Multiwalled carbon nanotubes enhance human bone marrow mesenchymal stem cells' spreading but delay their proliferation in the direction of differentiation acceleration

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Investigating the ability of films of pristine (purified, without any functionalization) multiwalled carbon nanotubes (MWCNTs) to influence human bone marrow mesenchymal stem cells' (hBMSCs) proliferation, morphology, and differentiation into osteoblasts, we concluded to the following: A. MWCNTs delay the proliferation of hBMSCs but increase their differentiation. The enhancement of the differentiation markers could be a result of decreased proliferation and maturation of the extracellular matrix B. Cell spread on MWCNTs toward a polygonal shape with many thin filopodia to attach to the surfaces. Spreading may be critical in supporting osteogenic differentiation in pre-osteoblastic progenitors, being related with cytoskeletal tension. C. hBMSCs prefer MWCNTs than tissue plastic to attach and grow, being non-toxic to these cells. MWCNTs can be regarded as osteoinductive biomaterial topographies for bone regenerative engineering.

Cellular interaction with substrate and neighboring cells plays a critical role in osteoblast survival, proliferation, differentiation as well as bone remodeling. Regulated biophysical cues, such as nanotopography, have been shown to be integral for tissue regeneration in the stem cell niche. Multiwalled carbon nanotubes (MWCNTs) represent a nanomaterial that has won enormous popularity in nanotechnology, exhibiting extraordinary physicochemical properties and supporting the growth of different kinds of cells.[1-3]

Simultaneous enhancement of osteoblast cells' proliferation and differentiation,[4,5] decrease of proliferation rates along with decreased differentiation[6] or increased differentiation accompanied with decreased proliferation[7] have been reported. Contradictory results concerning osteoblast cell adhesion, and morphology have also been reported. Osteoblast cell lines on CNTs have been found to elongate but not widen or displayed a spindle-shaped morphology.[8,9] Spreading and surface area covered were reduced.[8-10] On the contrary, Tutak et al.[7] reported robust spreading on medium roughness CNTs networks.

This variable behavior on CNTs is probably due to the various cell types used in these works. It is reported that primary human marrow stromal cells and cell lines use substantially different mechanisms to regulate adhesion and spreading on the substrate.[11]

In a recent work of ours, published in Annals of Biomedical Engineering,[12] it was found that MWCNTs can create an osteogenic environment for human bone marrow mesenchymal stem cells (hBMSCs), even without addition of exogenous factors, representing a suitable reinforcement for bone tissue engineering scaffolds.

In the following, we will highlight and discuss some aspects of this work's results, in the context of literature findings, and provide additional material in order to elucidate issues on the influence of MWCNTs on hBMSCs' proliferation, morphology, and differentiation into osteoblasts.

MWCNTs Delay the Proliferation of hBMSC Cells but Increase their Differentiation

Previous studies have shown that nano- or micro-rough Ti surfaces reduce osteoblast cells proliferation but enhance differentiation and local factor production, supporting a mature secretory osteoblast-like phenotype. On tissue culture plastic (TCP) and smooth Ti surfaces cells preserved a rather immature, dividing osteogenic phenotype (high proliferation rates, low integrin levels, and low specific osteogenic cell differentiation).[13-14] This enhancement is additive, if not synergistic, with the introduction of surface nanoscale structures (PLLA nanofiber scaffolds, electrospun poly(ε-caprolactone scaffolds).[15-16] Similar behavior of human mesenchymal stem cells has been observed on PLLA nanofiber scaffolds[17] or on electrospun poly(ε-caprolactone) scaffolds.[18]

Cells, not only respond to the presence of topographical features, but also to the dimensions of these features. A number of studies try to answer the question what is the optimum micro-nanodimension for enhanced response. Oh et al.[19] reported that small (30 nm diameter) nanotubes promoted hBMSC adhesion without noticeable differentiation, whereas larger (70 to 100 nm diameter) nanotubes elicited a selective differentiation into osteoblast-like cells.

In all above works, various types of osteoblast cells, stages of osteoblast maturation and chemistries have been used and the comparison was in respect to different substrates, making direct comparisons difficult. The general conclusion from all these results is that cell differentiation on micro- or nanostructured surfaces takes place at the expense of proliferation.

