The effect of osteogenic medium on the adhesion of rat bone marrow stromal cell to the hydroxyapatite

Ismet Deliloglu-Gurhan, PhD, Ibrahim Tuglu, MD, PhD, Hafize S. Vatansever, MD, PhD, Feyzan Özdal-Kurt, MSc, Hayati Ekren, PhD, Muzaffer Taylan, PhD, Bilge H. Sen, PhD.

ABSTRACT

Objective: To investigate the adhesive properties of bone marrow stromal cell (BMSC) on the hydroxyapatite (HA) particles and analyze their behavior.

Methods: The study took place in the Department of the Histology and Embryology, Celal Bayar University, Manisa and in the Department of Bioengineering, Ege University, Izmir, Turkey between 2004 and 2005. We cultured BMSC from the mature rat tibia and differentiated to the osteoblasts by osteogenic medium. The BMSCs were subcultured and were taken to the HA substrate. We measured their proliferation capacity and viability with MTT assay using the spectrophotometric method. Furthermore, we identified the osteoblast-like cells by immunohistochemical staining of osteonectin and osteocalcin and we analyzed the behavior of the cells on different sized HA particles by SEM at the end of 3 days incubation.

Results: Osteogenic medium caused the proliferation capacity of BMSC to speed up and the effects appeared earlier. We confirmed the osteoblastic differentiation by staining of most cells with osteoblastic markers. Subcultured cells were similarly adhesive to the HA particles and the osteogenic medium did not alter this behavior. They spread on the substrate similarly. Most of the cells demonstrated the cytoplasmic protrusion. Morphology of the cells did not change much with or without osteogenic medium. Different sizes of HA particles did not affect the adhesive properties of these cells except HA gel. The spreading and attachment ratios of the cells on HA gel were more than the others.

Conclusion: We found that there was heterogeneity in BMSC on differentiation capacity to the osteoblast, which was a sign of a subpopulation. Adhesive cells showed similar morphology and behavior under the effect of osteogenic medium. The only difference was the spreading capacity on the HA gel where cell used this substrate more effectively for adhesion.

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Bone fracture is a major problem due to difficult and expensive treatment. Various bone cement and inert materials have been used for treatment but the results are still not good enough. Thus, bioactive materials have been developed and their interaction with bone became an important issue for bone healing.[1-3] Hydroxyapatite (HA) ceramics have been used for a long time to increase adhesion to and implantation in bone tissue due to their bioadaptation capacity and bioactivity.[4,5] The physical and chemical properties of HA permits its use as bone graft where they cause angiogenesis and cell repair activation.[6-8]

From the Department of Bioengineering (Deliloglu-Gurhan), Department of Endodontıcs (Sen), Ege University, Inova Limited (Ekren, Taylan), Izmir, Department of Histology and Embryology (Tuglu, Vatansever), and the Department of Biology (Özdal-Kurt), Medical School, Celal Bayar University, Manisa, *Turkey.*

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Address correspondence and reprint request to: Dr. Ismet Deliloglu-Gurhan, Bioengineering Department, Ege University, Izmir, *Turkey*. Tel/Fax. +90 (232) 3884955. E-mail: ismetgurhan@yahoo.com

The interaction between cells and these materials cause adhesion, migration and proliferation of the cells, and therefore the repair.[9,10]

It has been shown that bone marrow stromal cell (BMSC) after differentiation can behave like osteoblast and express osteoblast markers. These cells can also mineralize the tissues.[11,12] Hence, it has been thought that these cells can play an active role during in vivo bone repair where they interact with bioactive materials.[13,14] Previous studies have shown that BMSC can produce confluent, multilayer bone nodule in culture.[15,16]

Bone repair require division of mesenchymal precursor, then their differentiation and matrix deposition and mineralization.[17] It has been shown that BMSC can achieve these repairs in cultures[18,19] and apoptotic cells in the beginning of the culture leave after confluency.[20] It is very important to know how BMSC behave in culture, how they react with biomaterials for their capacity to repair bone defect in vivo. Therefore, we investigated in vitro the behavior of BMSC on HA materials for their possible role in in-vivo osteogenesis.

