FLOW CYTOMETRIC MEASUREMENT OF REACTIVE OXYGEN SPECIES PRODUCTION IN RED BLOOD CELLS FROM FISH AFTER EXPOSURE TO HEAVY METALS

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ABSTRACT. Organisms react to environmental pollutants by disturbance of living processes at subcellular levels resulting in cells death. To understand the mechanisms underlying the process of cell death by heavy metals action, we measured by flow cytometry the reactive oxygen species (ROS) generation by heavy metal (AI, Cd, Hg, Pb, Zn) in fish nucleated erythrocytes correlated with morphological changes by scattered light flow cytometry in the mode FSC/SSC. Optical and scanning electron microscopy were accompanied the research. The results we obtained show that heavy metals generates a significant amount of ROS that induce significant morphological changes characteristic for the phenomenon of cell death by apoptosis. The present results demonstrate that oxidative stress assessment and the use of fish nucleated erythrocytes "in vitro", can be a good tool for ecotoxicological evaluation of the presence of pollutants, heavy metals in particular.

Keywords: nucleated erythrocytes, fish heavy metals, apoptosis, reactive oxygen species, flow cytometry, ESEM, optic macroscopy, ecotoxicology

INTRODUCTION

In the last decade, eutrophication caused by global industrialization and anthropogenic impacts on ecosystem can lead to biological damage. The aquatic environment represents the largest sink for accumulation of xenobiotics and nucleated red blood cells of fish are directly exposed to pollutants. Despite their structural simplicity, the erythrocytes of lower vertebrates preserve nucleus and mitochondria, both the sensors of the PCD machinery. Recently, we found that PCD can be rapidly induced in Rana esculenta erythrocyte by calcium influx and staurosporine (Bratosin et al., 2004). As well as playing a central role in the physiology of respiration, these cells can represented an outstanding model to study xenobioticinduced damage to different cellular compartments. Little is known about the effect of environmental toxicants on apoptosis induction.

Reactive oxygen species (ROS), unstable reactive molecular species possessing an unpaired electron are produced continuously in cells as product of metabolism and they has focused attention on physiological and non-physiological mechanism for their generation. (De Zwart et al., 1999). Their elevated levels can oxidise various molecules leading to cell death and tissue injury (Hershko et al., 1998). Oxidative stress is considered in tissues one of important general toxicity mechanisms of many xenobiotics. Induction of oxidative stress after exposure to numerous xenobiotics was revealed experimentally by anthropogenic contaminants as persistent organic pollutants, heavy metals, by pesticides (Livingstone, 2001) and also by toxins produced during massive blooms of cyanobacteria (Ding et al., 1998, van der Oost et al., 2003; Blaha et al., 2004).

Heavy metal analysis demonstrated the presence of nickel, zinc, aluminum and manganese, as a clear demonstration of water quality deterioration. Copper, zinc and iron are trace essential metals for different physiological functions (various enzymes and other cellular proteins), even though their excess can lead to biological damage by excessive intracellular accumulation (Alessia et al., 2007). An initial common event in the action of all toxic metals seems to be the generation of oxidative stress that is characterized by: i) depletion of intracellular antioxidants (largely GSH) and free-radical scavengers (vitamins E and C), ii) inhibition of the activity of various enzymes that contribute to the metabolism and detoxification of reactive oxygen species (ROS), such as glutathione peroxidase (GPx), GSH-reductase, GSH-transferase, catalase (CAT) and superoxide dismutase (SOD), and iii) increased production of ROS (superoxide anion radical, hydrogen peroxide, peroxyl radical, hydroxyl radical, nitric oxide, peroxynitrite radical, etc). In this context, oxidative stress can contribute to the development of apoptosis.

Measurement of reactive oxygen species (ROS) is extremely difficult, because of the short lifetime of these species (De Zwart et al., 1999) and methods such as electron spin resonance and spin trapping are complicated and provide average values that can skew results when heterogeneous populations are being studied. Flow cytometry has been used to measure oxidative stress in various types of cells, including human normal and thalassaemic erythrocytes (Amer J. et al., 2003).