Few contrary results have been reported. Knabe et al.,[20] comparing the effect of various bioactive glass ceramics on the expression of bone-related genes and proteins found that all novel glass ceramics supported cellular proliferation together with expression of bone-related genes. However, these results did not show consistent tendency of lower cell numbers along with expression of the osteoblastic phenotype to a higher degree.

Our results[12] showed that MWCNTs substrates decreased cell numbers but displayed an accelerated progression of osteoblast phenotype development, indicated by early and enhanced expression of alkaline phosphatase activity (ALP) and osteocalcin (OC) and osteopontin (OP) levels. In the absence of additional biochemical inducing agents, ALP on MWCNTs increased about fold4- as that on the control.

The above results, taken together, show that the ordered expression of genes during development of the osteoblast phenotype can be altered (osteoblastic maturation prematurely upregulated) because of micro-nanotopography. Nanotopography is probably the additional cellular signaling necessary for developmental expression of genes to pass the restriction points during osteoblast differentiation.[21] Differentiation markers could increase as a result of decreased proliferation and maturation of the extracellular matrix. Boyan et al.[14] concluded that microrough Ti surfaces can alter the maturation state of the cell, creating a microenvironment conducive to new bone formation on. This mechanism can possibly be extended to nano-topographies on different chemistries. To understand the relationship between the nano-dimensional cues and hMSC cell response, further research is required with excessive care not to misinterpret the value of various surface features to cell response *in vitro* and *in vivo* if one examines only cell attachment and proliferation without considering the ability of those cells to differentiate into competent osteoblasts in a timely manner.
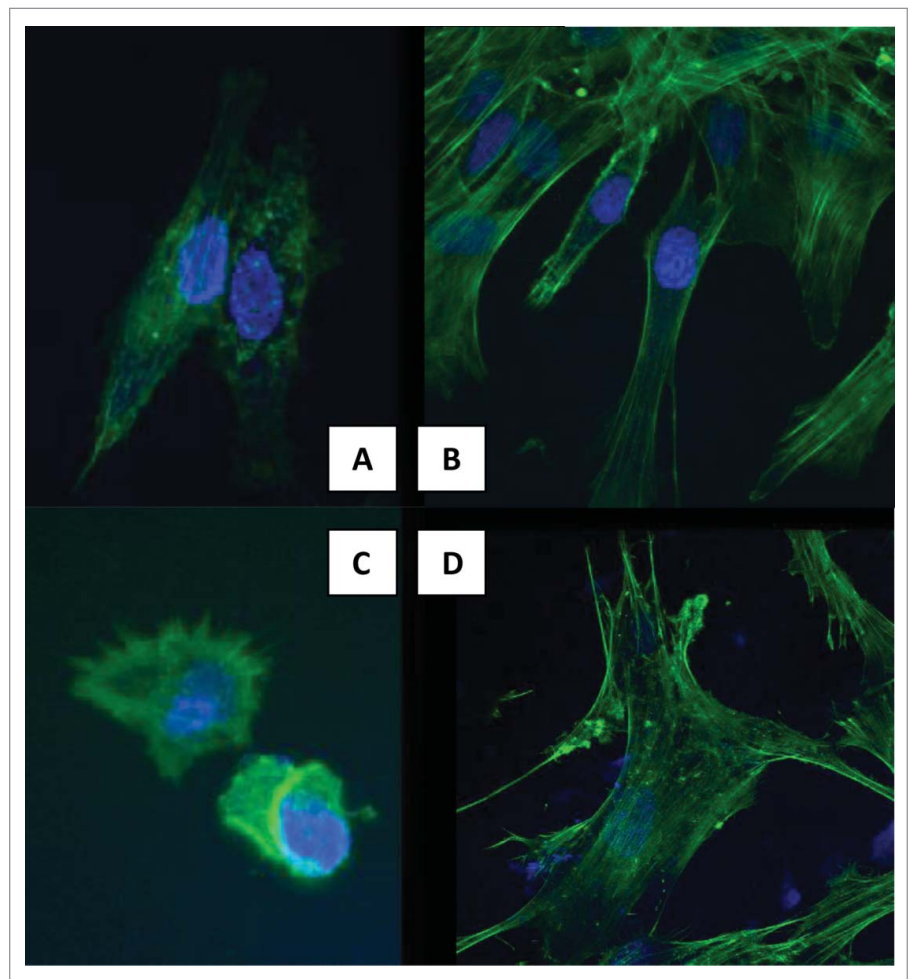

