating cancer cells. In apoptosis series of biochemical events like nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation takes place leading to alteration of cell morphology. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented.

In a process referred to as karyorrhexis the nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA.

The importance of studying apoptosis in aging and age-related disorders has been recognized by many scientists. Apoptosis may be a feature of neurodegenerative diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis. In most postreplicating cells the rate of apoptosis increases with age and thus may be a factor in many age-related diseases including that of the heart and kidney. Apoptosis is also thought to be responsible for the depletion of subsets of T lymphocytes, which are crucial in fighting against infections.

Commonly used techniques for the estimation of apoptosis are as follows. Agarose gel electrophoresis is used to demonstrate the ladder pattern of DNA (a hallmark of apoptosis) which is generated by endonucleolytic cleavage of genomic DNA into nucleosomal size DNA of approximately 180 bases long (monomers) or oligonucleotides, which are multiples of 180 bases

(oligomers). The technique usually involves a DNA isolation procedure from millions of cells and obtained results cannot be quantified. Caspase-3 quantification is used for the estimation of apoptosis in cell lysates. Both of these assays require large numbers of apoptotic cells and thus are relatively insensitive for the detection of low levels of apoptotic events.

Many different methods have been devised to detect apoptosis such as The TUNEL (TdT-mediated dUTP Nick-End Labeling) analysis, ISEL (in situ end labeling), and DNA laddering analysis for the detection of fragmentation of DNA in populations of cells or in individual cells, Annexin-V analysis that measures alterations in plasma membranes, detection of apoptosis related proteins such p53 and Fas.

Morphological estimation for apoptosis is based on cell characteristics such as chromatin condensation, formation of apoptotic bodies from one cell (each

having a fragmented piece of nucleus surrounded by a viable cell membrane), shrinkage of cytoplasm, and blebbing of plasma membrane with an irregular outline. LPS can induce DNA damage by generating free radicals. Among the reactive molecular species which might injure nucleic acids, superoxides and NO might be the most important molecular species: the former is generated by such enzymes like xanthin oxidase (XO) and NADPH oxidase, and the latter by NOS. Hydrogen peroxide (H_2O_2) is also potent. Biosynthesis of NO as well as superoxides generation are expressed in defense-oriented white blood cells such as macrophage and polymorphonuclear cells, endothelial cells and other tissue cells. These cells are activated, or these enzymes are induced to produce free radical species most commonly in microbial infections. It has also been shown that activated macrophage-derived NO and its oxidative metabolite, peroxynitrite, play key roles in hepatocyte injury during inflammation, and cause subsequent DNA damage in surviving hepatocytes.

The excessive production of reactive oxygen species (ROS), associated with inflammation, leads to a condition of oxidative stress. Oxidative stress is a major contributing factor to the high mortality rates associated with several diseases such as endotoxic shock. This condition can be controlled to a certain degree by antioxidant therapies. Immune cells use ROS in order to support their functions and therefore need adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of ROS. The review discusses the toxic effects of endotoxin, paying attention to immune function.

Oxidative stress in cells is produced by oxygen derived species resulting from cellular metabolism and from interaction with cells of exogenous sources such as carcinogenic compounds, ionizing radiations, and redox-cycling drugs.

LPS can induce DNA damage by generating free radicals. Among the reactive molecular species which might injure nucleic acids, superoxide and NO might be the most important molecular species; the former is generated by such enzymes like xanthine oxidase (XO) and NADPH oxidase, and the latter by NOS. Hydrogen peroxide (H_2O_2) is also potent. Biosynthesis of NO as well as superoxide generation are expressed in defense-oriented white blood cells such as macrophage and polymorphonuclear cells, endothelial cells, and other tissue cells. These cells are activated, or these enzymes are induced to produce free radical species most commonly in microbial infections. It has also been shown that activated macrophage-derived NO and its oxidative metabolite, peroxynitrite, play key roles in hepatocyte injury during inflammation, and

cause subsequent DNA damage in surviving hepatocytes. (Watanabe et al, 2001).

