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Research Article

Osteoblasts and osteoclasts

Development of Methods for Safe In Vitro-Differentiation of Different Progenitor Types in Osteoblasts/Osteoblast-Like and Osteoclasts/Osteoclast-Like Cells with Human and Mouse Origin

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Purpose: Development and application of techniques for safe derivation of cells from osteoblast and osteoclast lineages from progenitors of different sources and origin. **Methods:** Osteoblasts and osteoclasts with human origin were obtained from adult human bone marrow stem/stromal cells. Normal human mesenchymal stromal/stem cells (MSCs) were derived from human bone marrow material. Isolated nucleated cells were washed twice and resuspended in basic α-MEM (Minimum Essential Medium, alpha modification), supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS), 50μg/ml freshly prepared Ascorbic acid (Vitamin C) and antibiotics (100μg/ml Penicillin G). **Results:** In both cases of human bone marrow material used, derivation of mature osteoclasts and osteoblasts was proved, when respective appropriate conditions/growth factors were present. Probably, separated stem/progenitor cells in general 3T3 line differentiated in each one of respective lineages, when appropriate cultivation conditions are available. **Conclusions:** Besides factors of cultivation and components of extra-cellular matrix, role of cryo-protector DMSO as a stimulator of both cell differentiation in two cell types, but also of intra-cellular fusion in formation of osteoclast-like cells, was suggested.

Keywords: Osteoblasts/Osteoblast-Like Cells Osteoclasts/Osteoclast-Like Cells Growth Factors/Cytokines Extracellular Matrix

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Introduction

The knowledge and understanding on the role of different growth factors and cytokines, their mechanisms of action, but also signaling cascade pathways, have suggested probability for existing of novel therapeutic targets, including not only those molecules, but also their inhibitors and/or antagonists, which could influence their function both directly or indirectly, by targeting specific step of respective signaling mechanisms [20, 45].

The manifestation of cancer by aberrant Wnt-signaling has been proposed as a result of inappropriate gene activation, mediated by stabilized β-catenin, which, together with the factors as Runx2 and Osteorix in the role of co-factors, has been characterized as essential for osteoblast differentiation [25]. Other results have shown the leading role of factors as Osteocalcin and Bone Morphogenic Protein-2 (BMP2) in this process [43].

Osteoblast cell *in vitro*-differentiation by direct and/or indirect influence of Insulin-Like Growth Factors and their receptors (IGFs/IGFBPs) has also been demonstrated [1].

The influence of FGFs and their receptors has also been discussed [22, 24, 33, 36], as well as of other components of the extracellular matrix (ECM), as metalloproteinases (MMPs), microtubule-activated protein-kinases (MAPKs), Nuclear Factor kappaB (NF-κB), as well as nuclear factor-induced kinases (NIKs) and IκB kinases (IKKs) [7, 16, 34].

In this connection, the importance of relationship between intracellular calcium phosphate in the osteoblasts and their role in the mineralization of ECM about the normal and pathological events in this processes, has been underlined [3, 44].

On the other hand, pro-inflammatory activation by the influence of NF-κB on the expression of appropriate genes in neutrophils has also been suggested [7]. Signals from Transforming Growth Factor-beta (TGF-β) have been found to up-regulate Wnt5A expression directly through the Smad-complex, as well as through Smad-induced CUX1 and MAP3K7-mediated NF-κB [2, 13]. In this connection, a possibility for further differentiation of osteoclast cell lineages from derived cells with monocytic characteristics has also been proved [39].

According another study, large numbers of osteoclasts could be derived from embryonic stem cells in the presence of appropriate growth factors and co-factors [30]. Furthermore, multi-nucleated osteoclast-like giant cells have been observed in the presence of tumors in different anatomic organs, as for example in the pancreas [41].

Messages about enhanced cell differentiation to one or another direction on the influence of cryo-protector Dymethylsulfoxide (DMSO) have been obtained [9, 12, 23]. Moreover, the role of this substance [27, 29], as well as of other organic detergents [8, 17, 21, 39, 46], in the activation of fusion process between the cells, has also been indicated.