In the last time, the use of biological responses (biomarkers) as relevant test in assessment of the early detection of overall effects of contaminants, providing information on the sub-lethal level, is very commonly.

In the present study, we investigate the ROS generation obtained after heavy metals actions on fish nucleated erythrocytes by flow cytometric analysis correlated with analysis of morphological changes by scattered light flow cytometry in the mode FSC/SSC. Flow cytometric analysis is a powerful technique to rapidly and simultaneously analyze several parameters for a large numbers of cells. Optical and scanning electron microscopy were accompanied our research.

MATERIALS AND METHODS

Chemicals

Fluorescein-conjugated annexin-V and propidium iodide were from PharMingen (San Diego, CA, USA), the fluorogenic dye calcein acetoxymethyl ester (calcein-AM), was purchased from Sigma Aldrich (Saint Louis, Mo, USA).

Erythrocytes collection and cell treatments.

Erythrocytes from fish species Cyprinus carpio or Carassius auratus blood collected under ether anaesthesia on heparin were sedimented hv centrifugation (1000 g, 4°C, 5 min) and washed three times with Dulbecco's phosphate buffered saline solution pH 7.4 (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4 . In order to assess the toxic effects induced by heavy metals, 2x107 red blood cells were incubated in 1 ml of serum physiologically at 20° C for 3 h, in the plates with wells for cell culture in the absence (control incubation) and in the presence of heavy metals ranging from 0.5 mM and 0.0009 mM (ten serial dilutions).

Flow cytometric analysis

Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest Pro software for acquisition and analysis. The light-scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale, a minimum of 5 000 cells being analysed in each condition. Erythrocyte size and density were assessed using forward and side-angle scatters (FSC versus SSC).

Analysis of morphological changes by scattered light flow cytometry in the mode FSC/SSC

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents (Darzynkiewicz et al., 1997).

Flow cytometric measurement of reactive oxygen species

Intracellular level of reactive oxygen species was measured using an oxidation-sensitive fluorescent probe, 2',7'-dichlorofluorescein-diacetat (DCFH-DA) after the methods of Bass et al., 1983. In the presence of various intracellular reactive oxygen species, 2',7'dichlorofluorescein-diacetat (DCFH-DA) is oxidized to the highly fluorescent compound 2',7'dichlorofluorescein (DCF). The erythrocytes (5x105/ml PBS) were incubated for 1h at 37°C with 5 µM DCFH-DA dissolved in DMSO. The appearance of reactive oxygen species were measured by flow cytometry using an excitation and emission settings of 488 and 530 nm respectively compared to a positive control that had been incubated with 2mM H2O2 for stimulated RBCs production and consequently a positive fluorescence.

Optical and scanning electron microscopy

Direct light microscopic analysis was performed using an inverted microscope equipped with the Olympus BX 43 Olympus VC-30 and the room visualization software CellSens Dimension. ESEM analysis was performed using scanning electron microscope Fei Quanta 250. Fish erythrocytes were fixed for 1 hour with a working solution of 2.7% glutaraldehyde in 0.1M phosphate buffer pH 7.4 and stored at 40C until examination. Erythrocyte pellet was mounted on a Millipore filter nylon 0,45 mm, then the examination room is closed and the examination was performed at a temperature of -3 °C, relative humidity of 100% and a pressure of 910 Pa. Working with Spot 4:01 accelerating voltage of 15KV, using GSED detector (gaseous secondary electron detector).

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Examination time was between 10 to 30 minutes, order the examination in 1500 mag, 3000 mag, 6000 mag.

RESULTS AND DICUSSIONS

To understand the mechanisms underlying the process of cell death by heavy metals action, we measured by flow cytometry the reactive oxygen species (ROS) generation by heavy metal (Al, Cd, Hg, Pb, Zn) in fish nucleated erythrocytes correlated with morphological changes by scattered light flow cytometry in the mode FSC/SSC. Optical and scanning electron microscopy were accompanied the research.