The excessive production of reactive oxygen species (ROS), associated with inflammation, leads to a condition of oxidative stress. Oxidative stress is a major contributing factor to the high mortality rates associated with several diseases such as endotoxic shock. This condition can be controlled to a certain degree by antioxidant therapies. Immune cells use ROS in order to support their functions and therefore need adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of ROS. This review discusses the toxic effects of endotoxin, paying particular attention to immune function. It continues by analyzing the mechanism to which specific cells of the immune system recognize endotoxin, and the resulting pathways leading to nuclear factor- κ B activation and proinflammatory gene transcription. Focus was also given on the involvement of reactive oxygen and nitric oxide (NO) and the protective role of antioxidants. Therefore, DNA repair is regarded as one of the essential events in all life forms. There is an increasing awareness of the importance of oxidative DNA damage and its repair to human health. Thus, it becomes exceedingly important to understand, at the fundamental level, the mechanisms of oxidative DNA damage, and its processing by DNA repair enzymes as well as how unrepaired DNA lesions may lead to cytotoxicity, mutagenesis and eventually to diseases and aging. More detailed knowledge of mechanisms of DNA damage and repair might allow us to modulate DNA repair. This could lead to drug developments and clinical applications including the improvement of cancer therapy by inhibiting DNA repair in drug- or radiation-resistant tumors and/or the increase in the resistance of normal cells to DNA damage by over-expressing DNA repair genes. The role of oxidative DNA damage in chromosomal breakage in mammalian cells has been studied by focusing on genetic changes which modulate sensitivity to reactive oxygen species (ROS). These genetic changes may be inborn, as in cells from ataxia-telangiectasia (A-T) patients, or may be induced in normal cells by mutation at critical loci involved in prevention and/or repair of chromosomal damage by ROS. (Miral Dizdar). Lipopolysaccharide (LPS) causes direct pulmonary endothelial injury that can precipitate cell death. Collagen is found to be a survival factor against LPS-induced apoptosis in cultured sheep pulmonary artery endothelial cells (SPAEC) when these cells were grown in monolayer on plastic or collagen. The protective effect of collagen was not due to inactivation of LPS. It was concluded that LPS-induced apoptosis

occurs in SPAEC after genotoxic damage and this process is suppressed by the extra cellular matrix.

Lipopolysaccharide:

Lipopolysaccharide (LPS) is an endotoxin which induces a strong response from normal animal immune systems. LPS is a highly immunogenic molecule which stimulates the production of endogenous pyrogen interleukin-1 and tumor necrosis factor. Lipopolysaccharide (LPS) is a toxic component of cell walls in gram-negative bacteria and is widely present in the digestive tracts of humans and animals (Jacob et al., 1997). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase permeability of LPS from gastrointestinal tract into blood (Zhou et al., 2003). High levels of LPS have also been detected in women with bacterial vaginosis (Platz-Christensen et al., 1993). In human, Gram-negative bacterial infections are a recognized cause of fetal loss and preterm labor (Romero et al., 1988). Mimicking maternal infection by exposing the pregnant rodents to LPS at early gestational stages resulted in embryonic resorption and fetal death (Gendron et al., 1990; Ogando et al., 2003). Maternal LPS exposure at middle gestational stages caused fetal death and preterm delivery (Leazer et al., 2002). We and others found that maternal LPS exposure at late gestational stages led to fetal death, growth restriction, skeletal development retardation, and preterm labor and delivery (Buhimschi et al., 2003; Rivera et al., 1998; Xu et al., 2005, 2006a, 2007).

Relatively few studies have investigated LPS-induced teratogenicity. Several earlier studies found that maternal LPS exposure resulted in the development of malformed fetuses in rats (Ornoy and Altshuler, 1976) and golden hamsters (Collins et al., 1994; Lanning et al., 1983). Recent studies showed that subcutaneous injection of LPS led to fetal malformation including exencephaly and eye deformities (Carey et al., 2003; Chua et al., 2006). However, the exact mechanism of LPS-induced teratogenesis remains unclear.

LPS function has been used for experimental research for several years due to its role in activating many transcription factors. LPS acts as the prototypical endotoxin, because it binds the CD14/TLR4/MD2 receptor complex. LPS is known to induce endotoxin shock via the production of inflammatory modulators such as Tumour necrosis factor (TNF- α) and NO. Besides being an endotoxin, LPS also possesses a powerful adjuvant activity. Previously, it has been shown that changes in the chemical composition of the lipid A domain of LPS modulate its biological activity. For example, monophosphoryl lipid A (MPL) has been

shown to be a non-toxic immunostimulatory compound.

