Exposure of Mouse Peritoneal Resident Macrophages to Pristine MWCNT Promote Nitric Oxide Synthase Production and Acute Cytokine Response

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Abstract

The potential biomedical applications of carbon nanotubes are greatly limited due to the growing evidences of their toxic effects on consumers. Recently, the cells of the monocyte-macrophage-system were observed to be the main structures of accumulation and toxicity of these nanomaterials. This study aimed to determine the changes in the inflammatory status and apoptosis suffered by macrophages following exposure to pristine multi-walled carbon nanotubes (MWCNT). Macrophages were isolated from the mouse peritoneum and cultivated *in vitro* following a protocol previously described by Zhang et al. (2008). The immunocytochemical expression of iNOs, IL-6 and apoptosis was measured by fluorometry using Confocal laser scanning microscopy (CLSM).

Exposure of peritoneal macrophages to MWCNT promotes iNOs production and IL-6 expression in a dose-related manner. The exposure to 10 and 25 µg/dl MWCNT does not induce apoptosis in macrophage culture, although a significant increase in numbers of cells undergoing apoptosis is observed for the group exposed at 50 µg/dl MWCNT. In the present research we demonstrated that exposure of mouse macrophages to pristine MWCNT promotes nitric oxide synthase production and an acute cytokine response characterized by the elevation of IL-6 levels. Apoptosis is increased only at high levels of MWCNT exposure, being in respect of our results, a late expression of toxicity.

Keywords: carbon nanotubes, inflammation, peritoneal macrophages

INTRODUCTION

Carbon nanotubes (CNT) are nanoscale cylindrical graphene sheets from the fullerene family being discovered by Iijima Japanese scientist in the early nineties. Since then, due to extraordinary properties, remarkable work was made in the implementation of these new generation materials in various fields, from basic material science to nanomedicine. This increasing production and the widespread application of these nanomaterials provides multiple sources of contamination for humans, thereby raising concern about the interaction between nanomaterials and living beings mainly about the risk that

these materials have on humans. This concern is maintained by the conflicting conclusions of the toxicological studies, results which are difficult to be grouped under a single, definitive conclusion. Thus, the toxicological results varies between studies from mesotheliomas (Poland, 2008) and acute lethality following airborne exposure (Chiu-Wing, 2004) to the lack of any changes in the body that could indicate toxicity (Kam 2005).

Nevertheless, one of the main ideas which are constantly validated in the pristine carbon nanotube toxicology reports are their accumulation in the cells of the monocyte-macrophage system (MM), mainly in fixed macrophages, the main

active-phagocytic cells of the system (Muller 2005, Deng 2007). We also observed that after systemic administration, CNT in their single-walled or multi-walled have a strong tropism for the macrophages, this nanoparticle biodistribution being intimately linked with the presence of phagocytic-active macrophages.

Macrophages are highly dynamic cells with a key role in both innate (mainly by the phagocytocactivity) and acquired defence processes. After birth, macrophage has beside the phagocytic function and initialization of the inflammatory process also a secretory role for the coagulation factors and complement component (Ringler 1997, Lewis 2006).

MATERIALS AND METHODS

Carbon Nanotubes; Short multi-wall carbon nanotubes (MWCNT) were purchased from Sigma-Aldrich (product code 636495), the standard carbonaceous material being produced by *Chemical vapor deposition* (CVD) method. The assessed purity of the material was >95%, the length range between 0.5 and 2 µm (short MWCNT), the Outside Diameter 20-30 nm and the wall thickness (carbon sheet thickness) ranging between 1 and 2 nm.

Suspension and dispersion of MWCNT

Since carbon nanotubes are highly hydrophobic and in biological environment tend to form micron-sized bulks that will largely interfere with the experiment results, for a proper assessment of their interaction at the nano-scale with living cells the carbonaceous material was suspended in cell cultured medium (in our case RPMI media) enriched with Dimethyl sulfoxide (DMSO) (the final concentration of the DMSO in cell medium was 0,5%) and repeatedly ultrasonicated, following the protocol previously described by Asakura et al. (2010).

Mouse Resident Peritoneal Macrophages Isolation and Culture

Adult pathogen-free male Swiss mice weighing between 28 and 42 g were kept in individual ventilated cages at 22-25 °C, 40-50 humidity, and 12:12 light/dark cycle. Animals were allowed ad libitum water and standard mouse chow.