In this aspect, the main goal of the current study was directed to a possibility for derivation of mature cells from osteoblast and osteoclast lineages from common progenitors with different origin, by providing of appropriate conditions of *in vitro*-incubation.

Material and Methods

Native cell cultures

Normal human mesenchymal stromal/stem cells (MSCs) were derived from human bone marrow material. After washing with Phosphate Buffer Saline (PBS), the material was resuspended in PBS and layered over an equal volume of Ficoll-Hypaque, for density gradient separation. After centrifugation at 1400 rpm for 30 minutes, the obtained layer of stem cells was retrieved from the Buffy coat and placed in a sterile conic tube.

The so isolated cell material was washed again with PBS and centrifuged at 1400 rpm for 10 minutes. The supernatant was taken-off, and the pellet, eventually containing the stem cells, was resuspended in PBS. The so isolated nucleated cells were washed twice and resuspended in basic α-MEM (Minimum Essential Medium, alpha modification), supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS), 50μg/ml freshly prepared Ascorbic acid (Vitamin C) and antibiotics (100μg/ml Penicillin G).

The so received cell suspension was put in Petri dishes at a concentration 3 x 10^4 cells/cm^2, and the so seeded cells were cultivated at 37^0C in incubator with 5% CO_2 and 95% air humidification.

For further cell differentiation in osteoblasts, after 24 hours the cultural fluid, containing the non-adherent cells, was discarded. The received monolayer of adherent cells was washed twice with PBS, after which cultural fluid, previously prepared by addition of 10^{-8}M Dexamethazone and 10mM β-Glycerophosphate to the described above basic α-MEM, was added. The media were changed twice a week, and during this time the cell growth and proliferation was followed. When the formation of confluent cell monolayer was observed, the cells were trypsinised (by treatment with trypsin/EDTA solution), tested for viability by eosin staining, and pre-seeded for cultivation in appropriate conditions, depending of the goals of experiment.

For osteoclasts differentiation, method, successfully applied by Susa et al. (2004) [40] was used. Briefly, after 24-48 hours cultivation in basic α-MEM, separate cell sub-populations were pre-cultured in respective volumes of supplemented α-MEM, to which 25ng/ml M-CSF and 50ng/ml RANKL (receptor activator of NF-κB ligand) were added. The media were changed twice a week, and the cells were observed on every 24 hours. The appearance of osteoclasts was observed around the 7-th-10-th day after seeding in medium, supplemented with factors M-CSF and RANKL.

Normal fibroblasts from embryonic mouse Balb/c 3T3 line (1 x 10^6 cells/ml) were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification Dulbecco's Modified Minimal Essential Medium (DMEM) (high glucose), supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25mg/ml Streptomycin and 0.25mg/ml Amphotericin-B. The media were changed twice a week, and during this time the cell growth and proliferation was followed by observation at every 24 hours. When the formation of confluent cell monolayers was observed, the cells were trypsinised (by treatment with trypsin/EDTA solution), and tested for viability by Trypan Blue Dye Exclusion Test. Separate sub-populations from 3T3 fibroblast cell line, derived from Balb/c mouse embryos, were subsequently cultivated in the presence of supplemented cultural fluid, in which mouse malignant myeloma cells were previously cultivated, after its centrifugation and filtration. The so pre-cultured cells were freezed at -80^oC for 2-4 weeks after addition respective volume from the cryo-protector Dymethylsulfoxide (DMSO). After thawing of the so co-cultivated Cells, the received cell suspension was centrifuged, the supernatants, containing DMSO, were taken off, the pellets, containing the cells, were resuspended, and the cells were re-cultivated by application of the cultivation techniques, described above. After centrifugation and filtration, this so supplemented cultivation medium was added to newly prepared 3T3 fibroblast cultures, after formation of semi-confluent mono-layers. For differentiation in osteoclast-like cells, after the described procedure, separated sub-populations of mouse embryonic fibroblasts were subsequently pre-cultured in the presence of supplemented cultural fluid, in which malignant mouse myeloma cells were previously incubated.

