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In vitro studies of multiwalled carbon nanotube/ultrahigh molecular weight polyethylene nanocomposites with osteoblast-like MG63 cells

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Abstract

Carbon nanotubes are highly versatile materials; new applications using them are continuously being developed. Special attention is being dedicated to the possible use of multiwalled carbon nanotubes in biomaterials contacting with bone. However, carbon nanotubes are also controversial in regards to effects exerted on living organisms. Carbon nanotubes can be used to improve the tribological properties of polymer/composite materials. Ultrahigh molecular weight polyethylene (UHMWPE) is a polymer widely used in orthopedic applications that imply wear and particle generation. We describe here the response of human osteoblast-like MG63 cells after 6 days of culture in contact with artificially generated particles from both UHMWPE polymer and multiwalled carbon nanotubes (MWCNT)/UHMWPE nanocomposites. This novel composite has superior wear behavior, having thus the potential to reduce the number of revision hip arthroplasty surgeries required by wear failure of acetabular cups and diminish particle-induced osteolysis. The results of an *in vitro* study of viability and proliferation and interleukin-6 (IL-6) production suggest good cytocompatibility, similar to that of conventional UHMWPE (WST-1 assay results are reported as percentage of control ± SD: UHMWPE = 96.19 ± 7.92, MWCNT/UHMWPE = 97.92 ± 8.29%; total protein: control = 139.73 ± 10.78, UHMWPE = 137.07 ± 6.17, MWCNT/UHMWPE = 163.29 ± 11.81 µg/mL; IL-6: control = 90.93 ± 10.30, UHMWPE = 92.52 ± 11.02, MWCNT/UHMWPE = 108.99 ± 9.90 pg/mL). Standard cell culture conditions were considered as control. These results, especially the absence of significant elevation in the osteolysis inductor IL-6 values, reinforce the potential of this superior wear-resistant composite for future orthopedic applications, when compared to traditional UHMWPE.

Key words: Nanocomposites; Carbon nanotubes; Wear particle; Osteoblast; Orthopedic; MG63 cells

Introduction

Carbon nanotubes (CNTs) have unique physical, chemical, thermal, and optical properties that encourage their use in the electronics, computer, aerospace, and biomedical applications. For instance, their semi-conductive property makes CNTs useful for electrical stimulation (1). Cherukuri et al. (2) and Bianco et al. (3), among many others, have also suggested utilizing CNTs as translocators in image-based diagnostic procedures or in drug-delivery systems. Liu et al. (4) have described the high efficiency of single-walled CNTs (SWCNTs) for targeting tumors, thus anticipating the possibility of further therapeutic applications. Some CNTs can also be used to optimize the mechanical properties of polymers by tailoring and building anisotropic nanocomposites (5,6). One possible area of application for CNT composites is their use in artificial joints.

One of the major factors leading to failure of total hip

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replacements is osteolysis induced by particles formed from ultrahigh molecular weight polyethylene (UHMWPE) when the replacement joint is subjected to wear, and by *in vivo* oxidation and aging phenomena. Concerns about retarding aging and oxidation of UHMWPE have led to the use of technological solutions, such as formulating the material with vitamin E and other antioxidants, and combining heating and irradiation to produce highly crossed-linked polyethylene (HXLPE). However, these strategies also cause some degradation of mechanical properties, leading to wear, fracture, and loss of fatigue resistance (7). HXLPE may be suitable for some of the orthopedic uses of conventional UHMWPE, but not necessarily for all of them; thus, there is a need to study other solutions. Tribological studies of the mechanical properties of multiwalled carbon nanotube (MWCNT)/UHMWPE composites suggest that they are a promising alternative to HXLPE or UHMWPE. Kanagaraj et al. (8) have developed a novel material for acetabular cups using an MWCNT/UHMWPE composite that has favorable tribological characteristics. The reinforcement of the UHMWPE with MWCNT improves its mechanical and tribological characteristics for biomedical application, such as in the acetabular cups used for total hip replacements. Though the resulting MWCNT/UHMWPE material could reduce the number of revision surgeries required by wear failure of acetabular cups, the biological response to this novel composite is, as yet, unknown. Thus, we have attempted to study the interaction of particles derived from compressed sheets of UHMWPE polymer and MWCNT/UHMWPE composites with cultured cells.