Methods. The study was conducted at the Department of the Histology and Embryology, Celal Bayar University, Manisa and the Department of Bioengineering, Ege University, Izmir, Turkey between 2004 and 2005.

Collection of bone marrow. We used 6 adult male wistar rats weighing 200 ± 50 g in this study, for which approval was given by the Ethics Committee for Animal Experiments of Celal Bayar University. The rats, anesthetized with ketamine (90 mg/kg, Pfizer Warner Lambert®) + xylazine (8 mg/kg, Alfasan International B.V.), were sacrificed with cervical dislocation. After cutting from distal to head edges of bone, femurs and tibias were removed under sterile conditions and briefly immersed in alcohol. The rat bone marrow stromal cells were collected and cultured as described previously.[17,21,22] The rat BMSCs were obtained from the tibia and femur washed 4 times in Minimal Essential Medium (α-MEM); (MEM Gibco BRL, Life Technologies B.V. Breda, The Netherlands) with 0.5 mg/ml gentamycin and 3 g/ml Fungizone. Epiphyses were cut off and diaphyses flushed out with 15 ml α-MEM, supplemented with 15% fetal calf serum (FCS), (St. Louis, MO, USA), 50 μg/ml gentamycin, 100U/ml penicillin, 100U/ml streptomycin, 100U/ml amphotericin B (Sigma, Chemical Co., St. Louis, MO, USA). The cells were concentrated by centrifugation at 800 rpm for 5 minutes and resuspended with medium. Cells were counted by using a hemocytometer (Bürker, Germany).

Cell cultivation and differentiation. Cells were cultured in T25-flasks (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C (HeraCell, Kendro Laboratory, Germany). After 3 days, the floating cells in the medium (blood cells and fat cells mainly) were removed and replaced by fresh complete medium with osteogenic supplements which composed of 10 mM Na-ß-glycerophosphate, 50 μg/ml ascorbic acid and 10-8M dexamethasone (Sigma, Chemical Co., St. Louis, MO, USA). The medium was changed every other day. Cells were microscopically observed under inverted microscope with a phase-contrast attachment and photomicrographs were obtained. [23-30]

Cell viability and proliferation tests. The MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product was used to estimate cell viability and proliferation. Cells were incubated with 0.5 mg/ml of MTT in the last 4 hours of the culture period tested; the medium was then decanted. Formazan salts were dissolved with dimethylsulphoxide (DMSO) and the absorbance was determined at 570 nm in an UV-visible spectrophotometer multiplate reader (Versa Max, Molecular Device, USA). Cells were measured 3 times every other day for 24 days.[31,32]

Immunohistochemistry. Cultures were also assessed immunohistochemically using antibodies against osteocalcin (BT-582; Biomedical Technologies, Stoughton, MA), or osteonectin (AON-1; Developmental Studies Hybridoma Bank) on 30th day as follows. Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4). They were treated with 0.5% trypsin solutions and endogenous peroxidase was inactivated by incubation with 3% H_2O_2 for 30 minutes. After incubation with primary antibodies, the sections were incubated with biotinylated secondary antibodies and reacted with peroxidase-conjugated streptavidin using the protocol from a HISTOSTAIN kit (Zymed Histostain kit 85-9043 San Francisco, USA). Samples were stained with diaminobenzidine (Zymed 00-2020, San Francisco, USA) for immunoreactivities reaction. They were counterstained with Mayer's hematoxylin. The primary antibody was omitted for negative control.[33]