LPS induces dialation of blood vessels and this contributes to hypotension during septic shock via production of NO in the blood vessel wall which relaxes vascular smooth muscle cells (Deutz et al., 2003). LPS stimulates the secretion of pro-inflammatory cytokines such as IL-1 β . Proinflammatory cytokines such as interferon- γ , interleukin-1 β , and other cytokines are modulators of the inflammatory reactions and facilitate induction of the inducible isoform of NO synthase (iNOS), thus they could mediate excessive production of NO. Biosynthesis of NO as well as superoxide generation are expressed in defense-oriented white blood cells such as macrophage and polymorphonuclear cells, endothelial cells, and other tissue cells. These cells are activated by NOS induction, or these enzymes are induced to produce free radical species most commonly in microbial infections and especially when exposed to endotoxins like LPS. Besides NO it also induces the production of free radicals like hydroxyl, peroxynitrite radical. Peroxynitrite is much more reactive than NO or superoxide caused diverse chemical reactions in biological system including nitration of tyrosine residues of proteins, triggering of lipid peroxidation inactivation of aconitases inhibition of mitochondrial electron transport and oxidation of biological thiol compounds. Peroxynitrite also exerts a potent DNA cleaving activity. (Maeda and Akaike, 1998). These chemical reactions of peroxynitrite will lead to a profound biological consequence such as apoptosis, and even mutation of various cells. Once these reactive molecular species (or free radicals) are generated during the host's inflammatory responses against microbial pathogens or their products, they can induce damages to the host's DNA, and might become the cause of cancer. Agents able to neutralize the effects of LPS may be of clinical importance in therapeutics of septic shock and other inflammatory diseases.

Fig. - 2 Activation of signalling pathway via LPS

Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. CD14 is a 55-kD protein found as a glycosylphosphatidylinositol (GPI)-anchored protein on the surface of monocytes, macrophages, and polymorphonuclear leukocytes, and as a soluble protein in the blood. Both forms of CD14 participate in the serum-dependent responses of cells to bacterial lipopolysaccharide (LPS). While CD14 has been described as a receptor for complexes of LPS with LPS-binding protein (LBP), there has been no direct evidence showing whether a ternary complex of LPS, LBP, and CD14 is formed, or whether CD14 binds LPS directly. Alternatively, the LBP-LPS

complex can be recognized by a soluble version of CD14 binds LPS in the absence of LBP. Lipopolysaccharide (LPS)-binding protein (LBP) is a lipid transfer protein that catalyzes two distinct reactions: movement of bacterial LPS (endotoxin) from LPS micelles to soluble CD14 (sCD14) and movement of LPS from micelles to reconstituted high density lipoprotein (R-HDL) particles. Initially there is movement of LPS from LPS-sCD14 complexes to R-HDL particles. This action of LBP is catalytic, with one molecule of LBP enabling the movement of multiple LPS molecules into R-HDL. LBP-catalyzed movement of LPS from LPS-sCD14 complexes to R-HDL neutralizes the capacity of LPS to stimulate polymorphonuclear leukocytes. Findings show that LPS, complexes of LPS and rsCD14 formed in the absence of LBP or other serum proteins strongly stimulate integrin function on PMN and expression of E-selectin on endothelial cells, demonstrating that LBP is not necessary for CD14-dependent stimulation of cells. These results suggest that CD14 acts as a soluble and cell surface receptor for LPS, and that LBP may function primarily to accelerate the binding of LPS to CD14. It is likely that CD14 acts to present LPS to a distinct transmembrane receptor ie;TLR (Toll like receptor). (Wurfel et al, 1994).

OPA:

OPA is 2-oxo-1-pyrrolidine acetamide and is a cyclic derivative of GABA. It is one of the racetams. OPA was first synthesized in 1964 by scientists at the Belgian pharmaceutical company UCB led by Dr Corneliu E. Giurgea. The drug was the first of the so-called nootropics ("smart drugs" or "cognitive enhancers"), that is, substances which purportedly enhance mental performance. OPA is a dietary supplement which is claimed to enhance cognition and memory, slow down brain aging, increase blood flow and oxygen to the brain, aid stroke recovery, and improve Alzheimer's, Down syndrome, dementia, and dyslexia etc. OPA appears to increase communication between the two hemispheres of the brain, and increases activity of the corpus callosum. It stimulates the central nervous system without any toxicity or addictive properties. It facilitates long-term potentiation (Molnar et al., 1994), increases neurotransmitter release from presynaptic terminals and increases the amount of neurotransmitter, particularly acetylcholine, in the brain, but does not act directly on choline transport or metabolism (Nishizaki et al, 1998). Although many direct and indirect effects of OPA have been reported, its mechanism of action remains unclear. OPA has been used successfully to treat alcoholism and alcohol withdrawal syndrome in animals and man. OPA has improved recovery from