Only a few studies are available on composites using CNTs as reinforcement. In addition, there is controversy regarding the toxicity of CNTs: *in vitro* and *in vivo* studies present apparently contradictory results (9-32). After culturing the osteoblasts and fibroblasts with polysulfone and MWCNT/polysulfone, Chlopek et al. (9) reported that the cytocompatibility of the MWCNT/polysulfone composite is similar to that of the polysulfone alone. These investigators examined the viability of cells in the presence of MWCNTs and described a slight increase of collagen I production but no induction of interleukin-6 (IL-6, a pro-inflammatory marker) or free radicals. Meng et al. (10) reported improved fibroblast growth and collagen synthesis on a nanofibrous scaffold made of MWCNT/polyurethane compared to that of controls and polyurethane alone. Sitharaman et al. (11) confirmed that the *in vivo* tissue response of ultra-short SWCNT/poly(propylene fumarate) and poly(propylene fumarate diacrylate) composite scaffolds was similar to that of the polymers alone.

The various production methods and different catalysts involved in the synthesis of CNTs can explain some of the wide variability of CNT properties. From the results of previous studies, the length of the CNTs seems to be an important determinant of the biological response (11,26-30). If the relation between bioactivity and polymer/composite wear particles were clearly understood, an alternative engineering solution could be found to the development of bio-nanocomposites that would prolong the life of implants. In the present study, we investigated the response of human osteoblast-like MG63 cells after 6 days of culture in contact with particles artificially generated from both UHMWPE and MWCNT/UHMWPE nanocomposites.

Material and Methods

Materials

Medical grade UHMWPE was supplied by Ticona, Inc. (Germany); the trade name of the material used in this study was GUR® 1020. The manufacturer specifications of UHMWPE powder were: density = 0.930 g/m^3, mean particle size 140 µm, and average molecular weight = 3.5 million g/mol. The MWCNTs were purchased from Shenzhen Nanotech Port Co., Ltd. (China). The manufacturer specifications of CNTs were: diameter range = 60-100 nm, length of the tubes = 5-15 µm, and purity >95%.

The chemically treated CNTs (8) were mixed with UHMWPE using a ball milling technique to produce the homogeneous mixture that was used as the raw material in a compression molding machine. The compressed sheets of both UHMWPE polymer and MWCNT/UHMWPE nanocomposites (0.02 wt% of MWCNT) were prepared under optimized testing conditions of pressure, temperature, and time, as determined from our previous studies (8,33). The optimized milling time for UHMWPE and CNT powder mixture was 45 min and the best cooling medium for compressed sheets was water compared to air and liquid nitrogen (33). The wear particles from the polymer and the nanocomposites were generated by the surface-texturing technique on a roughened stainless steel plate under water. After draining the water from the system, the particles were collected and oven-dried, and then sterilized by γ rays at a dose of 100 kGy. The sterilized particles were used as the raw material in this study.

Micrographs of the generated wear particles show that those from both polymer and composite are in the form of fibrils and have an irregular shape (Figures 1, 2A,B). The size of the particles was comparable to the phagocyte range for biological response studies, although most were above the “critical size”, associated with secretion of cytokines inductive of osteolysis, as described by Green et al. (34).

Measurement of tribological properties. During a patient’s day-to-day activity, the femoral head and acetabular cup will have a long sliding contact; therefore, we decided to study the tribological properties of the materials at different sliding distances. The load applied to the sample was calculated on the basis of the maximum Hertzian contact pressure developed at the contact between the femoral head and acetabular cups. The tribological characterization of UHMWPE and MWCNT/UHMWPE nanocomposites was done at room temperature and under dry conditions

with an alumina ball on the sample plate at a normal load of 12 N for the sliding distance of 43.2, 86.4, and 230.4 m using a reciprocating sliding test rig (Model TE 67/R, Plint and Partners, UK). The wear volume was calculated from the profilometry of the wear scar obtained from the experimental study.

Cell culture

The control group comprised MG63 osteoblast-like cells that were seeded in the wells of 12-well plates at a density of 7600 cells/well and cultured in the presence of growth medium. The growth medium was prepared with alpha minimum essential medium (Cambrex, USA), 2 mM L-glutamine (Cambrex), 10% fetal bovine serum (Gibco, USA), 0.5% gentamicin (Cambrex), and 1% amphotericin B (Gibco). The sterilized UHMWPE and MWCNT/UHMWPE composite particles were suspended in the growth medium at a concentration of 500 µg/mL, and stirred for 1 min to ensure a homogeneous suspension of particles immediately prior to pipetting. The UHMWPE was designated as the polymer group, and MWCNT/UHMWPE as the composite group. The medium (control group) and suspensions (polymer and composite groups) were renewed every 48 h and the cultures were observed daily, both macroscopically and microscopically. The cells were maintained for 6 days in a 5% CO_2 humid environment at 37°C. All experiments were carried out three times and each experiment was done at least in triplicate.