Figure 1. Fluorescent staining of the f-actin cytoskeleton (green) and DNA (blue), showing cell spreading. (**A**) 6 h on MWCNTs. (**B**) 3rd day on MWCNTs. (**C**) 6 h on TCP. (**B**) 3rd day on TCP.
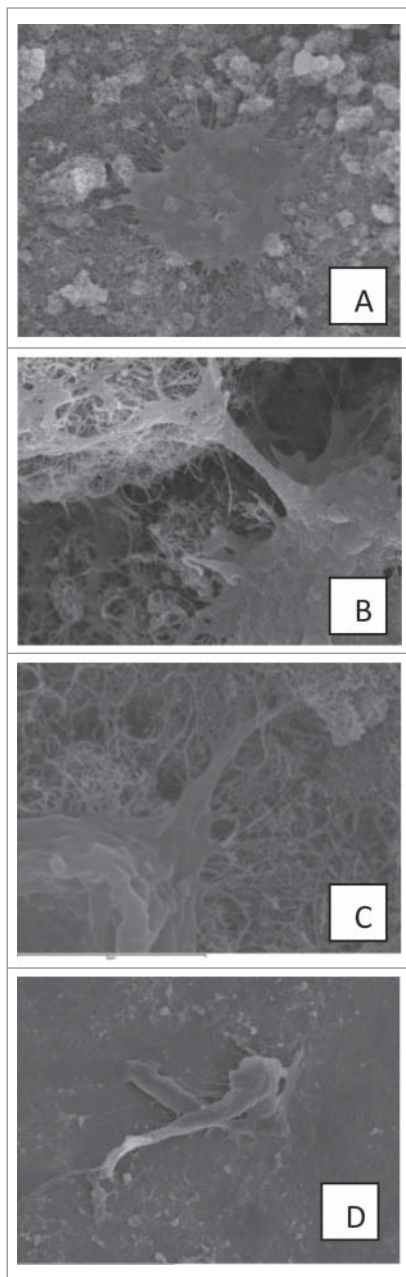
Cell Spread on MWCNTs Toward a Polygonal Shape with Many Thin Filopodia to Attach to the Surfaces

Cell shape is suggested to be a key regulator of MSC commitment.[22] The cell morphology correlates with the physiological behavior of the cells. It is admitted that cell growth better occurs when cell adhesion is decreased. On mirror-polished samples, the lower frequency of adhering pseudopodia and focal adhesions was correlated to an increase in cell proliferation.[23] On microrough surfaces, the cell bodies become more cuboidal and anchor themselves to the surface through long dendritic filopodia.[24] In contrast, on smoother surfaces, the cells flatten and spread, resulting in a fibroblastic appearance. Zhao et al.[25] reported that on smooth and low energy surfaces, the cells were elongated and formed spindle like shape; on rough and high energy surfaces, the cells were polygonal in shape with many thin filopodia to attach to the surfaces. This morphology was accompanied by lower cell numbers.

Additionally, cells grown in expansion media appeared spindle-shaped whereas cells cultured under osteogenic conditions showed a more flattened and polygonal morphology. Distinct changes found in cell architecture upon osteogenic differentiation, obtained by transfection of HBCs with an OC promoter gene, provided evidence for the connection between cell shape and functional state. The fibroblast-like phenotype of pre-osteoblasts changed to the flattened and polygonal shape of differentiated osteoblasts.[26]

The clear correlation between cell shape and differentiation leads to the assumption that changes in the assembly and disassembly of the actin cytoskeleton may be critical in supporting osteogenic differentiation.[27] It seems that cell spreading increases osteoblast differentiation in pre-osteoblastic progenitors. It is not yet clear if the change in morphology precedes the expression of a more mature physiology or if the differentiation of the cell is activated by another yet unidentified factor and the cell now responds differently to the surface microarchitecture.

