TGF-β1 on induced osteogenic differentiation of human dermal fibroblast[1]

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ABSTRACT

PURPOSE: To evaluate the role of transforming growth factor beta 1 (TGF-β1) on the induced osteogenic differentiation of human dermal fibroblasts.

METHODS: We performed four groups with cultured dermal fibroblasts according to the culture medium: CONTROL (DMEM culture medium); TGF-β1 (DMEM culture medium with 10 ng/ml of TGF-β1); OSTEOG (DMEM culture medium with 0.5 µg/ml of ascorbic acid, 10 mmol/l of β-glycerophosphate and 10 nmol/L of dexamethasone); and OSTEOG/TGF-β1 (osteogenic medium with 10 ng/ml of TGF-β1). Alkaline phosphatase (ALP) activity and the amount of osteocalcin (OC) in the supernatant, as well as the capability to form calcium phosphate deposits, were analysed for 28 days

RESULTS: There were significant differences ($p<0.05$) between CONTROL and TGF-β1 groups in comparison with OSTEOG and OSTEOG/TGF-β1 groups in the ALP activity and OC amount. Although, both osteogenic groups had the same behavior with regard the expression curve during the experimental time, the OSTEOG/TGF-β1 group achieved significantly higher ALP and OC levels and showed no significant difference in the levels of mineralized deposits and in comparison with the levels found in the OSTEOG group.

CONCLUSION: The addition of transforming growth factor beta 1 to the osteogenic culture medium increased the activity of alkaline phosphatase and the amount of osteocalcin, but TGF-β1 did not alter the presence of mineralized calcium phosphate deposits.

Key words: Fibroblasts. Transforming Growth Factor beta1. Dexamethasone. Cell differentiation.

Introduction

The repair of bone defects by means of grafting can be performed through autografts, allografts, and the employment of natural or biosynthetic materials[1]. Although grafting materials are adequate for the repair of bone tissue defects, there are inherent limitations associated with their use. Autogenic grafts involve a tissue source that is biologically active but is limited; thus, these grafts may cause complications due to morbidity of the donor area. Allogenic grafts are more available and may provide a framework for tissue growth, yet they are not biologically active and carry a potential risk for immune reaction and transmission of diseases[2]. Allografts, biomaterials are in abundant supply and provide support for tissue integration. However, these materials do not possess a component to assist in remodeling of the bone tissue. This issue has been solved by combining an understructure with a cell source and growth factors[1,2]. Strategies for bone tissue engineering to achieve restoration require productive sources of bone cells to functionally restore bone tissue[3]. Osteoblasts, the cells responsible for bone formation, are difficult to isolate and expand in vitro, leading to a less than ideal number of cells being used in bone tissue engineering. For this purpose, an alternative source of cells that could function as osteoblasts is desired.

This alternative cell population should be non-immunogenic, easily expanded in vitro, and obtainable by minimally invasive tissue sampling[21]. Dermal fibroblasts can be easily isolated by biopsy of small fragments of the patient's own skin and utilized for up to 14 subcultures without any decrease in collagen biosynthesis or growth level[5,6]. The ability of cells from the fibroblast lineage to undergo differentiation into the more specialized phenotypes of connective tissue, such as osteoblasts, was first suggested by Urist[9]. Reddi and Huggins[20] demonstrated that fibroblasts have the potential to differentiate into bone-forming cells[24]. Cells of fibroblast lineage, specifically gingival[2] and dermal fibroblasts[5], demonstrated an ability to express osteoblast activity markers. One approach for the incorporation of growth factors has been the employment of bone morphogenetic protein-2[4,7-9], but other growth factors, such as transforming growth factor (TGF) β1, platelet-rich plasma (PRP), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), are also found in bone tissue and play a relevant role in the formation of bone tissue[12].

Specifically, TGF-β1 has been considered a growth factor that controls the proliferation, migration, differentiation, and survival of many cell types. The presence of TGF-β1 influences bone tissue maintenance and development, in part by increasing the synthesis of the proteoglycan matrix and synthesis of collagen in the bone tissue[7,9,10]. Tissue engineering is also dependent on the use of signaling molecules that influence cell growth. Several factors have been associated with osteoblastic development, such as dexamethasone, which is a potent stimulator of osteoblastic differentiation and bone maturation and is known for increasing the expression of alkaline phosphatase, osteopontin, and osteocalcin in osteoblasts[14,16,17]. For this reason, the objective of this study was to evaluate the role of TGF-β1 on the induced osteogenic differentiation of human dermal fibroblasts.

Methods

This study was approved by the research ethics committee of the Federal University of Sao Paulo (UNIFESP) 1167/06, and free and informed consent was given by all patients.