aphasia (speech impairment) after stroke, and restored various functions (use of limbs, speech, EEG, state of consciousness) in people suffering from acute and chronic cerebral ischemia (decreased brain blood flow). OPA has improved alertness, co-operation, socialization and IQ in elderly psychiatric patients suffering from 'mild diffuse cerebral impairment. OPA is useful in the treatment of children with sickle cell disease. Studies indicate that cognition-enhancing properties of OPA are usually more pronounced in aged than in young animals. Chronic treatment of young and aged rats with OPA (300 mg/kg once daily) significantly increased membrane fluidity in some brain regions of the aged animals, but had no measurable effect on membrane fluidity in the young rats. The same treatment significantly improved active avoidance learning in the aged rats only. Nootropic drugs increase glucose uptake into anaesthetized brain and into Alzheimer's diseased brain. It is hypothesized that the OPA sensitive cellular plasticity mechanisms may make a significant contribution to its nootropic action at the behavioral level. OPA improves mitochondrial dysfunction following oxidative stress. Studies on PC12 cells and dissociated brain cells of animals the effect of OPA on mitochondrial dysfunction following oxidative stress. OPA treatment at concentrations between 100 and 1000 mM improved mitochondrial membrane potential and ATP production of PC12 cells following oxidative stress induced by sodium nitroprusside and serum deprivation. OPA treatment (100-500 mg/kg once daily) of mice was also associated with improved mitochondrial function in dissociated brain cells. Significant improvement was mainly seen in aged animals and only less in young animals. Thus it has been proved that therapeutically relevant in vitro and in vivo concentrations of OPA are able to improve mitochondrial dysfunction associated with oxidative stress and/or aging. Mitochondrial stabilization and protection might be an important mechanism to explain many of OPA's beneficial effects in elderly patients. OPA is an effective antimyoclonic agent useful for treatment of action myoclonus in Lance-Adams syndrome, progressive myoclonus epilepsy, Angelman syndrome, and Rett syndrome where VPA (valproate) was found to be ineffective. Chronic myoclonus is often associated with severe functional disability and is frequently intractable to currently used antiepileptic drugs. OPA at high doses is effective mainly in cases of myoclonus of cortical origin. Levetiracetam is a new antiepileptic agent, closely related to OPA and has a broad spectrum of activity including an antimyoclonic effect. It is well known that Nootropics may increase learning and

memory in healthy individuals through a distinctive power to promote what has been termed hemispheric-super-connection. (S.J. Dimond et al , "Effects of nootropics" *Psychopharmacol.* 64, 1979 341-348).

In present study we explore the sensitivity of DNA after LPS exposure at different concentrations. Effect of OPA was also investigated which could contribute in therapeutics of inflammatory diseases.

COMET ASSAY

Several man-made chemicals find their way into the environment and pose health risk to human population. These chemicals have been found to interact with the vital tissue macromolecules regulating the cellular functions leading to long lasting health disorders. Acute and chronic exposure to several of these environmental chemicals such as pesticide, metals, polycyclic aromatic hydrocarbons (PAHs), solvents etc. have been shown to produce marked toxicity at the target sites. Some of these chemicals affect the DNA, which is the carrier of inherited information and any gross change in its structure potentates serious biological changes. Hence there is a need to test the chemicals for their genotoxic potential before being released into the environment. The conventional methods for evaluating genetic damage include chromosomal aberration, micronucleus assay, sister chromatid exchanges. However these are time consuming, resource intensive and require proliferating cell population. Hence newer and more sensitive test systems have now been introduced for assessing the genotoxicity of chemicals.

The single cell gel electrophoresis or comet assay is one such state-of-the-art technique for quantitating DNA damage and repair in vivo and in vitro in any eukaryotic cell and some prokaryotic cells. This technique is rapid, non-invasive, sensitive, visual and inexpensive as compared to the conventional techniques and is a powerful tool to study factors modifying mutagenicity and carcinogenicity. It has rapidly gained importance in the fields of genetic toxicology and human biomonitoring.

