Evidence for a direct interaction between poly-dispersed single walled carbon nanotubes and murine erythrocytes resulting in *in vitro* and *in vivo* cytotoxicity.

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ABSTRACT

Single wall Carbon Nanotubes (SWCNTs) are hydrophobic and do not disperse in aqueous solvents. Acid functionalization of SWCNTs results in attachment of carboxy and sulfonate groups to carbon atoms and the resulting acid functionalized product (AF-SWCNTs) is negatively charged and disperses easily in water and buffers. In the present study, effect of AF-SWCNTs on blood erythrocytes was examined. Incubation of mouse erythrocytes with AF-SWCNTs and not with control SWCNTs, resulted in a dose and time dependent lysis of erythrocyte. Using fluorescence tagged AF-SWCNTs, binding of AF-SWCNTs with erythrocytes and reticulocytes could be demonstrated. A time and dose dependent increase in externalization of phosphatidylserine on erythrocyte membrane bilayer was also found. Administration of AF-SWCNTs through intravenous route resulted in a transient anemia as seen by a sharp decline in blood erythrocyte count accompanied with a significant drop in blood haemoglobin level. By using a recently developed technique of a two step *in vivo* biotinylation of erythrocytes that enables simultaneous enumeration of young (age <10 days) and old (age>40 days) erythrocytes in mouse blood, it was found that the *in vivo* toxic effect of AF-SWCNTs was more pronounced on older subpopulation of erythrocytes. Subpopulation of old erythrocytes fell after treatment with AF-SWCNTs but recovered by third day after the intravenous administration of AF-SWCNTs. Taken together our results indicate that treatment with AF-SWCNTs results in acute membrane damage and eventual lysis of erythrocytes. Intravenous administration of AF-SWCNTs resulted in a transient anemia in which older erythrocytes are preferably lysed.

Introduction

Nano-sized particles are finding increasing practical applications and commercial use. Consequently, the possibility of occupational exposure to nano-particles has increased. Furthermore, as the use of nanoparticles increases, they may diffuse and accumulate in environment increasing the risk of environmental exposure. Health effects of exposure to nanoparticles are now under investigation by many researchers [1], [2], [3]. Nanoparticles have a tendency to agglomerate into particles of larger size that may not have the same biological effects as non-agglomerated nanoparticles. For studying the biological effects therefore it is necessary to test non-agglomerated forms of nanoparticles. For Single walled Carbon Nanotubes (SWCNTs) disaggregation can be achieved by acid functionalization that introduces negative charge, without changing the essential structural features of SWCNTs [4], [5]. We have previously reported cytostatic and toxic effects of acid functionalized SWCNTs (AF-SWCNTs) on LA4 lung epithelial cell line [5].

Since AF-SWCNTs were toxic to some types of nucleated cells that are capable of self repair, we hypothesized that the AF-SWCNTs may have a more aggravated toxic effect on erythrocytes that have no nucleus and lack substantial cellular repair mechanisms. This proposition was examined in the present study

Materials & Methods

Experimental model.

Inbred Swiss and C57BL/6 female mice (6-12 weeks old, 20-25 g body weight) were used throughout this study. Animals were bred and maintained in the animal house facility at JNU, New Delhi or obtained from the National Institute of Nutrition, Hyderabad.

Cells and Reagents

For in vitro studies, erythrocytes derived from mouse blood were suspended in RPMI complete medium (RPMI-CM) Alexa fluor 488/633 hydrazide was obtained from Molecular Probes (Carlsbad, CA). Biotin-X-NHS Ester (BXN) was from Calbiochem (La Jolla, CA) and Streptavidin Allophycocyanin (SAv-APC) and Annexin-V PE were obtained from BD biosciences (San Diego, CA). Single-wall carbon nanotubes (SWCNT's) were purchased from Sigma (Catalogue #: 636797, amorphous carbon <3%).

Acid functionalization of particles

Acid functionalized SWCNTs (AF-SWCNTs) were produced by suspending 20mg of powder SWCNTs in 20ml of 1:1 concentrated HNO_3:H_2SO_4 in 100 ml high-pressure

vessels in a microwave digester as described previously [5]. Briefly microwave power was applied at 50% of 900 watt total and the pressure was controlled at 20±2 psi for 3 min resulting in an internal temperature of 138-150^{0}C. Suspensions were cooled, diluted five-fold with H_2O and dialyzed four times against 5 liter water over a two day period. Dialyzed suspensions were freeze dried, weighed and resuspended in 5 ml water. Particle size distribution and surface charge on AF-SWCNTs were as reported before [5]. The particle preparations were sonicated for 1 min in ice using a sonicator (Branson sonifier, VWR Scientific) prior to use.