Few contradictory results, showing that an increase in the hMSCs cellular elongation induced cells' differentiation into osteoblast-like cells, have been reported.[19]

Fig. 1 shows cell spreading on MWCNTs and TCP by fluorescent staining of the cytoskeleton f-actin (green) and DNA (blue). From data presented in Kroustalli et al.,[12] areas of lower cell density were selected to facilitate observation of individual cell shapes. The images of the cells shown in the selected micrographs are typical of cells throughout the culture. The confocal images of hMSCs on carbon nanotubes showed the formation of more filopodia, lamellipodia, and cellular extensions compared with those on flat TCP. They also show that, on TCP, cells tend to have a more elongated shape and long, thin actin stress fibers are running in parallel to the longitudinal cell axis. Cytoplasmic processes and filopodia are slightly concentrated at the narrow cell endings. On the contrary, cells cultured on MWCNTs spread to a larger area and displayed a more flattened and polygonal morphology. They also show marked interactions through extending cytoplasmic processes and filopodia, which enabled the anchorage of the cells (**Fig. 2**). The above findings on cell morphology conform with the results on proliferation delay accompanied by differentiation acceleration.
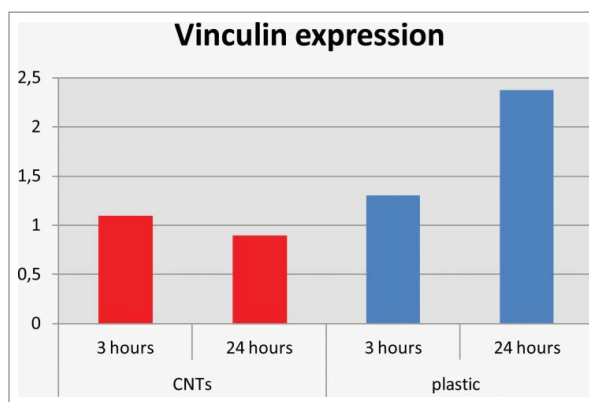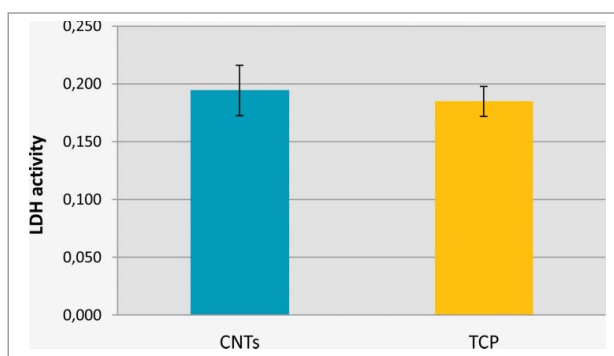
Figure 4. Lactate dehydrogenase (LDH) activity measured in cell supernatant after 24 h of culture on MWCTs and on TCP.

Adhesion blocking assays have demonstrated that integrins are mainly involved in osteoblast adhesion to MWCNTs. One of the hypotheses that Boettinger[28] makes for integrin-mediated signaling on how the signals get across the plasma membrane is that integrin mediated attachment to a solid surface allows cytoskeletal tensioning. Cell is tensioned using the integrin 'anchor' to pull against is required, along with specific linkage to the surface and integrin clustering. As a mechanism for the enhanced osteogenic differentiation connected with cell spreading, we can propose that when the stem cells are stressed, they tend to differentiate into a specific lineage to accommodate the stress. This hypothesis has been presented in the literature. It has been suggested that hMSC sense and transduce nanotopographical signals through focal adhesions and actomyosin cytoskeleton contractility to induce differential gene expression.[29]

Our results on vinculin gene expression on MWCNTs -TCP contribute to this direction. FAK and vinculin are major players in the focal adhesion processes activated by integrin-fibronectin interactions. In particular, vinculin transduces integrin-mediated intracellular signaling molecules that promote cell migration.[30]

We found that the vinculin gene expression for cells grown on MWCNTs was lower when compared with those attached on the flat TCP plate and it decreased after 24 hours (**Fig. 3**). It has

been reported that decreases in the levels of vinculin benefited cell migration by increasing the cell mobility.[31] In contrast, decreased migration is seen in cells overexpressing a-actinin and vinculin. Cells attached on MWCNTs reorganized to spread and create long extended filopodia and this rearrangement might result in lower vinculin expression.