Viability and proliferation assay. After 6 days, the culture supernatant was removed and frozen for IL-6 determination. Then, 900 µL medium and 100 µL WST-1 reagent (Quick Cell Proliferation Assay Kit, BioVision, USA) were added to each well and the cells were incubated for 2 h under standard culture conditions. The supernatants were then removed, centrifuged, transferred to 96-well plates, and read at 450 and 655 nm in an enzyme-linked immunosorbent assay (ELISA) autoanalyzer (CODA, Bio-Rad Laboratories Ltda., Portugal). Data are reported as percentage relative to medium control. The cells were washed three times with PBS and frozen, and later harvested using a cell scraper. The cell lysates were obtained using 0.1% Triton X-100 (Sigma, USA) in deionized water and by brief sonication in an ultrasonic bath. Total protein content was measured using

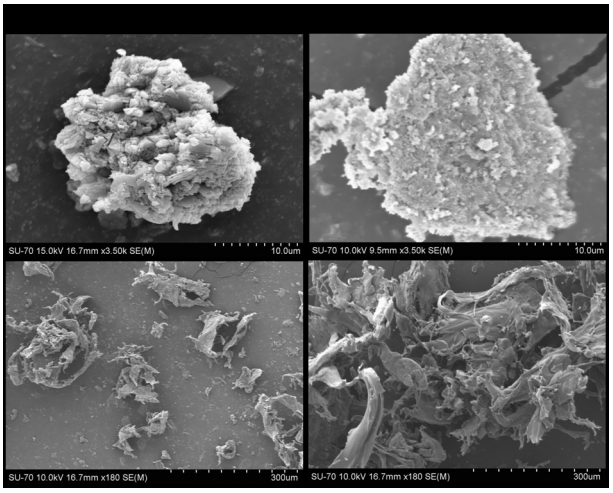

Figure 1. Polymer and composite microparticles. Scanning electron microscopy images of the microparticles of irregular shape (top) and fibrillar structure (bottom) generated from UHMWPE polymer (left panels) and from MWCNT/UHMWPE nanocomposites (right panels). UHMWPE = ultrahigh molecular weight polyethylene; MWCNT = multiwalled carbon nanotubes.

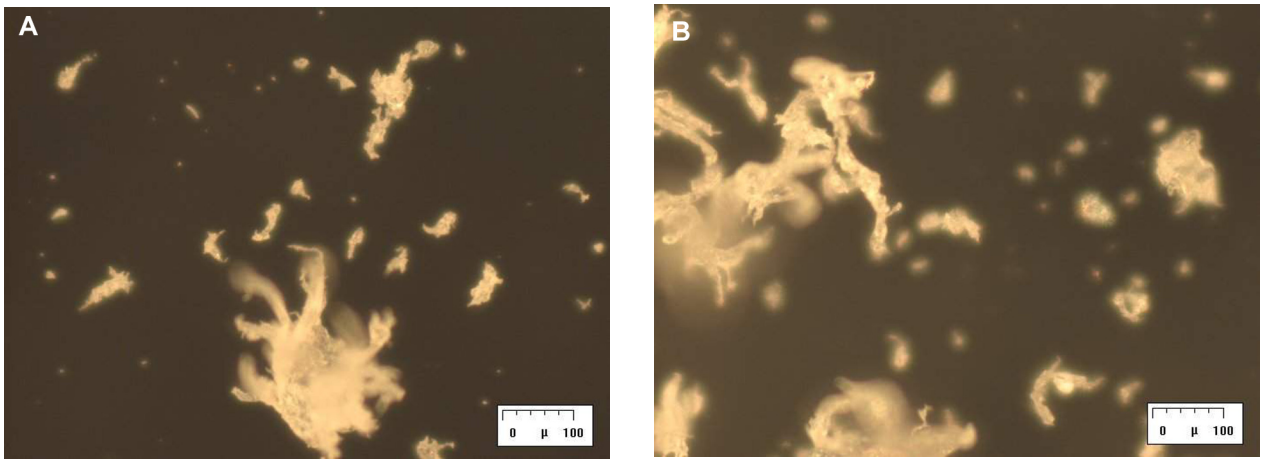

Figure 2. Polymer and composite microparticles. Optical micrographs of the microparticles generated from UHMWPE polymer (A) and from MWCNT/UHMWPE nanocomposites (B), evidencing irregular shapes, with fibrillar structure, and variable sizes. UHMWPE = ultrahigh molecular weight polyethylene; MWCNT = multiwalled carbon nanotubes.

the BCA method (Calbiochem, UK). All spectrophotometric measurements were done using an ELISA autoanalyzer reader (CODA, Bio-Rad). The experimental results were previously tested for normality using the Kolmogorov-Smirnov test and for homoscedasticity using the Levene test. Since the data were not normally distributed and the variances were not homogeneous, non-parametric procedures were used. A Kruskal-Wallis analysis was performed and the results were compared using the Monte Carlo test.