Attachment of fluorescent probes to AF-SWCNT

For covalent attachment of fluorescent probes to the nanotube surface, -COOH groups on AF-SWCNTs were exploited. AF-SWCNTs were treated with 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and N-hydroxy succinimide (NHS) in order to get a succinimidyl intermediate and were then incubated with Alexa Fluor 488/633 hydrazide (Molecular Probes, Carlsbad, Ca) in dark with continuous mixing, followed by dialysis to remove free dye [6]. Attachment of fluorescence tag to AF-SWCNTs was confirmed flow cytometry.

Particle Exposure

Erythrocytes were suspended in RPMI 1640 supplemented with 10% Fetal bovine serum and incubated with control and AF-SWCNT's at different concentrations over rotisserie (17 RPM) at 37ºC for different time intervals. For *in vivo* studies, mice were given 100 µg AF-SWCNTs or saline control through intravenous route and blood samples were taken at several time points thereafter. Blood erythrocyte count was determined by automated hematology analyser (Melet Schloesing Laboratories MS4e, Osny, France).

In vivo biotin labelling

I*n vivo* biotinylation of circulating erythrocytes was done as described previously [7], [8]. All blood erythrocytes were biotinylated by three daily intravenous injections of 1 mg of biotin-X-NHS Ester (BXN) dissolved in 20µl of dimethylformamide (DMF) and 250 µl of phosphate buffered saline (PBS). Thirty days later, a second lower dose of B-X-NHS (0.6 mg) labelled all erythrocytes generated over thirty days with low intensity biotin. Blood erythrocytes derived from these mice 10 days after the second biotinylation step were used to enumerate old (biotin^{high} population, age>40 days), intermediate (biotin^{low}, age 10 to 40 days), and young (biotin^{negative}, age <10 days) populations of erythrocytes. Biotin high, low and negative erythrocyte populations could be identified and enumerated by staining with streptavidin-APC followed by flow cytometry as described before [7].

Flow cytometry

To enumerate erythrocytes with high, low or no biotinylation, 1x10^6 erythrocytes were stained with streptavidin allophycocyanin (APC) as described before [7]. For PS externalisation studies, erythrocytes were stained with annexin V-PE, by the procedure recommended by the manufacturer (BD Biosciences). Attachment or internalisation of AF-SWCNTs was confirmed by incubating erythrocytes with alexa fluor 488/633 tagged AF-SWCNTs, as described previously [6]. Erythrocytes were incubated with fluorescenated AF-SWCNTs for 1h and unbound/loosely bound particles were removed by three extensive washes with PBS, after that suspensions were analysed by Stained erythrocytes were immediately analysed on FACS Calibre flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cell Quest software for acquisition and analysis. A minimum of 10,000 events were recorded for each sample.

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis by two-way ANOVA and student's t-test were done using Sigma plot and Sigma stat software. Data are presented as means ± SEM.

Results

Effect of control and acid functionalized SWCNTs on the recovery of murine erythrocytes in culture

The effect of control and acid-functionalized SWCNTs was examined on murine erythrocytes. For this purpose, blood erythrocytes derived from female Swiss or C57BL/6 mice were cultured with control and acid-functionalized SWCNTs. To facilitate the interactions between carbon nanotubes and erythrocytes, the culture tubes were gently rotated (17 RPM) throughout the culture duration. Erythrocyte recoveries at different time points are shown in Figure 1(panel B). Treatment with control SWCNTs had no significant effect on the recovery of erythrocytes. A dose and time dependent decline (70 to 90%) in erythrocyte recovery was however seen in cultures treated with AF-SWCNTs . At 50 µg/ml concentration, a significant decline in erythrocyte recovery was seen even at the earliest 4 h time point. At the 24 h time point, erythrocyte recovery in AF-SWCNT treated cultures fell by 80-90%.

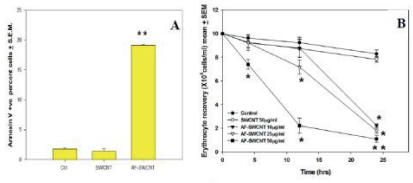

Figure 1. Effect of Control and AF-SWCNT's on erythrocytes *in vitro*. Erythrocytes from C57BL/6 mice were incubated with control and acid functionalized SWCNTs in RPMI+10% FBS at 37°C. Panel A shows PS externalization on erythrocytes (50µg/ml) and Panel B shows erythrocyte counts after different doses and time intervals of incubation with particles.