For test the influence of the derived cells by both procedures, to each other, after formation of full monolayers of the osteoblast-like cells, they were co-cultivated with osteoclast-like cells. For this goal, after taking-off the cultural fluids from the osteoblast-like monolayers and their washing with PBS, the half from the volume of suspension, received by their trypsinization and resuspendation, was added to the cultures of the obtained osteoclast-like cells, derived from mouse embryonic progenitors. The so prepared mixed cell cultures were cultivated at 37^0C, in incubator with 5% CO_2 and 95% air humidification.

All cell cultures were observed as native light-microscopy preparations by inverted light microscope, supplied with mega-pixel CCD-camera.

Preparation of fixed light microscopy slides

For proof of osteoblast phenotype of the cells, derived from human bone marrow stromal/stem cell sub-populations, incubated in the presence of respective appropriate factors, staining with Alizarin Red Dye (specific for osteoblasts) was used.

Generally, after taking-off of the cultural fluids, cells were washed twice with PBS and fixed with 10% formalin for 1 hour at room temperature. After washing with distilled water (3 times), dehydration subsequently in 70%, 80% and 90% ethanol, each one for 5 minutes at room temperature, was made, and the preparations were then incubated in the dye solution was for 2-5 minutes at room temperature in a dark, after which they were washed with tap water and dried at room temperature.

For proof of osteoclast phenotype, derived from human bone marrow stem/progenitor cell sub-populations, subjected in osteoclast differentiation conditions, separate staining both by TRAP technique, as well as by Hematoxylin dye, was performed. After turning-off the cultural fluids, the cells were washed with PBS, and subsequently fixed with fixative solutions, prepared previously by mixing of 5ml Citrate solution, 13ml Aceton and 1.6ml 37% Formaldehyde. TRAP solution was prepared by mixing of 100μl Fast Garnet with 100μl Nitrite solution. After incubation for 2 minutes at room temperature, 100μl Naphtol, 400μl Acetate solution, 200μl Tartrate solution, and 9.1ml distilled water, were added. The cells, treated with the so prepared mixture, were incubated for 10-20 minutes at 37oC, after which were washed 2 times with distilled water. For comparison of the results, sub-populations of the so derived cells were stained with Hematoxilin dye (instead of TRAP solution) after previous washing. Fixed light microscopic slides from both normal mouse embryonic fibroblasts 3T3 and mixed cultures, were prepared by fixation with 95% Ethanol, washing with PBS, after which they were stained by Giemsa and/or Hematoxilin/Eosin, after which they were washed and dried at room temperature. The so prepared slides were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

Preparation of fixed slides for transmission electron microscopy (TEM) assay

After turning-off the cultural fluids, the cells were washed twice with PBS and fixed in 10% Fomaline. The so prepared probes were put on netts on drops from the obtained liquid suspensions. For ultrastructural assay, TEM – JEOL JEM2100 microscope, with maximal tension of 200 kV and magnification 200÷1 500 000x was applied.

Results

In the presence only of cultural fluid, supplemented by incubation of malignant mouse myeloma cells, normal embryonic cells acquire round cell shape, but also light-stained cytoplasmic content with appearance of granules, centrally-located nuclei, as well as changed nuclei/cytoplasm ratio in many of the cells (Fig. 1 - b) were noticed, which are signs of initial myeloid differentiation, in comparison with the untreated controls (Fig. 1 - a).

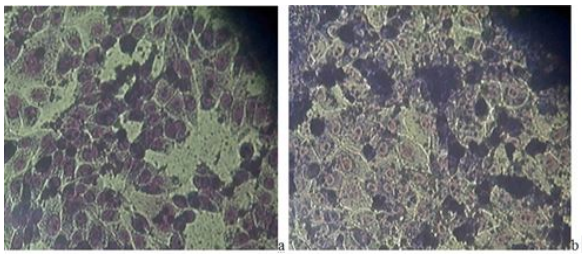

Fig. 1 In vitro-cultivated normal mouse embryonic 3T3 fibroblasts: a) control embryonic cell culture; b) mixed cell culture of 3T3 normal embryonic fibroblasts and malignant mouse myeloma cells – signs of early myeloid differentiation, as increased, rounded shape, but also light-stained cytoplasmic content with appearance of granules, centrally-located nuclei and changed nuclei/cytoplasm ratio, could be noted in comparison with the control fibroblasts (stained by Giemsa dye - magnification: x100)

In application of both techniques about differentiation in osteoclast direction of cells from the two sources used, appearance of many multi-nuclear cells could be seen, mainly with giant sizes, both derived from human bone marrow stromal/stem cells and mouse embryonic progenitors, respectively (Fig. 2).