Peritoneal macrophages (Mφ) were recovered from the mice peritoneal cavity following a protocol previously described by Kim et al. (1997) and Zhang et al. (2008), optimized for our laboratory conditions. Briefly, immediately following euthanasia and abdomen asepsis with 70 % isopropyl alcohol, 10 ml of ice cold, sterile, calcium and magnesium chloride free Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, product code **D8537**) was gently injected in the caudal-right quadrat of the abdominal cavity. After 20 seconds of softly massage of the abdomen, the medium was recovered and centrifuged at 1500 rpm for 5 min. The cell pellet was suspended in RPMI-1640 Medium (Sigma-Aldrich, product code R8758) and brought at an optimal number of 2×10^6 cells/mL. For an appropriate purity, the cells were assayed by immunocytochemistry for macrophage markers (F4/80, CD11b, CD11c, CD115, CD117, CD135, CD138, CD144, CD166, CD180, CD206, CD244, CD268, CD301, CD302, CD303, CD304, CD305, CD306, CD307, CD308, CD309, CD310, CD311, CD312, CD313, CD314, CD315, CD316, CD317, CD318, CD319, CD320, CD321, CD322, CD323, CD324, CD325, CD326, CD327, CD328, CD329, CD330, CD331, CD332, CD333, CD334, CD335, CD336, CD337, CD338, CD339, CD340, CD341, CD342, CD343, CD344, CD345, CD346, CD347, CD348, CD349, CD350, CD351, CD352, CD353, CD354, CD355, CD356, CD357, CD358, CD359, CD360, CD361, CD362, CD363, CD364, CD365, CD366, CD367, CD368, CD369, CD370, CD371, CD372, CD373, CD374, CD375, CD376, CD377, CD378, CD379, CD380, CD381, CD382, CD383, CD384, CD385, CD386, CD387, CD388, CD389, CD390, CD391, CD392, CD393, CD394, CD395, CD396, CD397, CD398, CD399, CD400, CD401, CD402, CD403, CD404, CD405, CD406, CD407, CD408, CD409, CD410, CD411, CD412, CD413, CD414, CD415, CD416, 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Olympus SP 350 and processed with Olympus stream basic soft.

Immunocytochemistry

For the assessment of the cell response to MWCNT exposure we used the immunohistochemical expression of inducible Nitric oxide synthase (iNOS) and IL6. The antibody source and dilution can be consulted in table 1.

Briefly, following exposure the media containing RPMI and MWCNT was repetitively washed with PBS and cells were fixed with ice-cold acetone for 7 minutes. Primary antibody incubation was conducted at 4°C overnight, and the secondary fluorescein isothiocyanate (FITC) conjugated antibodies for 30 min. at room temperature. In the end of the staining process, the cells nuclei were stained with blue pseudocolor (Draq5, Cell Signaling Technology, code 4084) and mounted with a water-compatible medium (Mowiol 4-88, Carl Roth, nr. 0713).

Confocal Scanning Laser Microscopy (CSLM) assessment of cell cultures

Immunofluorescent staining results were observed and quantified of cell fluorescence we used an Zeiss LSM-7 mounted on a AxioVision inverted microscope. For the detection of the fluorochromes we used the 495 nm laser line for FITC and 543 nm laser line for Draq5. For the entire length of the experiment the capacity of both Argon and HeNe laser was kept at 5% of their potential.

Quantification of iNOS and IL6 expressions

FITC signal measurement (the fluorescent label for iNOS and IL6) was carried by meta-analysis on the acquired LSM images using the point-by-point fluorescence quantification functions of the ZEN CLSM software provided by Zeiss.

At least different 20 microscopic fields of arbitrary chosen regions were obtained for each group using an 63x oil apochromatic objective. For constant quantification conditions, identical scan speed, output power of the lasers, master and digital gain were used for all scans.

Each microscopic field was scanned on the in x, y and z axis, resulting an scan volume of 182120 μm^3 /field. Based on the image quality and cell surface contained for each field was chosen a representative image which was further used for quantification.

Quantification of apoptotic cells

Caspase3 positive cells were counted in ten, randomly chosen, high power fields (obx63), being reported at the total number of nuclei presented in the analyzed.

Statistical analysis of results

Measurements were compared using ANOVA statistical system on the Origin 8.5 software. The level of statistical significance set at p value <0.05.

RESULTS AND DISCUSSIONS

Immunocytochemical expression of peritoneal macrophages for iNOS suffers significant changes following exposure to different doses of MWCNT, thus, a significant increase in the expression of iNOS are observed following exposure at concentrations of 10 and 25 $\mu\text{g}/\text{dl}$ MWCNT. But even if both values are significantly different from the control, these changes are not observed by comparing the two groups between them. The highest values of iNOS expression is observed in the group exposed to 50 $\mu\text{g}/\text{dl}$ MWCNT, were significantly different both the control group and the groups exposed to 10 and 25 $\mu\text{g}/\text{dl}$ MWCNT.