Cell culture

Human dermal fibroblasts (HDF) were isolated from skin fragments discarded in surgical procedures for the six males adults donors and cultured to 80% confluence in DMEM (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 1% penicillin, 100 U/streptomycin, and 100 mg/ml solution[10,11], designated the standard culture medium. At this stage the adherent cells were detached (0.01% trypsin), counted in a Neubauer chamber, and seeded at a density of $2.5x10^4$ cells/cm2 in 24-well plates containing collagen sponge discs (B. Braun, S.A., Germany) measuring 16x2 mm for 28 days under the following experimental conditions: CONTROL group, standard culture medium; TGF-β1 group, standard culture medium supplemented with 10 ng/ml TGF-β1 (Sigma); OSTEOG group, standard culture medium supplemented with 50 μg/ml ascorbic acid (Sigma), 10 mmol beta-glycerophosphate (Sigma), and 10 nmol of dexamethasone (Sigma); and OSTEOG.TGF-β1 group, standard culture medium supplemented with 50 μg/ml ascorbic acid, 10 mmol beta-glycerophosphate, 10 nmol dexamethasone, and 10 ng/ml TGF-β1[18-20].

The culture was analyzed on days 2, 7, 14, 21, 28 for functional characteristics (i.e., alkaline phosphatase (ALP) activity, ability to form calcium phosphate deposits, amount of osteocalcin and expression of the ALP. The cells were incubated at a temperature of 37°C in an atmosphere composed of 95% oxygen and 5% CO2. The culture medium was exchanged every 48 hours, and the culture was monitored daily using an inverted optical microscope.

ALP activity

The medium was removed from the culture plates and submitted to alkaline phosphatase activity readings with the QuantiChrom® Alkaline Phosphatase Assay Kit (DALP-250) (BioAssay Systems, CA, USA). A direct measurement of the alkaline phosphatase activity in the supernatant was conducted using p-nitrophenyl phosphate, which is hydrolyzed by alkaline phosphatase, producing a yellow substance with a maximum absorbance of 405 nm, where the intensity of the reaction is proportional to the activity of the enzyme. For this, 200 µl of distilled water and 200 µl of calibrating liquid (Kit) was transferred to a 96-well plate, 50 µl of the medium to be analyzed was transferred to the other wells, and 150 µl of the working solution (Kit) was transferred to the wells containing the medium. The final amount of solution in each well was 200 µl. After gently closing the plate cover without mixing, the solution was read by a spectrophotometer (Multiskan, CA, USA) at 405 nm. Four minutes later, the plate was again read to obtain an alkaline phosphatase activity curve.

OC amount

At each evaluation point of this study, the medium was removed and subjected to ELISA for quantitative dosing of osteocalcin. The ELISA OSTEOCALCIN (ZYMED LAB, Inc., CA, USA) kit was used as follows: solution 1 contained horseradish peroxidase-conjugated antibody (HRP-antibody). Vial 2 was reconstituted with 11 ml of distilled water gently mixed for 10 minutes with few intervals avoiding foam formation. Solution 2 was prepared with standard solution containing 16 ng Gla-OC/ml. Vial 3 was reconstituted with 1 ml of distilled water. Next, 100 µl of the medium sample and 100 µl of solution 2 were transferred to a 96-well plate from the kit. After gently mixing the solution, the plate was covered and kept at room temperature for two hours. The medium was removed from the wells and rinsed three times with 400 µl of phosphate-buffered saline (PBS). Between each rinse, the plate was placed on sterile paper to completely remove the liquid. Next, 100 µl of solution 1 was added with a pipette and incubated 1 hour. After this time, the solution was removed, and the wells were rinsed four times with PBS. Then 100 µl of substrate solution was added (vial 5) and left for 5 minutes. Subsequently, 100 µl of interruption solution was added, followed by gentle mixing. The solution was immediately read by spectrophotometer at 450 nm. The reading unit was ng/ml.

Formation of mineralized deposits of calcium phosphate

Slides with 0.5-µm-thick section cuts of collagen sponge were prepared[21]. First, a dewaxing process with xylol was performed, followed by hydration with distilled water, which was exchanged at every rinse (using at least two rinses). Next, the slides were immersed in a 5% silver nitrate solution for 30 to 60 minutes with exposure to UV light or a 100-watt incandescent light bulb. Subsequently, the slides were rinsed with distilled water, rinsed with a solution of 5% sodium thiosulfate for 2 to 3 minutes, rinsed with distilled water, immersed in nuclear fast red staining for 5 minutes, rinsed with distilled water, quickly dehydrated with three rinses of absolute alcohol, and finally rinsed with xylol. The Von Kossa staining protocol for calcium was utilized to demonstrate deposits of calcium or calcium salts specific for calcium ion in the sponges.