Measurement of IL-6. The level of IL-6 in the culture supernatant was measured after centrifugation using a commercially available human IL-6 ELISA kit (Peprotech, USA). Briefly, each well of the 96-well plate (Greiner Bio-One, Germany) was coated overnight with capture antibody before being washed with Dulbecco's PBS containing 0.05% Tween 20. The IL-6 standards and the samples were then added in triplicate and incubated for 3 h at room temperature. The wells were again washed and a detection antibody was added. The wells were then incubated for a further 2 h at room temperature and washed once more before adding the horseradish peroxidase-conjugated avidin complex. After 30 min of incubation and washing, the color was developed by adding a peroxidase substrate to each well, and the quantity of IL-6 was measured at 405 and 655 nm with an ELISA autoanalyzer (CODA, Bio-Rad). Inter-assay variation was 3.43% for the MWCNT/UHMWPE group and 3.01% for the UHMWPE group, always showing the same trend.

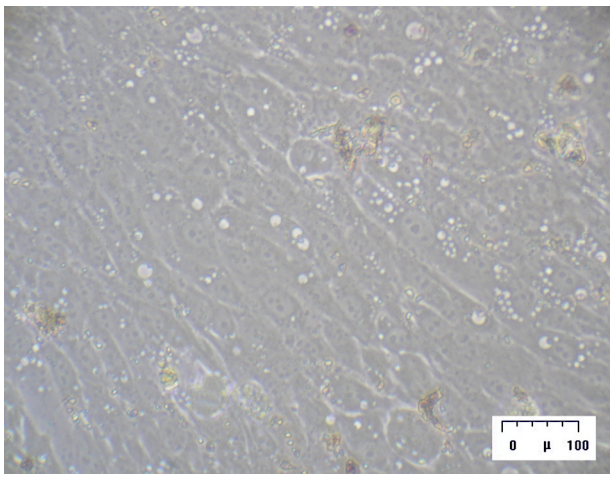

Figure 3. Cell culture. Cells after 6 days in contact with polyethylene particles (100X).

Table 1. Total protein (24 wells) and interleukin-6 (IL-6, 12 wells) measurements in MG63 cells, seeded at an initial concentration of 7600 cells/well.

	Total protein (µg/mL)	IL-6 (pg/mL)
Control	139.73 ± 10.78	90.93 ± 10.30
UHMWPE	137.07 ± 6.17	92.52 ± 11.02
MWCNT/UHMWPE	163.29 ± 11.81	108.99 ± 9.90

Data are reported as means ± SEM. Values were determined after 6 days of culture in a standard 5% CO_2 humid environment at 37°C. UHMWPE = ultrahigh molecular weight polyethylene; MWCNT = multiwalled carbon nanotubes.

Results

Cell culture

The microscopic observation of cultured cells showed morphological changes in the cells that were cultured with UHMWPE particles: supranuclear vacuolization, a more spindle-like shape, and suggestion of engulfed particles (Figure 3). Such changes were not evident in the composite group. At 144 h all groups had reached confluence.

Viability and proliferation assay and measurement of IL-6. No significant differences were found between the control, UHMWPE, and MWCNT/UHMWPE groups in the WST-1 assay (results are reported as percentage of control ± SD): UHMWPE = 96.19 ± 7.92, MWCNT/UHMWPE = 97.92 ± 8.29%. The total protein content and the levels of the pro-inflammatory cytokine IL-6 were also similar in these three groups (Table 1).

The materials

Measurement of tribological properties. In the composite tested, the reinforcement effect of CNTs helps to restrict the plastic deformation of the sample; thus, the roughness of the composite sample is higher than that of the UHMWPE under the same operating conditions. We observed that the wear volume decreased with the addition of CNTs, and that the decrease of wear volume increased with increasing sliding distance. The wear volume can be correlated with an increase in toughness of the polymer composites compared to that of the pure polymer. The decreased wear volume of nanocomposites was observed to be 28% at 43.2 m, 28% at 86.4 m, and 35% at 232.2 m, compared to that of UHMWPE. The wear study of the composite sample was also carried out at 965.6 m. Interestingly, the wear volume was found to be 0.123 mm^3, which was about 14% higher than the wear volume of the UHMWPE sample at 232.2 m sliding distance. Though the sliding distance was increased by 315%, the enhancement of wear volume was observed to be only 14%. We observed that the coefficient of wear decreased with the reinforcement of CNTs in UHMWPE, and that it followed the same trend as that of the wear volume.