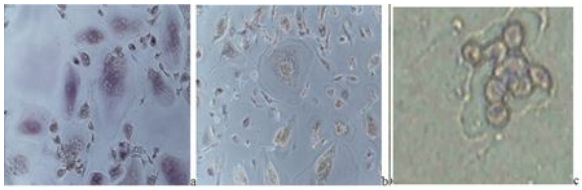

Fig. 2 Cell differentiation in osteoclasts and osteoclast-like cells: a) differentiation of human stroma/stem progenitor cells from bone marrow material, stained by Hematoxillin (magnification: x100); b) differentiation of human stroma/stem progenitor cells from bone marrow material, stained by TRAP-technique (magnification: x100);

C) osteoclast-like cell, derived from embryonic 3T3 fibroblasts by subsequent pre-incubation in cultural fluids, supplemented after cultivation of fibroblasts-derived osteoblast-like cells and of malignant mouse myeloma cells, with consequent freezing in the presence of DMSO, thawing and cultivation in the same supplemented medium (staining by Hematoxilin & Eosin, magnification: x200)

Signs of osteoclasts could be seen in both stained by Hematoxilin (Fig. 2 - a) and by TRAP technique (Fig. 2 - b).

In the mouse embryonic cells, incubated in supplemented cultural fluid after previous cultivation of mouse myeloma cells, subsequent freezing in the presence of DMSO, thawing and re-cultivation in the same conditioned cultivation media, signs of osteoclast-like differentiation were observed, the most typical of which was the presence of many nuclei, as well as the giant cell sizes (Fig. 2 - c). These results could be confirmed by data from transmission electron microscopy (TEM) assay (Fig. 3).

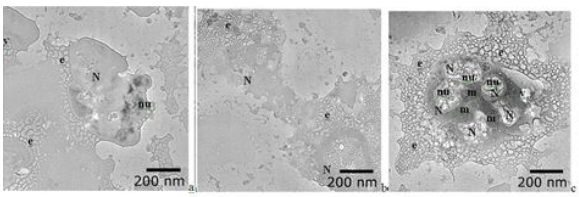

Fig. 3 TEM of osteoclast-like cells, derived from mouse embryonic 3T3 fibroblasts, after freezing in the presence of DMSO, subsequent thawing and cultivation on respective appropriate medium supplementation procedure: initial myeloid differentiation (a); fusion of two myeloid cell progenitors

(B); osteoclast-like cell, with several nuclei and endoplasmic reticulum invaginations near each one nuclei and around them

(C). Nucleus (N), nucleola (nu), endoplasmic reticulum (e), mitochondria (m), vesicles (v), could be noted (magnification: x40000).

A giant cell, possessing clear puffed border with lots membrane pseudopodia (Fig. 3 - a), components of fusion between two cells (Fig. 3 - b), and a formation of a giant cell in process of multi-nuclear appearance (Fig. 3 - c) could be noted.

In all cases, endoplasmic reticulum components could also be seen.

In the mouse embryonic cells, incubated in the presence of supplemented cultural fluid, signs of osteoblast-like phenotype were noticed (Fig. 3 - b).

These features noted were similar with the osteoblast characteristics, which were visible in the osteoblasts, obtained from human bone marrow material, which are visible by Alizarin Dye staining (Fig. 4 - a).