The differentiation ability of MSCs could be influenced by cytoskeletal rearrangement.[32] Live cell analysis of human bone marrow mesenchymal stem cells on transparent titanium demonstrated rapid cytoskeletal re-organization on the nanoscale surface features, which ultimately induced higher expression of osteoblast phenotype genes.[15] Born et al.[26] found that during osteogenic differentiation the actin cytoskeleton was reorganized, resulting in thick non-aligned actin stress fibers. It is likely that the reorganization of the intracellular link is responsible for the transformation of the mechanical force into a biochemical signal, which in turn triggers cytoskeleton assembly.

Cells Prefer MWCNTs Than TCP to Attach and Grow

Several studies have described contrasting results regarding cytotoxicity of CNTs.[33-35] Such different results are probably caused by variations both in the specific characteristics of the CNTs tested (single versus multi; length and diameter; concentration; and impurities) and the type of cells used. Regarding our substrate, after very careful washing, the lactate dehydrogenase (LDH) activity was measured in cell supernatant after 24 h of culture, which was not significantly different from a positive control TCP (**Fig.4**).

Moreover, total protein synthesis experiments with various concentrations of MWCNTs (80, 160 and 320 µg/mL) for 1, 3 and 7 d at cell seeding densities of 3000 cells/cm^2 have showed that the total protein increased with concentration at each culture time point. Images of cultures displayed a very low presence of cells on TCP substrate among CNTs (**Fig. 5**). These results lead to the conclusion that cells prefer CNTs than TCP to attach. The BMSCs, even at very low seeding
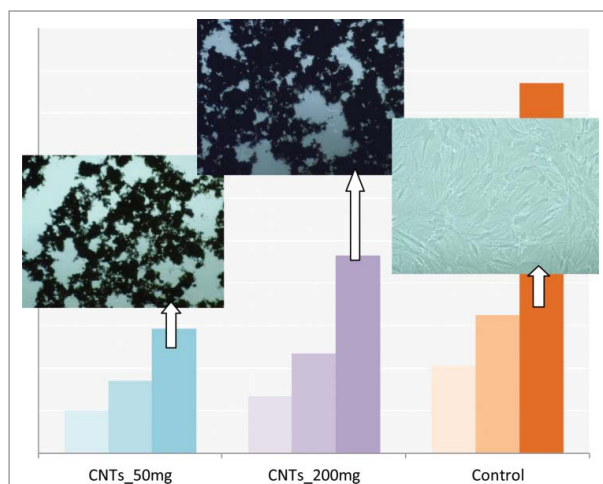

Figure 5. Total-protein of the hMSCs cells after 1, 3 and 7 d of culture on 2 different concentrations of MWCNTs. CNTs_50: 50 µg/mL, CNTs_200: 200 µg/mL and on TCP. Images of cultured cells on the substrates are also shown.

densities, grow and tend to seek CNTs in order to adhere and spread, and not TCP. Thus, increasing concentration of CNTs resulted in increased adhered cells and, consequently, more total protein.

Perspectives

MWCNTs represent a structure that provides the sustained effects on the organization of the extracellular matrix to modify the progression of differentiation of proliferating cells of the osteoblast lineage.

Despite literature evidence supporting the nanostructures' ability to be both osteoconductive and osteoinductive, there is still disparity regarding how nanostructures regulate the progression toward an osteoblastic phenotype. It is necessary to explore unique micro- and nano-architectures, to understand how they initiate osteoinductive signals through pathways similar to BMPs, and how these unique geometries can be translated to the clinic. More fundamental questions are related to defining the specific mechanisms operative in proliferating cells that allow for increased phenotypic alterations and the signals that promote progressive differentiation of the hBMSCs.

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