Discussion

The present viability and proliferation studies suggest a cellular response to the novel composite particles similar to the one elicited by conventional UHMWPE particles.

Several investigators have raised the question of possible interactions between CNTs and the various dye markers that are used in viability and metabolic assays. With this in mind, the choice of the WST-1 assay was deliberate, and the significantly higher protein values of the composite group may be attributable to these interferences. The results of the present study may contribute to a critical analysis of some of the discrepancies found in the literature that has reported cytocompatibility of CNTs (17,21,35).

In the present study, the absence of a significant increase of IL-6 levels in the composite group when compared to the levels in the control and polymer groups suggests good cytocompatibility. IL-6 is generated mainly by macrophages and monocytes, and also by fibroblasts, endothelial cells, lymphocytes, and osteoblasts, and is a powerful inductor of bone resorption through osteoclast activation (36). Previous studies have emphasized the importance of the number and size of particles as critical factors in macrophage activation and cytokine production (34).

Brown et al. (26), based on their *in vitro* studies, have suggested that the response of the monocytic cells is strongly dependent on the morphology and state of aggregation of the CNT. Long, straight, well-dispersed nanofilaments induced the production of more tumor necrosis factor-alpha and reactive oxidative species than highly curved and entangled aggregates. These investigators also described incomplete uptake or frustrated phagocytosis induced by CNT. The same mechanisms of frustrated phagocytosis increase the production of pro-inflammatory cytokines and oxidative stress apparently explains the *in vivo* findings described by several investigators who conducted studies with longer implantation times. These pro-inflammatory cytokines may eventually lead to carcinogenesis (28-30). These studies, considered as a whole, emphasize the need for careful re-evaluation and research before enlarging the field of CNT application. However, the findings of Fraczek et al. (27), Lacerda et al. (30), Sitharaman et al. (11), and Schipper et al. (31) suggest that the behavior of CNTs under *in vivo* conditions depends on their length, functionalization, and degree of agglutination. The presence of CNT has been shown in the liver, lung, and feces after intravenous injection, although several studies have reported low or undetectable liver and systemic toxicity in mice (27,31,32). Since the potential applications for the UHMWPE/MWCNT composite are weight-bearing and involve sliding friction, it is well known that the size and rate of the particle generation determine the cellular response (34).

Other studies have addressed different aspects of the question of protein adsorption onto materials and how this influences both the inflammatory response *in vivo* and the results of the *in vitro* assays. UHMWPE induces lower protein binding relative to polystyrene plate wells (controls) (37). The question of protein adsorption onto carbon-based materials is far more complex: SWCNTs cause dose-dependent adsorption of culture medium amino acids and vitamins, and show higher affinity for planar aromatic or conjugated structures and positively charged solutes (38). Functionalization of SWCNTs and MWCNTs with terminal or surface-specific groups alters solubility and protein adsorption, including those of the cytokines IL-6 and IL-8, in a dose-dependent manner, and at CNT concentrations higher than the ones in the present study (19). In the absence of specific chemical affinity between the nanotube surface and the protein, one cause of error would be the trapping of the molecule inside the nanotube, dependent on molecular size, unless CNTs are functionalized with specific groups that promote chemical binding. The active surface issues of CNTs are equally important: in a composite, the CNT surface available for interaction is reduced because the nanotubes are embedded in a matrix. There is growing evidence that MWCNTs can inhibit osteoclast differentiation and activity *in vivo* and *in vitro*. The results reported in another study are consistent with absence of significant stimulation of IL-6 production by osteoblasts (39).

A thorough study of how carbon nanotubes interact with cytokines and other molecules present in the culture medium is justified. However, results should be examined considering the variable chemical structure and dimensions of CNTs, the dose present, and the surface characteristics and area available for interaction. The present study shows a decrease of the wear coefficient with increasing CNT content in the polymer. The same trend is observed for wear volume. This effect may result from a high interfacial strength between the polymer and the CNTs resulting in good load transfer into the nanotube network from the polymer. This could be inferred from our earlier results regarding the mechanical properties of UHMWPE/CNT composites (8,33). The results also suggest that with 0.02% MWCNT reinforcement, MWCNT/UHMWPE cytocompatibility is similar to that of the presently used UHMWPE.

Although several studies point to serious detrimental effects of CNTs *in vitro* and *in vivo*, we firmly believe that more studies must be done before abandoning these multifunctional, highly promising molecules. The superior wear behavior of this novel composite suggests its potential use in orthopedic applications.

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