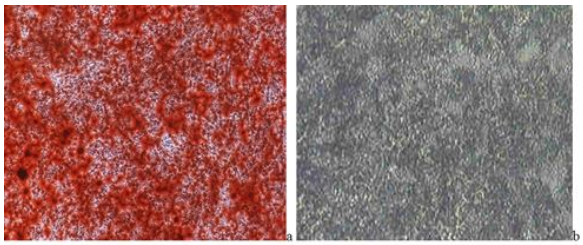

Fig. 4 Osteoblast lineage differentiation of progenitor cell sub-populations from different sources:
a) osteoblasts, derived from human adult bone marrow stromal/stem cells (stained by Alizarin Red dye);
b) osteoblast-like cells, derived from mouse embryonic 3T3 fibroblasts by pre-incubation in cultural fluid, supplemented by cultivation of fibroblasts, after freezing in the presence of DMSO, conditioned additionally after incubation of osteoclast-like cells, and subsequent freezing in the presence of DMSO, thawing and cultivation in the supplemented on the same way medium (stained by Giemsa dye). In both cases, mineral depositions (darker stained regions) could be seen (magnification: x100)

In both cell types, derived from human bone marrow material, as well as from mouse embryonic progenitors, respectively, accumulations of mineral depositions as dark stained regions were observed, which is proof of osteoblast/osteoblast-like phenotype (Fig. 4). These data were supported by the results, obtained from the performed TEM analysis (Fig. 5).

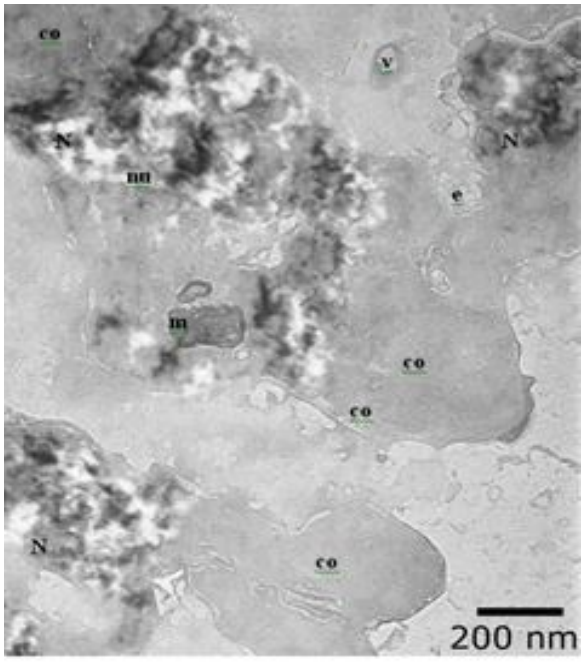

Fig. 5 TEM of osteoblast-like cells, derived from mouse embryonic 3T3 fibroblasts, by pre-incubation in cultural fluid, supplemented by cultivation of fibroblasts, and subsequent freezing in the presence of DMSO, thawing and cultivation on the same procedure. Parts from three osteoblast-like cells could be seen. Despite nucleus (N) and endoplasmic reticulum components (e) in each one of the cells, also nucleoli (nu), mitochondria (m), vesicles (v), but also collagen fibers (co) could be observed (magnification: x40000).

Besides the role of the extracellular matrix components, eventually containing in the supplemented cultural fluids, the role of the cryo-protector DMSO for further cell derivation of both osteoclast-like (Fig. 2 – c) and osteoblast like (Fig. 4 - b) cells, as well as in the process of cell-cell fusion about differentiation in osteoclast lineage was proposed.

In co-cultivation of osteoblast-like and osteoclast-like cells, both derived by us from mouse embryonic progenitors, zones of destroyed osteoblast-like cells monolayer and of mineral depositions were noticed (Fig. 6).

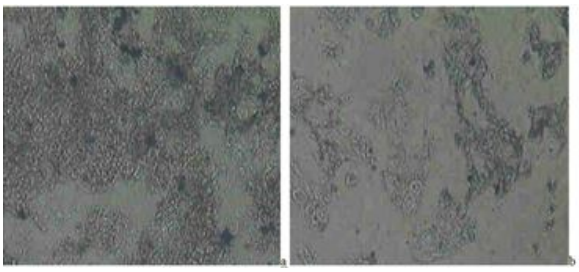

Fig. 6 Mixed preparations of co-cultivated osteoblast-like and osteoclast-like cells, both derived from normal mouse embryonic cells: a – zones of destroyed osteoblast-like cells monolayer and of mineral depositions, could be noted; b – almost full destruction of osteoblast-like cells, but also multi-nuclear osteoclast-like cells, as well as debris of destroyed monolayer of osteoblast-like cells, both in and out of the cytoplasm of the osteoclast-like cells, could be seen (Giemsa staining, magnification: x100)

The noted changes varied from large regions of degraded material (Fig. 6 - a) to almost full destruction, where only single small parts and debris of degraded material, as well as multi-nucleated osteoclast-like cells (Fig. 6 - b) could be noted.

