MWCNTs enhance hBMSCs spreading but delay their proliferation in the direction of differentiation acceleration

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In investigating the ability of films of pristine multiwalled nanotubes (MWCNTs) to influence human mesenchymal stem cells’ proliferation, morphology, and differentiation into osteoblasts, we concluded to the following: A. MWCNTs delay the proliferation of hBMS cells 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. hBMS cells 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.

MWCNTs Delay The Proliferation of hBMSC Cells But Increase Their Differentiation

Our results 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 4-fold as that on the control.

In our recent work, published in Annals of Biomedical Engineering [2013 Jul 3, Epub ahead of print], we found that MWCNTs can create an osteogenic environment, even without addition of exogenous factors, representing a suitable reinforcement for bone tissue engineering scaffolds. We will highlight some aspects of our results, connecting them with others’ findings, to conclude that 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.

Abstract

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 nanometer scale and extraordinary physicochemical properties, supporting the growth of different kinds of cells.1-3

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containing PLLA nanofiber scaffolds, with a diameter of 0.217 μm, accompanied by an increase in gene expression of ALP, OC, and collagen-I8 or on electrospun poly(ε-caprolactone) scaffolds composed of aligned or cross-aligned fibers, with 1–1.2 μm diameter, in respect to TCP and flat substrate.9

Is it micro-nanoscale surface roughness6 or nanoscale features10 that elicit the promotion of osteogenic maturation through the decrease of cell number and the increase of important markers of mature osteoblast phenotype? 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.11 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.

On the contrary, Knabe et al.12 compared the effect of various bioactive glass ceramics on the expression of bone-related genes and proteins by human bone-derived cells. All novel glass ceramics supported cellular proliferation together with expression of bone-related genes.

However, the results did not show consistent tendency of lower cell numbers along with expression of the osteoblastic phenotype to a higher degree.

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.13 Differentiation markers could increase as a result of decreased proliferation and maturation of the extracellular matrix. Boyan et al.5 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.

Cell Spread on MWCNTs Toward A Polygonal Shape With Many Thin Filopodia to Attach to The Surfaces

Figure 1 shows cell spreading on MWCNTs and TCP by fluorescent staining of the f-actin cytoskeleton (green) and DNA (blue). From our data, 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

Figure 1. Fluorescent staining of the f-actin cytoskeleton (green) and DNA (blue), showing cell spreading. (A) 6h on MWCNTs. (B) 3rd day on MWCNTs. (C) 6h on TCP. (B) 3rd day on TCP.
elongated shape and long, thin actin stress fibers are running in parallel to the longitu-
dinal 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).

Cell shape is suggested to be a key regulator of MSC commitment. 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.

On microrough surfaces, the cell bodies become more cuboidal and anchor themselves to the surface through long dendritic filopodia. In contrast, on smoother surfaces, the cells flatten and spread, resulting in a fibroblastic appearance. Zhao et al. 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.

Cells grown in expansion medium appeared spindle-shaped whereas cells cultured under osteogenic conditions for 10 days 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.

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. 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.

Contradictory results have been also reported. 100-nm diameter TiO2 nanotubes elicited a 10-fold increase in the hMSCs cellular elongation compared with 30-nm nanotubes, which induced hMSCs’ differentiation into osteoblast-like cells. As demonstrated by adhesion blocking assays, integrins are mainly involved in osteoblast adhesion to MWCNTs. One of the hypotheses that Boettinger 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. As an explanation for the enhanced proliferation and osteogenic differentiation, mechanotransduction pathways triggered by high cytoskeletal tension in the aligned hMSCs have been proposed.

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.

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. In contrast, decreased migration is seen in cells overexpressing α-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. 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. Born et al. 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.

Several studies have described contrasting results regarding cytotoxicity of CNTs. 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 days at cell seeding densities of 3000 cells/cm² 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 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.

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.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 5. Total protein of the hMSCs cells after 1, 3 and 7 days of culture on two 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.

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