Hydroxyapatite specimens. The commercial HA (BoneMIX®) was supplied by INOVA (INOVA Ltd., Izmir, TURKEY). BoneMIX®-HA Granules, Ca-P macro porous ceramic powders consisting of HA [$Ca_{10}(PO_4)_6(OH)_2$] and ß-TCP [$Ca_3(PO_4)$] with constant HA /ß-TCP ratios [70:30] were obtained by calcinating (1050°C for 4 hours) 4 calcium-deficient apatites with different Ca/P ratios prepared by

aqueous precipitation method. Particles Sample#1 250-500 μm (HA-1), Sample#2 500-1000 μm (HA-2) and Sample#3 1000-2000 μm (HA-3) in size were selected. Bone MIX-HA Gel, Ca-P nonporous ceramic nanopowders consisting of HA [$Ca_{10}(PO_4)_6(OH)_2$] was prepared by aqueous precipitation method. The chemical composition and crystalline structure of the Bone MIX Hydroxyapatite Gel corresponds to the calcium phosphate component of the natural bone. The fineness of the crystals gives a very large surface area of approximately 175 m^2/gr for dry gel. Particles Sample#4 50-500 nm (HA-gel) in size was selected. All of the material samples were sterilized by gamma radiation at 25 kGy. Three scaffolds types, HA-1, HA-2, HA-3 and nonporous HA-gel was investigated. Each of these units was subjected to treatments with or without osteogenic medium.

Cell adhesion assay. The BMSCs were isolated by their attachment to culture dishes. The media were replaced on the third day. The BMSCs were digested with 0.25% trypsin plus 0.02% EDTA (Sigma, Germany) and subcultured for 2 days on different size of HA materials in 24-wells plates. Substrates were seeded with 1 ml (500.000) of cell suspension.[34-36] At the end of the inoculation period, unattached cells were removed and the culture surface was rinsed 3 times with PBS to collect any unattached cells remaining on the surface. The unattached cells were counted using a Coulter counter (Coulter Electronics Ltd., Luton, Beds, England) and the percentage of attached cells on each HA substrate disk was calculated according to the equation below:

$$\text{Attachment (\%)} = \frac{\text{Initial cell number} - \text{unattached cell number}}{\text{Initial cell number}} \times 100$$

Experiments were performed in triplicate with 5 disks of HA substrate each. The results of BMSCs adhesion were expressed as relative cell adhesion (%) ± standard error of the mean.

Scanning electron microscopy (SEM). Cell morphology was examined by SEM. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 minutes, post-fixed in 1% osmium tetroxide in distilled water for 30 minutes, dehydrated through a graded ethanol series, desiccated 5 minutes in hexamethyldizilasane, followed by 30 minutes air drying, incubated in desiccator with phosphor pentoxide overnight. Finally, samples were coated with a thin layer of gold by ion sputtering. Observations were carried out with a JEOL JSM-5200 scanning electron microscope (Tokyo, Japan).[37,38]

Results. The difference between osteogenic and normal medium for percentage of cell attachment to the tissue culture plastic at 3 days was not significant. There was a little difference in cell spreading within the osteogenic medium at 7 days. The BMSCs have assumed their characteristic polygonal morphology at 14 days, but some cells remained more rounded in both medium. bone marrow stromal cells achieved more cubical morphology in osteogenic medium.

Cell viability and proliferation were determined using an MTT assay, in the presence or absence of osteogenic medium every other day for 2 weeks. There was a significant increase in the cell number ($p \geq 0.001$): 2-fold at 7 days and 7-fold at 14 days. There were no alterations at 2 weeks for proliferation capacity but osteogenic medium seemed to cause early proliferation.