Those results could be approved as a confirmation of the osteoblast-like and osteoclast-like phenotypes both cell types, derived from mouse embryonic progenitors in appropriate laboratory conditions.

Discussion

Taking in consideration the results obtained, together with appropriate literature findings in the respective direction, we proposed that maybe separated stem/progenitor cells in the general 3T3 line differentiated in each one of the respective lineages, when appropriate cultivation conditions are available. As main molecules, underlining the osteoblast differentiation of cells with non-osteoblastic nature, has been characterized factor Osf2/Cbfa1, known as a typical transcription activator and [14], as well as Runx2/Cbfa1 messenger RNA [38]. A lot of messages about the role of Fibroblast Growth Factors (FGFs) and their receptors for cell differentiation in osteoblast lineage, but also of the balance between the different types of those molecules, have been obtained [4, 22, 24, 36, 45]. Also, the appearance of osteoblast phenotype by proof of appropriate adhesion molecules in laboratory cultivation of osteoblasts [35, 46] and osteoblast-like cells [6, 18, 28, 37] on appropriate substrates has also been shown.

Tumor Necrosis Factor-alpha (TNF-α), characterized as one of the main cytokines, necessary for osteoclast differentiation, has been proved to be released from malignant myeloma cells [19, 32]. The same authors have also shown the role of cytokines, mainly interleukins and in particular, IL-6 and IL-1-beta, also secreted from these neo-plastic cells. Other literature data have confirmed the importance of cytokines TNF-α and IL-1-beta in the process of osteoclastogenesis [43], as well as of molecules IL-6 and Granulocyte-Macrophage Colonia Stimulation Factor (GM-CSF) [15, 26]. In a similar way, a new method for derivation of osteoclasts and osteoclast-like cells from bone marrow hematopoietic precursors has been developed in use of progenitors with rabbit origin [11], but also by myeloid differentiation of mouse embryonic stem cells in the presence of receptor for Vascular Endothelial Growth Factor - VEGFR-1 [30]. Despite the obtained messages about cell differentiation in osteoclast lineage on the influence of osteoblasts-derived metabolites [40], the

Opposite phenomenon, osteoblast differentiation in the presence of osteoclasts-conditioned cultural fluid has also been observed [44]. The fusion of monocytic cells in the late stages of differentiation has been characterized as another main mechanism, included in the derivation of osteoclasts and osteoclast-like multi-nuclear cells, both in vitro and in vivo [39]. In this connection, together with taking in consideration many literature findings, the suggestion about the eventual role of the cryo-protector DMSO as a stimulator of both cell differentiation in both cell types [9, 12, 23], as well as cell-cell fusion [27], in particular in stimulation of the osteoclast-like cells arising [29], has also been confirmed. The change in the properties of the cell membrane structures could be accepted as one of the eventual explanations [29]. These data could be supported by the established similar effects of other organic solvents [8, 17, 21, 39, 46]. Differentiation of malignant myeloid cells in osteoclast-like cells on the influence of autocrine molecules and signals has also been demonstrated [5]. In many literature sources, the destruction of osteoblasts has been indicated as one of the main indices for differentiation in osteoclast lineage [31] and bone structures [26], on the influence of the so derived cells. Signals from osteoblasts and osteobast-like cells in the stimulation of the osteoclast destruction activity have also been supposed [10].

Conclusion

Mature cells with osteoblast and osteoclast characteristics were derived in vitro from progenitors from two different sources: as adult bone marrow material and embryonic fibroblasts, with human and mouse origin, respectively. For this goal, appropriate techniques of laboratory cultivation were performed and applied, depending both of the source of cell precursors, as well as of the differentiation lineage in each one case. Besides of the laboratory conditions of incubation and the components of extra-cellular matrix, the role of the cryo-protector DMSO as a stimulator of differentiation in both cell types, as well as of fusion between the cells in the formation of osteoclast-like cells, was proposed.

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