

Osteostimulatory effect of bone grafts on fibroblast cultures

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Abstract

Objective: We analyzed the morphological changes and alkaline phosphatase (ALP) level in fibroblast, which is indicative of their functional ability when cultured in three different commercially available graft materials with osseointegrative property. **Materials and Methods:** Fibroblasts obtained from fifth passage were seeded within three different bone substitutes (bovine hydroxyapatite [HA] [Osseo-graft®], β -tricalciumphosphate [RTR®], bovine HA [Bio-oss®]) and incubated under standard cell culture conditions. 10 samples in each group were evaluated for cell morphology and alkaline phosphatase activity using scanning electron microscopy and spectrophotometric analysis on the 7th day of culture. **Results:** Fibroblast cultured with RTR® showed changes in morphology and increase in ALP activity when compared to fibroblast cultured with Osseo-graft® and Bio-oss®. **Conclusion:** Alkaline phosphatase activity was observed in fibroblasts when cultured with three types of commercially available bone grafts. ALP activity was highest when cultured with β -tricalcium phosphate graft material indicating its better bone regenerating capacity of this graft material.

Key words: Alkaline phosphatase, beta-tricalcium phosphate, fibroblast, osseo-graft, scanning electron microscopy study

INTRODUCTION

Tissue engineering is valuable in fabrication of graft materials and is based on principles of cell biology, developmental biology and biomaterials science.^[1] A cell-based approach is one of the most promising therapies for tissue engineering. Two subsets of stem cells of emerging importance for cell-based therapies are hematopoietic and mesenchymal stem cells (MSC). Among stem cells MSC like all other stem cells can give rise to mature cell types that have characteristic morphologies and specialized functions and these cells are also capable of self-renewal for the lifetime of the organism.^[2] MSCs offer great potential for regenerating defects in bone.^[3,4] Fibroblasts derived from primitive mesenchyme are morphologically

heterogeneous with diverse appearances depending on their location and activity. Fibroblasts can be induced to form calcified tissue by a number of factors such as bone morphogenetic protein-2^[5] and hydroxyapatite (HA).^[6] Under these conditions, fibroblasts produce a number of proteins common to bone and cementum including osteopontin,^[7] bone sialoprotein^[7] and alkaline phosphatase (ALP).^[8] Incidentally, fibroblasts express ALP,^[9,10] a marker for developing bone cells. ALP plays a key role in formation and calcification of hard tissues and its expression, and enzymatic activity are frequently used as a marker of osteoblastic cells. ALPs are considered to be involved in the transport of calcium, phosphate, or nutrients across cell membranes^[11-13] and/or in the formation of mineral.^[14-19] Fibroblasts chemotactically respond to appropriate growth factors^[20] and participate in defect repair at surgical sites.^[21] Thus, fibroblasts appear to play a significant role in the re-growth of bone following regenerative surgeries. Xenografts are routinely used in surgical reconstructions and could be valuable in regenerative medicine, provided their compatibility with the various stem cells is established. Using fibroblasts as our test system we evaluated the compatibility of two commercially available xenografts

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(Bio-oss® [bovine HA], Osseo-graft® [bovineHA]) and one alloplastic grafts (β -tricalcium phosphate [RTR®]) by assessing morphological changes and ALP activity level in fibroblast.

MATERIALS AND METHODS

Fibroblast cell culture

Cells from fifth passage of continuous MRC5 cell line was used.

Passaging cells (subculture of MRC5 cells) and monolayer culture

One T 25 flask with a confluent monolayer of MRC5 cells contains approximately 10^7 cells suspended in the storage medium. Storage medium was removed from the flask using 10-ml pipette and discarded. 5 ml of $\times 1$ Dulbecco's phosphate buffered saline (PBS) without Ca^{++} or Mg^{++} was added to wash cell layer. Then, PBS was pipetted and discarded. 5 ml of trypsin phosphate versene glucose (TPVG) solution was added on the side opposite to the cell sheet in 25 cm^2 flask. To disperse the TPVG solution evenly on the monolayer, the flask was laid flat with the cell surface side down on the table for 2-3 min. Then the TPVG was removed using a pipette and the flask was placed in an incubator until the cells detach from the surface (checked under microscope). Cells were resuspended in 5 ml of the growth medium-minimum essential Eagle's medium. The suspension was aspirated and diluted to achieve cell concentration of 1 lakh cells/1 ml. To prepare monolayer cultures, the cells were seeded onto 24-well culture plates in the above-mentioned culture medium and incubated under standard cell culture conditions.