Immunohistochemical studies were performed to characterize BMSCs, by using osteonectin (**Figure 1a**) and osteocalcin (**Figure 1b**) antibodies. Many cells in osteogenic medium that spread and contacted and also a few cells in normal medium were differentiated by changing their morphology from fibroblast to epithelioid type which expressed both markers at 30 days. The markers were not uniformly expressed, but found in subsets of cells, indicating that the BMSC population was heterogeneous. The attachment behavior of the cells on HA materials were examined by SEM. The BMSCs with or without OM cultured for 2 days on different HA materials are shown in **Figures 2a-2d**. Cells seemed to have adhered and attained a normal morphology on all materials. The SEM micrographs showed that cells with long cytoplasmic processes, ruffles, protrusions and microvilli attached to the surface of HA-1 (**Figure 2a**). Osteogenic medium did not alter the behavior of BMSCs (**Figure 2b**). The cytoplasmic processes or extensions were observed to have similar lengths in the SEM. The similar morphologies were observed on the other different-sized of HA materials HA-2 and HA-3. Similarly, these cells did not change their behavior by osteogenic medium. The only differences were seen on HA-gel (**Figure 2c**), which made BMSCs more adhesive and more spread compared to that of other HA materials. This difference was more obvious for cells within the osteogenic medium (**Figure 2d**). Morphology of the cells suggested that adhesion and viability on HA materials were considerably good in both media and materials. Interestingly, non-adhesive and rounded cells were also found in both media as well. In general view by SEM, adhesive BMSCs in

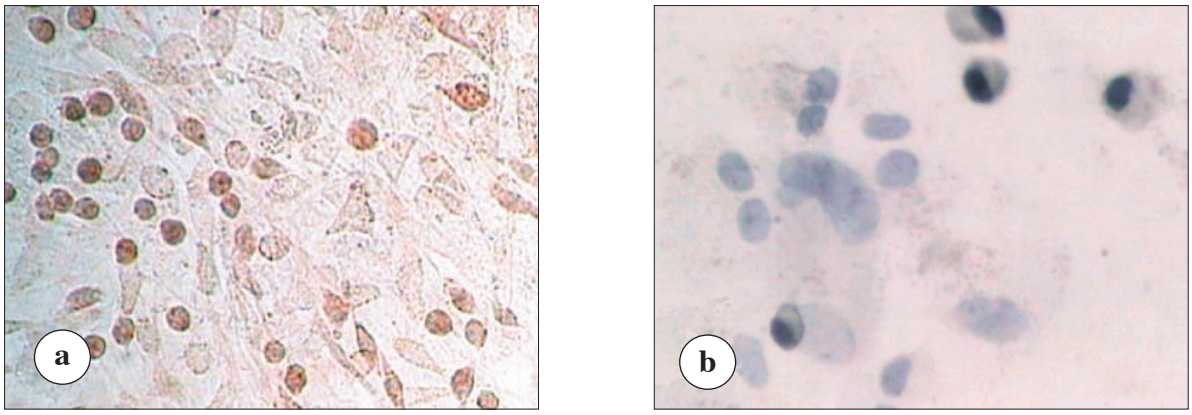

Figure 1 - Immunocytochemistry of bone marrow stromal cell (BMSC) after osteogenic differentiation in monolayer culture using anti-osteonectin **(a)** and anti-osteocalcin antibodies **(b)** as markers. Both immunoreactivities were detected after differentiation of the cells at confluency. ×400