WHAT COMET ASSAY MEASURES:

Comet assay measures, double strand breaks (DSBs), single strand breaks (SSBs), alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-Drug crosslinking and DNA repair.

Comet assay, also known as single cell gel electrophoresis (SCGE) , is a microgel electrophoresis technique which detects DNA damage and repair at the level of individual cells. It is a quantitative assay in which procedure is easy and the sensitivity is high. The damage is represented by an increase of DNA

fragments that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet. The DNA fragments are generated by DNA double strand breaks, single strand breaks and/or strand breaks induced by alkali-labile sites in the alkaline version of the assay. The length and fragment content of the tail is directly proportional to the amount of DNA damage .

Comet assay can be conducted in *ex vivo* , *in vitro* and *in vivo* test systems and is increasingly being used in genotoxic testing of industrial chemicals, agrochemicals and pharmaceuticals. Comet assay is rapid (results in days), simple to perform, requires small amounts of test substance (25-50 mg) and can be performed in almost any eukaryotic cells (different animal organs). Comet assay serves as an important tool in the early drug development compounds as a mechanistic and genotoxic predictor

HISTORY:

The comet assay and microgel electrophoresis (MGE) were first introduced by Ostling and Johanson in 1984. This was a neutral assay in which the lysis and electrophoresis were done under neutral conditions. Staining was done with fluorescent dyes like SYBR green , PI, PtBr . In present studies PI was used to stain DNA .The image obtained looked like a "comet" with a distinct head, comprising of intact DNA and a tail, consisting of damaged or broken pieces of DNA hence the name "Comet" Assay was given. The extent of DNA liberated from the head of the comet was the function of the dose of irradiation. However, in this procedure, only double strand breaks could be analyzed.

The above neutral assay was modified by two groups, Singh and co-workers (1988) and Olive et al (1989). Singh et al used microgels, involving electrophoresis under highly alkaline conditions ($pH > 13$). This enabled the DNA supercoils to get relaxed and unwind, which are then pulled out during application of electric-current which made possible the detection of single strand breaks in DNA and alkali labile sites expressed as frank single strand breaks in individual cells. This method was developed to measure low levels of strand breaks with high sensitivity.

Olive and co-workers conducted the electrophoresis under neutral or mild alkaline ($pH = 12.3$) to detect single stranded breaks. This method was optimized to detect a subpopulation of cells with varying sensitivity to drug or radiation. The technique of Singh et al was found to be one or two orders of magnitude more sensitive than the other techniques.

Since then a number of advancements have greatly increased the flexibility and utility of this technique for detecting various forms of DNA damage (e.g., single-

and double-strand breaks, oxidative DNA base damage, and DNA-DNA/DNA-protein/DNA-Drug crosslinking) and DNA repair in virtually any eukaryotic cell.

APPLICATIONS:

This assay has critically important applications in fields of toxicology ranging from aging and clinical investigations to genetic toxicology and molecular epidemiology.

Major applications of the Comet assay are in the following areas:

Genetic toxicology (DNA damage)

In vivo & in vitro evaluation of genotoxic chemicals

DNA damage:

SSB's, DNA crosslinking, alkali labile sites

DNA repair:

Strand break repair

Excision repair

Eco-toxicology: the assay has been used to monitor soil and aquatic toxicology

Nutrition

Bio-monitoring genotoxicity

Environmental biomonitoring

Evaluation of genotoxic pollutants from hazardous waste sites

Hypoxia assessment

Human epidemiology

For assessing levels of DNA damage in occupationally, clinically and environmentally exposed individuals or in evaluating the differences in DNA repair competency among control and exposed individuals.

(a) Sperm bank

(b) Blood bank

(c) Monitoring Radio- and chemo- therapy in cancer patients

The assay can be performed on a variety of samples which can be obtained as a single cell population e.g. peripheral blood lymphocytes, nasal and buccal epithelium from clinically or occupationally exposed human population and for in vitro studies on cell lines e.g. CHO, V79, mouse lymphoma or cultured human lymphocytes and bone marrow cells. Both DNA damage and repair studies can be conducted. Also a variety of information related to genetic toxicology, human epidemiology, patients undergoing radio/chemo- therapy, ageing and nutrition can be obtained. The assay has been used for environmental biomonitoring and has utilized earthworms, fishes, mollusks exposed to polluted environments.