After seeding monolayer, the grafts were added in duplicate and incubated for 37°C in a 5% CO_2 atmosphere.

Experimental groups

- Group-I (10 samples) — Cells were cultured with xenograft (Osseo-graft®).
- Group-II (10 samples) — Cells were cultured with β -tricalcium phosphate graft (RTR®).
- Group-III (10 samples) — Cells were cultured with xenograft (Bio-oss®).
- Osseo-graft and Bio-oss are xenografts, and RTR is an alloplastic material. All three grafts are osseoconductive in nature.

The following investigations were done on the 7th day of cell culture:

1. Morphology of the cells adherent on to the graft material was evaluated using – scanning electron microscopy (SEM).

2. Alkaline phosphatase activity was evaluated using spectrophotometer to determine the osteogenic potential of fibroblast cultured with the graft materials.

Preparation for scanning electron microscopy

The samples were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.5, stored at 4°C, and then rinsed 3 times with PBS and dehydrated in a graded ethanol series. The samples were critical point dried, gold sputtered, and examined with a Hitachi E1010 scanning electron microscope.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured by spectrophotometer using an ALP determination kit (Golechha diagnostics) according to the manufacturer's instructions. Briefly, after homogenization of the pieces of graft materials. Working reagent was prepared by dissolving one substrate tablet in 3.2 ml of buffer solution. Working reagent was added to the test and the control tubes and incubated at 37°C for 1 min and then the 0.02 ml of the sample was added to the respective tubes. The mix was mixed well and the initial absorbance (405 nm) was read after 1 min and repeated at 1, 2 and 3 min.

RESULTS

The adherence of the fibroblast cells cultivated with bone grafts were similar to all the three groups when examined under inverted cone microscope [Figure 1a-c].

Scanning electron microscopy analysis

Fibroblasts cultured with Osseo-graft® (Group-I) and Bio-oss® (Group-III) had morphology similar to typical fibroblasts grown without any grafts [Figure 2a-d]. These cells were long spindle shaped or star shaped. On the contrary, the fibroblasts cultured with RTR® (Group-II)

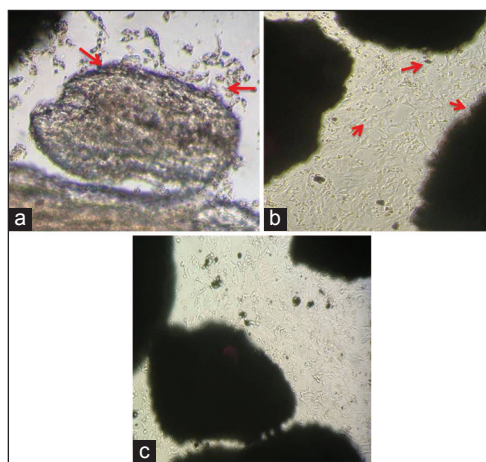


Figure 1: The adherence of fibroblasts on to (a) Osseo-graft (Group-I), (b) β -tricalcium phosphate (Group-II), (c) Bio-oss (Group-III) seen under inverted cone microscope. Arrows indicate the fibroblast adhesion

showed different morphology [Figure 3a and b]. These cells were round shape.

Spectrophotometric analysis

Alkaline phosphates activity in each group was estimated using the formula:

$$\text{Alkaline phosphatase activity in U/L} = \Delta A / \text{MIN} \times 2754.$$

Were $\Delta A / \text{MIN}$ - mean absorbance change per min.

An increase in ALP activity was observed in Group-II when compared to Group-I and Group-III which are approximately closure to the control value as shown in Table 1 and Figure 4.

DISCUSSION

A distinct subpopulation of fibroblast namely fibrocytes has been found to play a significant role in connective tissue healing including bone.^[22] ALP a hydrolyzing enzyme is a reliable indicator of active bone metabolism.^[22]

The present study evaluated ALP activity and morphology of fibroblast cell cultured with three commercially available bone grafts. Fibroblastic structure on Bio-oss[®] and Osseo-graft[®] bone graft material was normal. However altered spherical morphology of the fibroblast was observed on the surface of β -tricalcium phosphate. Fibroblasts and fibrocytes are two states of the same cells, the former being the activated state, the latter the less active state. Fibrocytes the less active form has the capacity to display distinct functional activity under specific environmental conditions to maintain structural integrity of the tissues. Fibrocytes like cells with altered morphology as seen under SEM evaluation in Group-II (RTR[®]) is significant finding in our study as fibrocytes are cited as an important cell in bone regeneration. Although subpopulation of fibroblast cells have morphological alterations,^[8] changes in morphology of cells in contact with HA particles may be influenced by the presence of HA to undergo transient dedifferentiation prior to redifferentiating into osteoblasts. This process may be important as a means by which HA acts as an osteoconductive material. The degree of adherence of

fibroblast showed normal filopodium and lamipodium projections of the fibroblast to all the three grafts, which is suggestive that all the three grafts provide a conducive environment for fibroblast proliferation and adhesion.