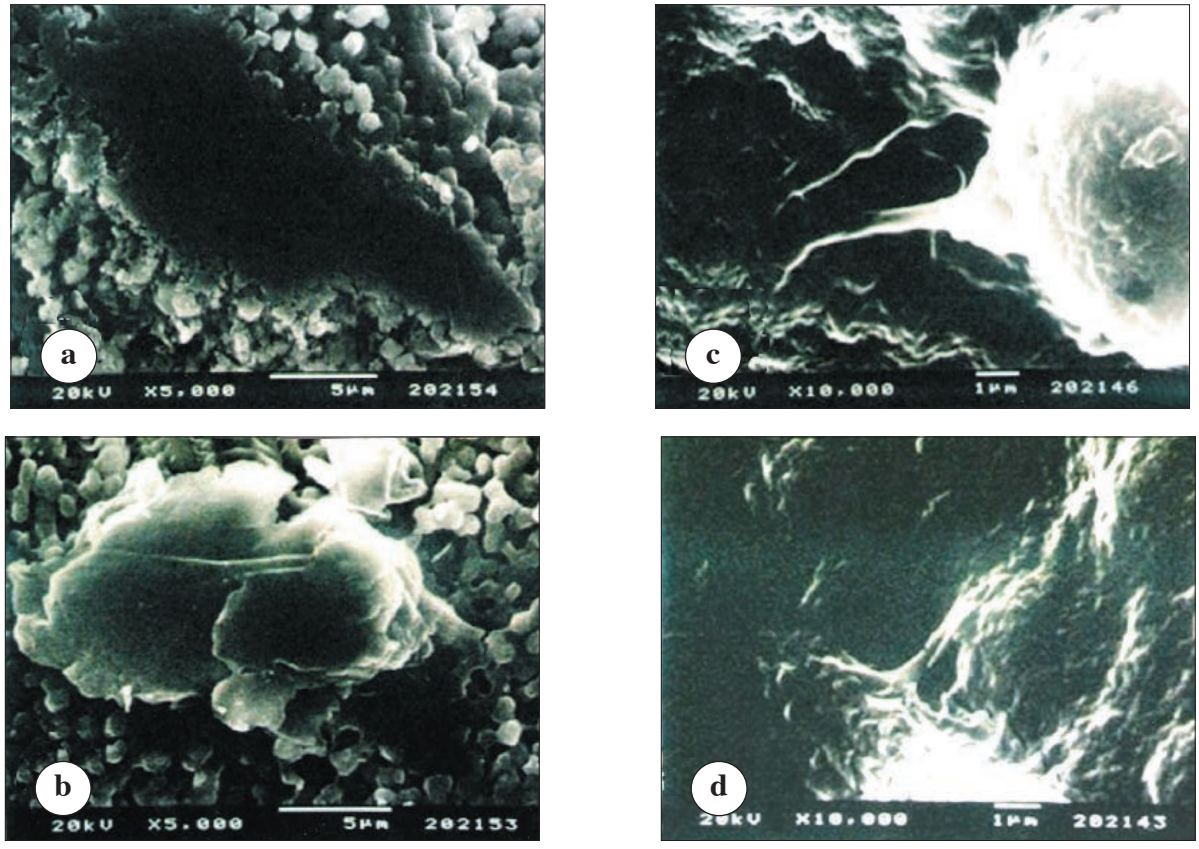

Figure 2 - SEM micrographs of **(a)** rat bone marrow stromal cell (BMSC), **(b)** BMSCs with osteogenic medium**, (c)** seeded onto HA-1, and **(d)** HA-gel**.** Bone marrow stromal cells on the all substrate were spread, migrative and cover the pores. The cell's behavior with osteogenic medium was similar without clear effect of the osteogenic medium for all substrate. The only differences for the cells behavior was seen with osteogenic medium and HA-gel materials. The cells with osteogenic medium on the HA-gel biomaterials seemed more spread and adhesive by the effect of osteogenic medium. This effect occurred as more spread cytoplasm and longer cytoplasmic protrusion (a, b ×5000, c, d ×10000).

both media had slightly flattened body with numerous cytoplasmic slender processes, numerous microvilli and a cell surface with slight protrusions of microvilli in contact with HA materials.