Immunocytochemical expression of peritoneal macrophages for IL6 undergoes significant changes following exposure to doses of MWCNT comparing with the control group. This change is observed for all the groups, but important changes in IL6 expression is observed especially for the cells exposed at 25 and 50 $\mu\text{g}/\text{dl}$ MWCNT. Interestingly, regarding the differences between the last two groups, no statistical relevant changes are observed between them.

Following the exposure to 10 and 25 $\mu\text{g}/\text{dl}$ MWCNT, the number of caspase3 positive cells suffers no significant changes comparing with the control group. For macrophage cultures exposed to 50 $\mu\text{g}/\text{dl}$ MWCNT there is a significant increase in cell apoptosis, this increment being observed by cooperation with control groups and also with those exposed at 10 and 25 $\mu\text{g}/\text{dl}$ MWCNT.

Studies carried out by He *et al.* (2011) indicates that the main source of CNT toxicity is the local increment of oxidative stress and afterward the induction of pro-fibrogenic cytokines (mainly IL-1 β , IL-6, IL-10 and MCP-1). This production of inflammatory cytokines occurs mainly in the local macrophages, resulting in a chronic inflammatory response and induction of fibroblast/myofibroblast proliferation. This is a critical step in CNT

Fig. 1. Mouse peritoneal macrophages exposed to 50 µg/dl MWCNT. Intracellular accumulations of CNT can be observed as brown-black material. Image A and B were acquired using an 40x objective, C and D using an 100x immersion objective. Scale bar represents 100 µm for images A and B, respectively 50 µm for images C and D. *Wright-Giemsa stain.*

Fig. 2. Immunocytochemical expression of mouse peritoneal macrophages for iNOs. Image a represents the control cells exposed to normal culture medium. Image B represents the cells exposed at 50 µg/dl MWCNT. Ob x63

toxicity, the local fibroblast/myofibroblast activation being based on the paracrine effect of the pro-fibrogenic cytokines. This activation of fibroblast and myofibroblast is the basis of the strong deposition of connective fibers (fibroplasia) observed in many nanotoxicologic studies carried out to elucidate the CNT (Lin 2012, Donaldson 2013).

For a better understanding of those exposed above, after exposure to nanomaterials, under important elevation in the cell oxidative stress (stages I or II on the scale proposed by Meng *et al.*) (2009), the macrophages will initiate

the expression of proinflammatory cytokines (TGF β , IL-1, TNF- α , etc.). These local molecules will activate the local response granulomatous response or fibroplasia.

The iNOs and IL6 expression is critical for the macrophage function also from another consideration. This cytokine is one of the most important stimuli for the transformation of macrophages from the M1 form ("inflammatory macrophage") into the M2 state ("reparatory macrophage") (Mantovani 2007), the last state of activity being importantly involved in local tumor progression (Mantovani 2002).

Fig. 3. Immunocytochemical expression of mouse peritoneal macrophages for IL-6. Image A represents the control cells exposed to normal culture medium. Image B, C and D represents the cells exposed at 10, 25 and 50 $\mu\text{g/dl}$ MWCNT. Ob x63

Fig. 4. Immunocytochemical expression of IL-6 –A, iNOs-B and median number of apoptotic cells -C

In nanomaterial toxicology the oxidative stress and inflammation are closely related and interrelated processes, being difficult to distinguish the initial and the most intensive process due to the vicious circle in which the: oxidative stress induces inflammation which, in turn produces oxidizing factors that maintain systemic and local (cellular) oxidative stress.

In addition to the mechanism of oxidative stress as an inductor of inflammation following CNT exposure, this inflammatory reaction can be due to direct influence of the internalized particles on cell organelles (Brown 2007). Incomplete capture of carbon nanotubes can lead to erratic coupling of lysosomes with early endosomes and in the outpouring of the lysosome enzymes into the cytosol or extracellular. This mode is one of most important associated phenomenon of incomplete phagocytosis (or “frustrated phagocytosis”) which appears secondary to incomplete internalization of long particle, this mechanism being well-characterized for particles such as asbestos (Cheng 2009, Ali-Boucetta 2013).

Partially, the inflammatory molecules expressed by macrophages consecutive to the exposure to CNT will action also as paracrine stimuli for other immune cells, as T and B cells or local Dendritic cells. This mechanism is the main way by which nanomaterials can boost the immune system response (Hussain 2012, Zhou 2014), this mechanism being speculated today more and more for the immunoprevention an immunotherapy in critical fields as oncology (Battigelli 2014).

CONCLUSION

In this study we demonstrated that exposure of mouse peritoneal macrophages to pristine MWCNT promote nitric oxide synthase production

and an cytokine response characterized by the elevation of IL-6 levels. Apoptosis is increased only at high levels of MWCNT exposure, being in respect of our results, a late expression of toxicity.

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