Detection of altered morphology by light scattering flow cytometry

Multiparametric flow cytometric analysis which discriminates and quantifies viable, apoptotic and necrotic cells via measurement of forward and side light scatter (proportional to cell diameter and internal granularity, respectively) is a method very rapid and sensible. Figure 1A and 1B shows that morphological changes of nucleated erythrocytes were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of characteristic features of apoptosis.

Flow cytometric measurement of ROS production after aluminium injury

In order to test how heavy metals concentrations influences ROS generation under the action of different concentrations, normal nucleated erythrocytes incubated at 20°C for 3h (control normal sample) were compared with erythrocytes stimulated by 2mM H2O2, as positive sample.



Fig.1. Analysis by flow cytometry as a "density plot" FSC / SSC (size cell/cell content) of nucleated erythrocytes of Cyprinus carpio incubated for 3h with different serial dilutions of of Aluminum (1-7) to determine morphological changes.

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Fig. 2. Optical microscopic analysis (A) of Cyprinus carpio erythrocytes at the time of sampling (T0a and T0B) and Carassius auratus (T0c) and scanning electron microscopy (B) of Cyprinus carpio erythrocytes at the time of sampling (T0). The results shown are representative of experiments.



Fig. 3. Optical (A) microscopy analysis of Cyprinus carpio nucleated red blood cells incubated 3h with dilution 1 Al (Al3h1) and 5 (Al3h5) and scanning electron microscopy (B) of erythrocytes incubated with dilution 2 Al (Al3h2) to determine changes induced morphology. Results are representative of change observed

In order to test the possible ROS production under the action of different concentrations of aluminum (Fig.4) and Cd, Hg, Pb and Zn, nucleated erythrocytes incubated at 20° C for 6 h were compared with the control incubated in the absence of heavy metals (Fig.5).



Fig.4. Flow cytometric analysis in FL1 histogram system (green fluorescent 2', 7'-dichlorofluorescein) to determine the level of ROS induced in erythrocytes of fish (Carassius auratus) incubated with serial dilutions (1-7) of Aluminum, after 6h incubation. The results shown are representative of three experiments.

The mean values of fluorescence for red cells incubated with different concentrations of different heavy metals compared with controls presented in Fig.5 suggests a high capacity to generate a significant amount of ROS, directly proportional to the concentration of heavy metals and differently from a metal the other.



Fig.5. Comparative analysis of the green fluorescence mean values (MFI) conferred by 2 ', 7' dichlorofluorescein corresponding to the amount of ROS in erythrocytes of fish (Carassius auratus) incubated with various dilutions of heavy metals for 6h.

CONCLUSIONS

To emphasize the toxic effect of heavy metals (aluminum presented us an example), we examined morphological change and oxidative stress. A particular attention has been accorded to the evaluation and correlation of morphological changes analyzed by flow cytometry compared with optical and electronic microscopy.

The results reported in the present study indicate that the exposure of nucleated erythrocytes to heavy metals (aluminum) induces a mediated oxidative stressdependent apoptosis. Measurement of reactive oxygen species (ROS) by flow cytometry proves a sensitive method that succeeds in quantify this production inside the cell, with the advantage of an accurate determination, knowing that free radical species have a long life short. The changes of all the erythrocyte parameters investigated appear to be strongly correlated with increasing concentration of the heavy metals.

Our results indicate that the sensitivity of fish nucleated RBCs to toxicants was further increased and the information could be potentially useful for the development of low cost and rapid ecotoxicity assays. Nucleated RBCs are good cellular biosensors for the ecotoxicological assessment of complex mixtures of pollutants and "oxidative stress biomarkers" could potentially develop new applications for the water ecosystem health evaluation providing significant information of environmental stress. In conclusion, the use of fish nucleated red blood cells as a cellular biosensor could be an alternative system for the ecological monitoring of aquatic environment.



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