Effects of a mixture of growth factors and serum proteins on human osteogenic cell cultures

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Introduction: Platelet rich plasma (PRP) has been used in a series of clinical procedures to promote bone healing. Although beneficial clinical results have been reported, PRP has not been demonstrated to enhance bone formation under experimental conditions [1,2]. The aim of the present study was to evaluate the effects of a mixture of growth factors (GFs) and serum proteins that is typical of platelet extracts on various parameters of *in vitro* osteogenesis in human alveolar bone-derived cell cultures.

Methods: Osteoblastic cells were obtained by enzymatic digestion of human alveolar bone fragments and cultured under standard osteogenic condition until subconfluence. First passage cells were cultured ($2x10^4$ cells/well) on polystyrene (24-well plates and Thermanox coverslips) up to 14 days. The mixture of GFs and serum proteins tested contained 0.27 µg/ml PDGF-BB, 0.22 µg/ml TGF-β1, 0.15 µg/ml TGF-β2, 37 µg/ml albumin, 20 µg/ml fibronectin, and 5 µg/ml thrombospondin, all purchased from Sigma-Aldrich (St. Louis, MO). Cultures were exposed during the first 7 days (proliferative phase) to the mixture GFs+proteins in which PDGF-BB concentration was 20 ng/ml. For control cultures, the mixture was replaced by Gibco α-MEM (Invitrogen, Carlsbad, CA). Cell morphology was observed by fluorescence labeling of actin cytoskeleton (Alexa Fluor-conjugated phalloidin; Molecular Probes, Eugene, OR) and nuclear stain (DAPI, Molecular Probes) at days 1, 4, 7, and 14; alkaline phosphatase (ALP) labeling was performed using a primary mouse anti-human ALP antibody (B4-78, Hybridoma Bank, Iowa City, IA) for cultures at day 7. Total cell number ($x10^4$) and cell viability (% viable cells) were determined at days 1 and 4 by the Trypan blue exclusion assay. The proportion of cycling cells at days 1 and 4 was determined by double nuclear labeling DAPI/Ki-67 using a primary rabbit anti-human Ki-67 antibody (Diagnostic Biosystems, Pleasanton, CA). Alkaline phosphatase (ALP) activity normalized for total protein at day 4 ($x10^{-7}$ mol p-nitrophenol/mg/min) and bone-like nodule formation (Alizarin red S (AR-S) staining) at day 14 were also evaluated. Data were compared by Mann-Whitney test.

Results/Discussion: At days 4, 7, and 14 epifluorescence clearly revealed that cultures exposed to the mixture of GFs and proteins exhibited a significantly higher number of adherent cells. In such experimental condition, the direction of the long axis of the cells did not significantly change throughout the culture mainly at days 7 and 14 (compare in Fig. 1, E with B). Although at day 1 there appeared to be less adherent cells in the control cultures, cell counts revealed no significant differences between groups (1.5±0.5 for control and 1.4±0.1 for GFs+proteins; $p>0.05$). Total cell number at day 4 was 5.8±0.8 for the control cultures and 25.2±5.6 for the treated group ($n=5$, $p<0.05$). At day 4, GFs+proteins cultures exhibited significantly higher values for total protein content (53.4±10.7; for control, 27.6±2.7; $n=5$, $p<0.05$) and reduced levels for ALP activity (0.25±0.2; for control, 3.9±0.25; $n=5$, $p<0.05$). Number of Ki-67 positive cells (cycling cells) were significantly higher both at days 1 and 4 (Fig. 1A,D) for treated cultures compared to control ones (respectively at day 1, 80±6.8 and 56.5±11.3; at day 4, 86.7±4.9 and 59.6±8.3; $n=3$, $p=0.05$ for both comparisons). Cell viability was significantly higher for GFs+proteins exposed cultures compared to control ones (96.5±1.1 and 91±5.1, respectively; $n=5$, $p<0.05$). Simple observation revealed more ALP positive cells with more intense labeling at day 7 (Fig. 1 C,F) and more AR-S stained nodules at day 14 for control cultures.

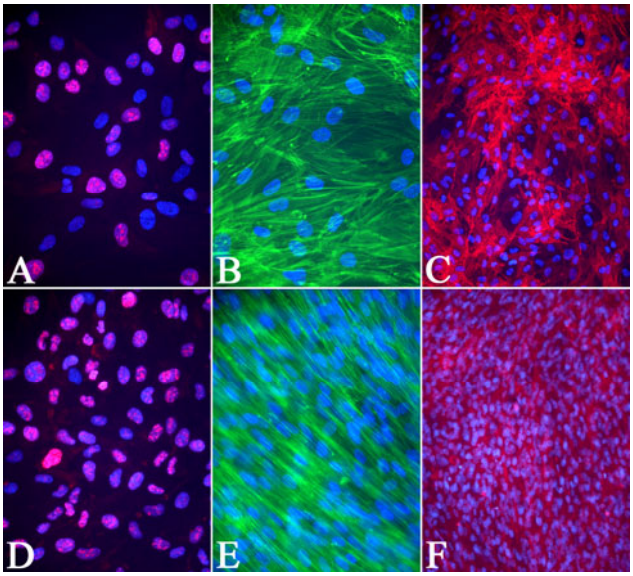

Figure 1. Epifluorescence of osteogenic cells cultured under control conditions (A-C) and in the presence of GFs+proteins (D-F). Note that higher number of cells are observed for treated cultures at days 4 (A,D) and 7 (B,E;C,F). (A,D) Double nuclear labeling DAPI(blue)-Ki-67(red) shows higher number of Ki-67 positive cells in GFs+proteins cultures at day 4. (B,E) Double labeling phalloidin(green)-DAPI(blue) reveals that cells are oriented in the same direction in treated cultures at day 7. (C,F) ALP labeling (red) is clearly more intense for control cultures at day 7. Objectives: A,B,D,E = X40; C,F = X20.

Conclusions: The present results point toward a significant influence of a mixture of GFs and serum proteins on cell cycle during the proliferative phase of human osteogenic cell cultures, leading to an increase in the cell population and a reduction in the differentiation process. Strategies to improve bone healing should also include the presence of osteogenic factors such as BMPs during the onset of differentiation phase of cell cultures.

References: [1] Ranly DM, McMillan J, Keller T, Lohmann CH, Meunch T, Cochran DL, Schwartz Z, Boyan BD. J Bone Joint Surg Am 87:2052-2064, 2005. [2] Klongnoi B, Rupprecht S, Kessler P, Thorwarth M, Wiltfang J, Schlegel KA. Clin Oral Implants Res 17:312-320, 2006.

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