ADVANTAGES:

There are many advantages of the comet assay, some of which are:

it is a non-invasive technique

it requires counting of 50-100 cells per individual /

treatment group, through a computerised image analysis software gives a robust statistics

that virtually any eukaryotic cell population is amenable to analysis

its sensitivity (1 break in 1010 daltons) for detecting DNA damage and repair

results obtained in a few hours compared to conventional cytogenetics techniques which take a few days

single strand breaks (SSB's) and alkali labile lesions (capable of being transformed into SSB's under alkaline conditions) in the DNA of individual cells can be assessed

only few microlitres of blood (5-10[?]l), nasal & buccal mucosal cells, epithelial cells, male germ cells, fine needle biopsy, etc. required human studies.

Method

Materials: Anesthetic ether, Lipopolysaccharide, OPA, Agarose, Low Melting point Agarose, Phosphate buffered saline, Sodium Chloride, EDTA, DMSO, Triton-X-100, Sodium hydroxide, Tris Base, Propidium iodide, DMEM and FK12 media, Sodium bicarbonate, HEPES, Penicillin – streptomycin, Fetal bovine serum, Fetal calf serum, cover slip (No.1 24 X 60 mm) microcentrifuge tubes Micropipettor and Tips, microscope slides (conventional) / Micro gel electrophoresis(MGE) slides, Coplin Jar(opaque) Horizontal gel electrophoresis Apparatus Technologies ,Electrophoresis power supply, microscope slide tray.

Experimental animals:

Male rats of SD strain weight ranging from 100-140g were used. These were bred and maintained at The Division of Laboratory of animals, CDRI, Lucknow. Rats were kept at conventional conditions housed in plastic cages on sterilized bran bedding. They were fed with water and food which is mainly ground nut pellets. The temperature of animal house was maintained at 22-25°C.

Treatment of Lipopolysaccharide and OPA:

Experiments were performed in three systems ex vivo and in vitro with different concentration of LPS with and without OPA treatment.

EX VIVO:

It involves the use of tissues from exposed animals for study. Whole blood was used to perform the experiment.

* The rat is anaesthetized with ether and body cut open to expose the still beating heart. Then blood was collected from heart and transferred to 1% EDTA coated eppendorf tubes.

* The blood was mixed slowly with EDTA to prevent

clotting.

* Then 10 μ L of blood was taken give different LPS and LPS+OPA treatment.

* The stock was 100 μ g LPS and 10 μ g./ml 50 μ g/ml,100 μ g/ml treatment was given separately for 1 hour according to the purpose of our study.

* Then the comet assay was performed and the slides were stained with the propidium iodide

Preparation of leukocytes:

* For Hoechst staining and ROS generation leucocytes were isolated to prevent the interruption of RBCs from rat's blood.

* The blood is collected from heart and added sodium citrate with 9:1(blood: citrate).

* Then blood was centrifuged at 3000rpm at 20°C for 20 minutes.

* The WBC will be appearing as buffy coat and it is collected discarding the supernatant. To this is added 2mL of Histopaque (H-1077).Mix this properly and centrifuged at 800g for 20 minutes at 20°C.

* The creamy supernatant is collected. It is centrifuged at 2000g for 20 min at 20°C to pelletise.

* It is washed with Ca²⁺/Mg²⁺ free PBS. This is resuspended in 100 μ L PBS.

* Then treatment of LPS is given as 10 μ g/ml,50 μ g/ml,100 μ g/ml for 1 hour.

* The cells are counted using hemocytometer.

* The remaining cells are fixed with paraformaldehyde and kept at 4°C.

* This is then proceeded for Hoechst staining.

IN VITRO

Tissue Culture Media Composition:

DMEM: FK12 -1:1

DMEM – 5 gm

FK12 - 5.3 gm

NaHCO₃- 2.2 gm

HEPES - 10 mM stock

Penicillin-streptomycin - 10mL

FBS - 150 mL

FCS - 25 mL

Make this up to 1L in sterile autoclaved water.