Discussions. This study examined the effects of the osteogenic medium on the differentiation of rat BMSCs. It has been shown that rat BMSCs differentiate to osteoblast-like cells in proper medium and produce matrix molecules which cause calcified nodules stained by von Kossa.[39] Therefore, we investigated the behavior of these cells on HA materials by SEM. In this study the adherent BMSCs in osteogenic medium behaved in a way similar to those observed in previous studies.[22,40,41] However, for cultures where osteogenic medium was used both the ratio of attachment and spreading on HA-gel materials was higher. This might reflect alterations in adhesion receptor display.[42] Previous studies have reported that osteogenic medium may affect the expression of various integrins[43,44] and cadherins.[45] The morphological observations could also indicate heterogeneity of these cells, which is consistent with phenotypic plasticity between osteoblastic and fibroblastic types.[46] The effect of osteogenic medium on BMSC proliferation has been shown to depend on cell density and state of differentiation. The MTT results showed that osteogenic medium caused the cell proliferation to occur earlier and faster. This effect may originate from the matrix production of the cells which shows contact inhibition. Cell proliferation decreases at confluence via accumulation of these molecules and mineralization.[40,41] The BMSCs cultured in the presence of both media initially exhibited a fibroblast-like spindle shape. As cultures reached confluence, cells merged into a morphologically heterogeneous state where both fibroblastic and osteoblastic types could be observed. The altered cell morphology was significant in the nonosteogenic medium; they were more flattened and strongly attached on the surfaces. The reason for his alteration could be cell-cell and cell-matrix interactions which may be regulated by osteogenic medium and HA materials. However, both cell types and behaviors were seen in both media and with different sized HA materials except osteogenic medium and HA-gel. Osteoblastic differentiation at confluent cultures was marked by the expression of 2 matrix proteins, osteonectin and osteocalcin.[47,48] In this study, we demonstrated the expression of osteonectin and osteocalcin from differentiated cells in both media. There were clearly more positive cells in osteogenic medium than normal medium, but the positive cells in normal medium suggested heterogeneity and subpopulation of BMSCs as it was shown in previous studies.[49,50] Another explanation for this behavior could be the effect of cell adhesion to both cells and matrix at confluence, which mimic the effect of osteogenic medium. Adipocyte subpopulation showed by previous studies still supports the idea of subpopulation.[40] Hydroxyapatite ceramic has been shown to be biocompatible, nontoxic material capable of forming a biochemical bond with bone owing to its chemical similarity to bone mineral. There are a number of studies concerning the properties of HA implants.[41] The responses of cells and tissues at implant interfaces have been shown to be affected not only by the chemical properties of the implant surface, but also by the surface topography or roughness of the implants. Methods of altering implant surface topography have also been found to affect the biological responses that occur at the implant surface and to affect implant fixation.[42] A plasma-spraying technique is commonly used to produce HA coatings on implant materials. It has been demonstrated that this HA coating on cpTi implants enhances rapid bone formation because of their improved osteoconductive properties compared with uncoated cpTi implants.[43] However, problems such as the low adhesive strength of the coating substrate interface and high biodegradation or bioresorption governed by the chemical composition, crystal structure, crystal and grain size, and microporosity have not been resolved.[44,45] However, the effects of these materials on cell migration, proliferation and differentiation have been shown.[29,46,47] In this recent study, we found that BMSCs interacted with HA materials causing the cells spread and adhered with cytoplasmic protrusions. Moreover, cells on these substrates produced matrix molecules and calcified nodules. Bone marrow stromal cell s showed mostly osteoblastic morphology on these substrates under the effect of both media. However, osteoblasts and spindle-shaped fibroblasts were seen in all HA materials, with or without osteogenic medium. This observation also supported the idea of subpopulation. This behavior may come from technical factors of culture conditions but it requires detailed investigations. Another important observation from this study was the effect of HA-gel materials on the BMSCs. This effect was dependent on the physical properties and chemical composition of HA materials. The gel form of HA seemed more effective on the cell morphology although there were both fibroblast and osteoblast even with this substrate. This is also another sign for the subpopulation. Morphometric studies are required to prove this hypothesis. The effect of HA materials on the repair capacity of BMSCs has been shown on the sheep tibia.[48,49] The size and combination

of HA is very important for their interaction with BMSCs, especially, gel form of HA was found more effective.[50]

In this study, it has been demonstrated that osteogenic medium may play a role on the proliferation, which occurs earlier and faster, and gel form of HA is more effective on the BMSCs adhesion. Our results suggest that there is heterogeneity and subpopulation among BMSCs. Detailed analyses and further research are needed to unravel the potential of these cells in the healing processes during bone repair.

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