AF-SWCNT induced membrane effects in erythrocytes

Phosphatidylserine (PS) externalisation on cell membrane is an early marker of apoptosis and cell death . Results in Figure 1(panel A) show a dose and time dependent increase in externalization of PS on erythrocytes incubated with AF-SWNCTs whereas no similar effect was seen upon incubation with control SWCNT preparation. Two hours after incubation with AF-SWCNTs, about 19% of the erythrocytes had externalized PS whereas in control erythrocytes as well as the erythrocytes treated with control SWCNTs, less than 2% cells expressed PS.

Uptake of AF-SWCNTs by erythrocytes and reticulocytes

For examining the interactions between AF-SWCNTs and erythrocytes, AF-SWCNT preparations tagged with fluorescence probe were used. Erythrocytes and reticulocytes freshly derived from female C57BL/6 mice were incubated with fluorescence tagged AF-SWCNT particles at 50µg/ml *in vitro* for 1 h and analysed on flow cytometer after washing the preparations. Results in Figure 2 (panel A) show that 28.6% reticulocytes and 18.40% of the erythrocytes (panel A) were positive for uptake of tagged AF-SWCNTs. These results suggest that AF-SWCNTs could associate with erythrocytes and reticulocytes in a relatively stronger manner.

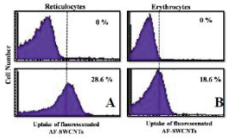

Figure 2. Uptake of fluorescenated AF-SWCNTs by erythrocytes and reticulocytes *in vitro*. Blood was drawn from C57Bl/6 mice and $10x10^6$ cells were incubated with fluorescenated AF-SWCNTs (50µg/ml) for 1h and were further analysed by flow cytometry for uptake by reticulocytes (panel A) and erythrocytes (panel B).

In vivo effect of control and acid functionalized SWCNTs on blood erythrocytes

In order to determine if AF-SWCNTs exerted toxic effect on erythrocytes *in vivo* too, mice were administered control or acid functionalized SWCNTs through intravenous route and the erythrocyte count in blood was monitored. Results in figure 3 show that treatment with AF-SWCNTs resulted in a transient anemia in mice. A second dose of AF-SWCNTs given 24 h after the first dose resulted in a more sustained anemia that continued the anemia upto 72 h time point as well as decline in haemoglobin content (Figure 3, panel B) Administration of two doses of control SWCNTs however still had no significant effect on the blood erythrocyte count.

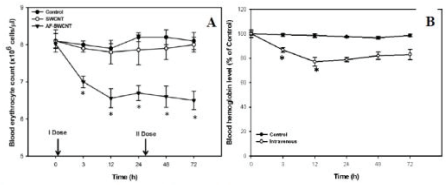

Figure 3. Effect of AF-SWCNT's on blood count of erythrocytes in mice. AF-SWCNT's were administered to 6-8 weeks old Swiss mice with control and AF-SWCNT's (100µg) and a booster dose of 50µg intravenously. Panel A shows, erythrocyte count at different time intervals and effect on hemoglobin content (Panel B).

Age dependence of susceptibility of blood erythrocytes to AF-SWCNTs.

Results so far indicate that the intravenous administration of AF-SWCNTs caused a marked and transient anemia in mice. It was of interest to determine if the toxic effect of AF-SWCNTs was generalize or was selective for erythrocytes of specific age groups. To determine the susceptibility of erythrocytes of different age groups, we used a technique that we recently developed to enumerate circulating erythrocyte cohorts of different age groups [7]. In this technique that involves a two step *in vivo* biotinylation of circulating erythrocytes, erythrocytes of different age groups can be identified as a biotin negative population (young erythrocytes, age <10 days), a biotin high population (Old erythrocytes, age>40 days) and a biotin low population (erythrocytes of age between 10 to 40 days). Results in Figure 4 show that just 3 h after a single dose of AF-SWCNTs, the proportion of old erythrocyte (age>40 days) in blood fell from 7.3% to 5.8%, whereas the proportion of young erythrocytes (age<10days) increased from 36.19% to 38.95%. No significant change occurred in the erythrocytes of intermediate age group (age 10-40 days). Time kinetics of changes in the proportion of old and young erythrocytes is shown in Figure 8B. These results suggest that the older erythrocytes in blood circulation may be most susceptible to the toxic effect of AF-SWCNTs.

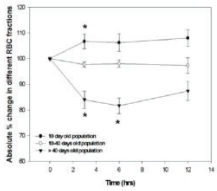

Figure 4. Effect of AF-SWCNTs on the proportions of erythrocyte cohorts of different age groups in mouse blood. Mice were prepared by Double In vivo Biotinylation technique, thereafter AF-SWCNTs (100 µg) were administered i.v. and proportions of erythrocyte cohorts of different ages in blood circulation were monitored by flow cytometry.