Trypsin- EDTA – 0.1%

Cell cultures:

In vitro systems are being used to study the toxicities of compounds now a days. This method does not involve exposure of animals; instead cells and tissues are The cell line used in present study is C6 (rat glial cells). Cell lines were grown in 1:1 DMEM: FK12 media at 37°C and 5% CO₂ using standard cell culture methods. About 80-90% confluent flasks were taken for experiments. After confluency, the media is discarded and cells were washed with PBS and serum free media using scrapper. The cells are collected in eppendorf by centrifuging at 800g for 10 minutes. The

supernatant is thrown and pellet is resuspended in 50-100 μ L media according to requirement and cell number. Then 10 μ L or about 1X 10⁶ cells were taken in eppendorf to give treatment with LPS and LPS+OPA. Therefore In case of ex vivo, treatment is given to 10 μ L of blood, which is already treated with LPS. In case of in vitro also the method is same as in exvivo. To about 10 μ L of C6 cells to which LPS treatment was given, OPA is given. In all cases 10mM OPA is given from a stock of 100mM. Following experimental groups were taken:

* 1.Control - 10of blood (without any treatment)

* 2.OPA - 10 μ L of blood + 1 μ L of 100mM OPA

* LPS - 1 μ L of blood + 1 μ L for 10 μ g concentration from 100 μ g/ml stock of LPS.

* 10 μ L of blood + 5 μ L for 50 μ g concentration.

* 10 μ L of blood + 10 μ L for 100 μ g concentration.

* LPS + OPA -1 μ L of blood + 1 μ L of OPA (100mM)+different concentrations of LPS (10,50 AND 100 μ g/ml.)

COMET ASSAY

Procedure

0.8% Low Melting point Agarose

Lysis solution-: NaCl- 5M (Stock) , 2.5 M Working

Na₂EDTA – 1M (Stock), 100mM (Working)

DMSO – 10%

Triton X 100 -1%

Alkaline buffer (pH- 13) -: NaOH - 300mM

Na₂EDTA – 1mM.

Tris Base (pH-7.5) -: 0.4M

Propidium Iodide

Comet assay is used to determine DNA damage in cells. It is the 'single cell gel electrophoresis' (SCGE) to determine DNA damage at the level of individual cells. This is a quantitative assay in which procedure is easy and sensitivity is high. This method detects DNA samples treated in vivo/in vitro/ex vivo with toxicants. It was first reported by Ostling and Johanson in 1984.To detect DNA use fluorescent dyes like SYBR green, PI, EtBr. In present study PI was used to stain DNA. Tail is observed due to damage of DNA while in control intact DNA was observed. Following five categories of DNA are possible to get:

* The DNA remains intact without any of it migrated out of cells.

* With a little of DNA migrated out of the cell but the tail is shorter than diameter of the cell.

* The length of DNA tail is similar to the diameter of cell.

* With DNA tail longer than diameter of nucleus.

* With no intact DNA and all stained DNA are in the tail.

Results and Discussion

SINGLE CELL GEL ELECTROPHORESIS / COMET ASSAY:

IN VITRO

blood cells were treated with LPS at different concentration for 1 hrs. OPA treatment offered significant protection against LPS induced DNA damage at 10 and 50µgm however at higher conc. 100µgm no significant protection was observedg.

EX VIVO

Ex vivo LPS treatment to Blood cells at different concentrations (10,50,100 µg/mL) lead to significant DNA damage. Treatment of OPA offered significant protection in DNA damage although protection was less with high concentration of LPS. Long tail is obtained in case of 100µg/mL.

1 Control LPS - 10 µg L+OPA-10µg

2 Control LPS - 50 µg L+OPA-50µg

3 Control LPS -100 µg L+OPA-100µg

Discussion

In the present study DNA sensitivity in peripheral blood leucocytes after LPS exposure was assess. LPS is well known inflammation-causing agent and rapidly used to explore the different mechanisms involved in inflammatory diseases. LPS exposure significantly led to increased nitric oxide production and increased levels of proinflammatory cytokines. Increased levels of cytokines caused signaling and release of several factors from their reservoir. These factors include several apoptotic and cytotoxic factors, which could lead to apoptotic or necrotic death of cells. LPS induced cytotoxicity was also reported in murine macrophage that is mediated through aldose reductase (Ramana et al, 2007). In present study we investigate the effect of LPS and OPA on peripheral leukocytes. LPS exposure to peripheral leukocytes caused significant DNA damage as observed through comet assay. OPA treatment offered significant protection at 10 and 50µg concentration of LPS though at higher concentration (100 µg) the protection was partial.

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