Tricalcium phosphate bone graft material cultured with fibroblast showed increased ALP activity when compared to Bio-oss[®] and Osseo-graft[®] material,

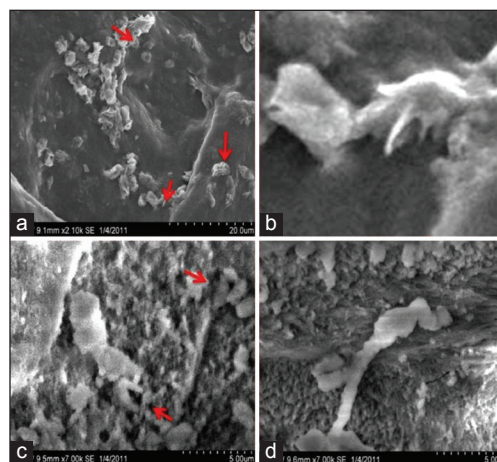


Figure 2: Scanning electron microscopic view of fibroblast cells. (a) Fibroblast cells adherent to Osseo-graft (Group-I) in day-7 culture, (b) indicates the spindle and star shaped cell adhered to Osseo-graft, (c) Cells adherent to Bio-oss (Group-III) in day-7 culture, (d) indicates the spindle shaped cells adhered to Bio-oss

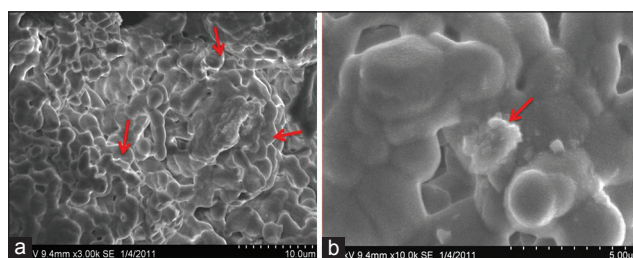


Figure 3: (a) The scanning electron microscope view of fibroblast cells adherent to β -tricalcium phosphate (Group-II) in day-7 culture, (b) shows the change in morphology of the cell

Table 1: Mean ALP activity calculated among the different groups

Samples (n = 10)	Mean absorbance change per min	ALP activity (mean values)
Control	0.164	451 U/L at 37°C
Group-I	0.191	526 U/L at 37°C
Group-II	0.301	828 U/L at 37°C
Group-III	0.148	407 U/L at 37°C

ALP: Alkaline phosphatase

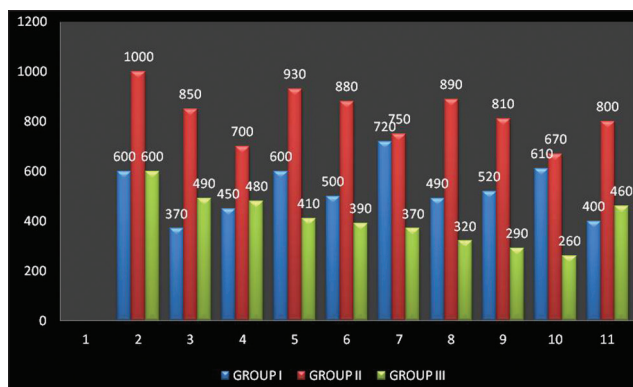


Figure 4: Bar chart which compares the alkaline phosphatase activity data among various groups

indicating an increased bone stimulatory effect. Which is consistent with the previous study showing positive staining for ALP activity in human-osteoblast like cells cultured on beta-tricalcium phosphate based membranes when compared to anorganic bovine bone membrane that is, Bio-oss®.^[23]

Within the limits of the study *in vitro* fibroblast cultures did show ALP activity when cultured with three types of commercially available bone grafts with increased activity observed when cultured with β -tricalcium phosphate graft material. Altered fibroblast morphology was noted with SEM on β -tricalcium phosphate graft surface, which is evident of formation of fibrocytes. Hence, altered morphology is associated with increased ALP activity as seen in β -tricalcium phosphate group indicates better bone regenerating capacity of this graft material.

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