In vitro differentiation of human bone marrow-derived mesenchymal stem cells towards the osteoblast lineage. A model to study the effects of cancer cells on bone formation.

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Introduction :

Breast carcinoma has a high predisposition to metastasize to bone and to induce a marked osteoclast-mediated bone destruction. However, osteoblasts indirectly participate in the process of tumor-induced osteolysis. Tumor cells can activate osteoclasts notably through the secretion of PTHrP that acts on osteoblasts and increase RANKLigand expression that will induce osteoclast differentiation and activity.

Osteoblasts are actually pivotal cells for the control of bone turnover. Immature osteoblasts regulate the differentiation and the activity of osteoclasts, while mature osteoblasts produce bone matrix (collagen synthesis and mineralisation).

Human bone marrow-derived mesenchymal stem cells (MSC) are pluripotent stem cells that can be differentiated into many mesenchymal lineages, including chondroblasts, adipocytes or osteoblasts lineage (osteoprogenitor, osteoblast and osteocyte) *(Figure 1)*, by appropriate stimulations. The effect of cancer cells on osteoblast differentiation is unknown.

Material and Methods :

MSC obtained from 9 donors were isolated from fresh healthy bone marrow specimens and identified by expression of SH2 and SH3 antigens and the absence of CD45 antigen. Undifferentiated MSC are expanded in alpha MEM supplemented with 20% FCS without any change in their differentiation potential. Briefly, MSC were expanded until subconfluence and then trypsinied and replated at low density for all subsequent passages (P1, P2, P3, …). MSC were incubated with dexamethasone, ascorbic acid and beta-glycerophophate-enriched medium (DAG) to induce MSC differentiation towards the osteoblast lineage. After 7, 14, 21 days of culture in the DAG-enriched culture medium, the extra-cellular matrix (ECM) were demineralised by HCl and the calcium content was evaluated by colorimetric assay (Quantichrom™Calcium Assay Kit, BioAssay Systems). In addition, cell-mediated matrix mineralization was determined by von Kossa or alizarin staining. The alcaline phosphatase (ALP) activity was measured using p-Nitrophenyl phosphate as substrate (LabAssay™ALP,WAKO) and semi-quantitative RT-PCR for osteoblast-typical markers (osteopontin (OPN), ALP and the PTH receptor) (Qiagen Multiplex) were also performed.

Results :

Five days after seeding of mononuclear cells, monocyte and round hematopoeitic cells are present and spindle-shaped fibroblast like cells apear adherent *(Figure 2, A)*. The differential attachement to plastic allows the selection of MSCs (strongly attached to the plastic dishes). After 2 weeks, homogeneous fibroblast-like MSCs were present in the cultures *(Figure 2, B)*. The treatment of MSCs for 21 days with DAG medium induces osteogenic differentiation , as revealed by calcium deposits *(Figure 2, C)* and the von Kossa reaction or alizarin red staining to demonstrate mineral deposition and nodule formation *(Figure 2, D, E)*.

Alkaline phosphatase (ALP) activity, a classical marker of osteoblastic lineage, was increased in MSC incubated with DAG *(Figure 3)* . The increase was already detectable at day 7 (2.6 fold versus untreated MSC), reached a maximum after 14 days of DAG stimulation (7.1 fold) and decreased after 3 weeks of culture (2.4 fold). The increase in ALP activity was low in MSC tested after the first passage, became significant after the second passage and dropped after the fourth passage.

After the first cell passage, DAG medium stimulated calcium deposition at day 14 and even more at day 21 (6.7 and 12.6 fold versus untreated MSC, respectively) in MSC from two thirds of the donors (6/9). After the second passage, DAG-stimulated matrix mineralization was observed in MSC from nearly all donors (8/9), again at day 14 and 21 *(Figure 4)*. However, after the fourth passage, matrix mineralization was stopped in all MSC tested, indicating loss of differentiation potential.

Furthermore, we are currently testing and comparing several other osteoblastic markers, assessing the expression of genes for early (type I collagen), middle (ALP), and late (osteopontin (OPN), PTH receptor) differentiation markers of the osteogenic lineage by semi-quantitative RT-PCR. We thus observed a 2.5-fold increase in ALP and PTHr mRNA expression in MSC 21 days of culture in DAG medium, while OPN was increased for more than 3 fold *(Figure 5)*.