Discussion

Our first set of experiments indicated that the AF-SWCNTs had a marked toxic effect on erythrocytes *in vitro*. The effect was dose and time dependent and after one day of incubation with AF-SWCNT, 70-90% of the erythrocytes were lost at all the doses (10, 25 and 50µg/ml) of AF-SWCNT. It was also interesting to observe that the damaging effect is seen earlier with the highest dose of

50µg/ml leading to 70-90% decline in recovery after just 10-12 h while interaction ensures the similar damaging effect on erythrocytes treated with lower doses also (10 and 25µg/ml) when kept for a longer duration (24 h). Interestingly control SWCNTs had no significant effect on survival of erythrocytes. The reason for this lack of effect of control SWCNTs could be that the control SWCNTs preparations are highly agglomerated and do not interact effectively with cells. These results corroborate our previous findings on the effects of control and acid functionalized SWCNTs on LA4 lung epithelial cells lines where too the AF-SWCNTs induced a marked cellular damage but control SWCNTs lacks a significant cytopathic effect [5].

Using fluorescence tagged AF-SWCNTs, we could demonstrate a significant binding of AF-SWCNTs with erythrocytes and reticulocytes. Flow cytometric analysis indicated that almost 28% of reticulocytes were bound with nanotubes. Since an active uptake of AF-SWCNTs by erythrocytes appear unlikely, the observed association of AF-SWCNTs could reflect loose binding of AF-SWCNTs with erythrocyte membrane or a stronger binding perhaps reflecting embedding of nanotubes in erythrocyte membrane or even some passive entry of the nanotubes into the cells. Our results showing that 18% of the erythrocytes retained the association with fluorescence tagged AF-SWCNTs even after three rigorous washings suggest that AF-SWCNTs may interact effectively with erythrocytes.

PS externalization seen in response to treatment with AF-SWCNTs could be a direct consequence of interactions of the AF-SWCNTs with erythrocytes

Results of *in vitro* studies established the toxic effect of AF-SWCNTs on erythrocytes, which was further confirmed by *in vivo* studies. Intravenous administration of AF-SWCNTs induced transient anemia in mice. A second dose of AF-SWCNTs given intravenously extended the duration of anemia. There could be several possible mechanisms of induction of anemia in response to AF-SWCNTs. A direct cytotoxic effect of nanotubes on erythrocytes is suggested by our *in vitro* experiments. It was important to understand whether the cytotoxic effect of nanotubes was exerted uniformly on all erythrocytes in circulation or it was related to the age of erythrocytes in circulation. This question could be addressed by help of a technique involving two steps of *in vivo* biotinylation of circulating erythrocytes that has recently been developed in our laboratory. By using this technique it became clear that the oldest subpopulation of erythrocytes (age > 40 days) was specifically sensitive to nanotubes. In conclusion, we have shown an acute cytotoxic effect of polydispersed SWCNTs *in vitro* as well as *in vivo*. Rapid induction of anemia within a day is followed by a recovery of blood count of erythrocytes. Effect of AF-SWCNTs on the erythropoietic activity in spleen and bone marrow will further clarify if the fall in blood count of erythrocytes is accompanied with changes in erythropoietic activity. These studies using fluorescence tagged AF-SWCNTs are currently under way in our laboratory.

References

1. Lam CW, James JT, McCluskey R, Hunter RL (2004) Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol Sci 77:126-134.

2. Lam CW, James JT, McCluskey R, Arepalli S, Hunter RL (2006) A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Crit Rev Toxicol 36:189-217.

3. Stoker E, Purser F, Kwon S (2008) Alternative Estimation of Human Exposure of Single-Walled Carbon Nanotubes Using Three-Dimensional Tissue-Engineered Human Lung. Int J of Toxicol. 27:441-448

4. Wang Y, Iqbal Z, Mitra S (2006) Rapidly functionalized, water-dispersed carbon nanotubes at high concentration. J Am Chem Soc 128:95–99.

5. Saxena RK, Williams W, McGee JK, Daniels MJ, Boykin E, Gilmour MI (2007) Enhanced in vitro and in vivo toxicity of poly-dispersed acid-functionalized singlewall carbon nanotubes. Nanotoxicology 1:291–300.

6. Zuxun Xu, PingAn Hu (2008) Biological functionalization and fluorescent imaging of carbon nanotubes. Applied Surface Science 254:1915-1918.

7. Khandelwal S, Saxena RK (2006) Assessment of survival of aging erythrocyte in circulation and attendant changes in size and CD147 expression by a novel two step biotinylation method. Exp Gerontol 41:855–861.

8. Khandelwal S, van Rooijen N, Saxena RK (2007) Reduced expression of CD47 during murine red blood cell (RBC) senescence and its clearance from the circulation. Transfusion 47:1725-732.