We finally determined the influence of the breast cancer cells (MDA-MB 231) conditioned medium (CM) on the proliferation of MSC. The proliferation was evalueted by crystal violet staining and as observed in figure 6, after 7 days, Secretory products of breast cancer cells appear to inhibit the proliferation of MSCs leading to senescence.

Figure 1 : Osteoblast lineage.

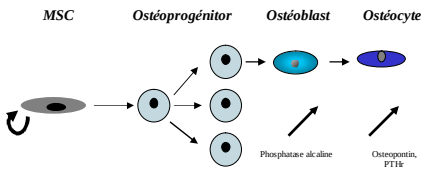
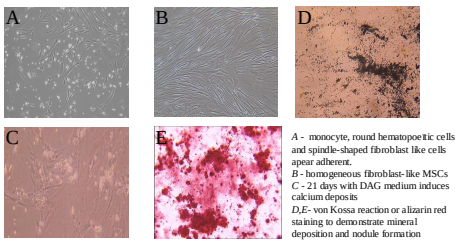


Figure 2 : Variation of bone marrow cells morphology during the culture.



A - monocyte, round hematopoeitic cells and spindle-shaped fibroblast like cells apear adherent.
B - homogeneous fibroblast-like MSCs
C - 21 days with DAG medium induces calcium deposits
D,E- von Kossa reaction or alizarin red staining to demonstrate mineral deposition and nodule formation

Figure 3 : Alcaline posphatase activity. N=9

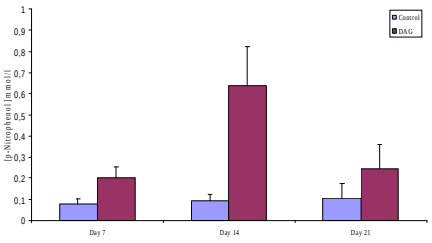


Figure 4 : Calcium deposition. N=9

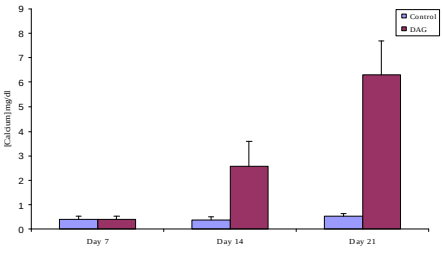
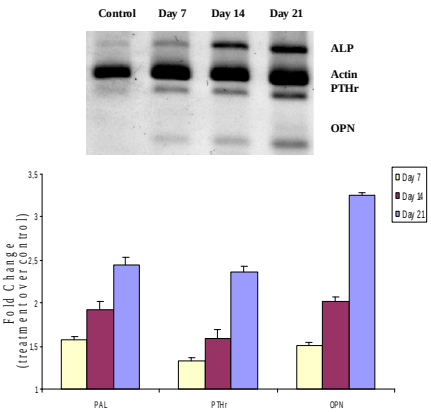
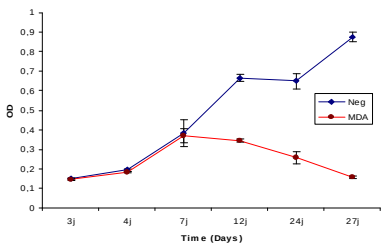


Figure 5 : Gene expression for ALP, PTH receptor and osteopontin (OPN). N=7



Amplified products were electrophoresed and analysed by densitometry using b-Actin as an internal control.

Figure 6 : Effects of the breast cancer cells conditioned medium on proliferation of MSCs



Proliferation evalueted by crystal violet staining

In summary, describes an *in vitro* model of DAG-induced osteogenic differentiation of bone marrow mesenchymal stem cells, using various osteoblastic markers. Our data indicate the need to use MSC during the second and third cell passages. The more passaged cells may lose their ability of differentiation into osteoblast. Human MSC should constitute a useful *in vitro* tool to study the effects of osteotropic cancer cells on osteoblast differentiation and to better understand the inhibitionof normal bone formation during the process of tumor-induced osteolysis.