Statistical analysis

The Friedman analysis of variance was used to evaluate the results of the triplicates in each group at the study measurement points. The Kruskal-Wallis test was used to compare the averages of the three repetitions among the study groups. The threshold for statistical significance was set at 0.05 or 5%. Statistical values were presented in the form of tables and graphics.

Results

The human dermal fibroblasts were cultivated in standard and osteogenic medium, supplemented or not with TGF-β1, for a period of 28 days under controlled experimental conditions.

ALP activity was assessed by a photometric curve based on p-nitrophenol and indicated the presence of protein in the supernatant, there was an increased activity from the second to the seventh day and after this period to the twenty-eighth day can observe a gradual decrease. This behavior was similar in both OSTEOG and OSTEOG.TGF-β1 groups. The ALP activity was significantly greater in the OSTEOG. TGF-β1 group 12.29±0.4 in comparison with OSTEOG group 10.15±0.2 ($p<0.05$), in seventh day [peak], and both control groups (CONTROL and TGF-β1) did not expressed ALP, as shown in Figure 1.

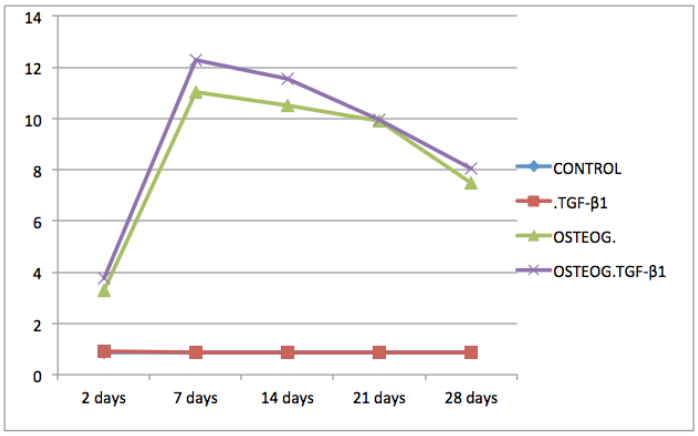

FIGURE 1 - Curve expression of ALP measured in IU/L, showing the peak of expression in the early period with decrease by the end of the study.

The amount of OC in the supernatant was measured by ELISA, which revealed the presence of protein in the supernatant, the OC was detected from the seventh reaching its maximum peak in the twenty-first day and soon after we were able to observe its decrease, both OSTEOG and OSTEOG/TGF-β1 groups exhibited the same behavior. The amount of OC was significantly greater in the OSTEOG/TGF-β1 group 25.32±0.8 in comparison with OSTEOG group 21.21±1.2 ($p>0.05$), in twenty-first day [peak] and both control groups (CONTROL and TGF-β1) did not expressed OC, as shown in Figure 2.

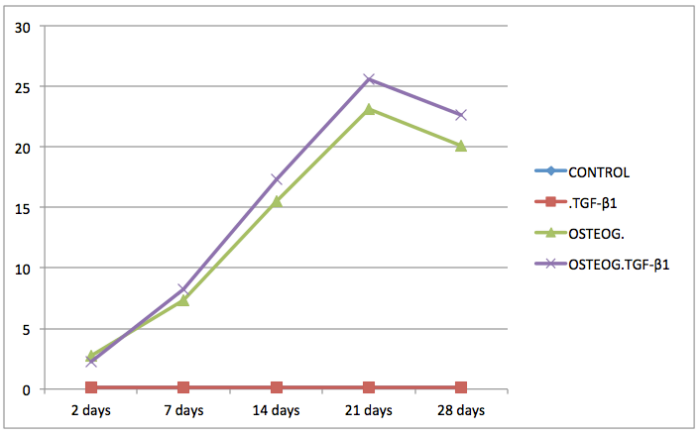

FIGURE 2 - Curve expression of OC measured in ng/mL, showing the peak of expression in the period later with decrease by the end of the study.

Mineralized deposits of calcium phosphate

The evaluation of the mineralized deposits on the collagen sponge was performed with Von Kossa staining. The results demonstrated the presence of mineralized deposits in the OSTEOG and OSTEOG.TGF-β1 groups after day 21 of culture. The presence of mineralized deposits was not verified in the CONTROL and TGF-β1 groups, as showed in Chart 1.

CHART 1 - Results of detection the presence of calcium phosphate deposits with Von Kossa stain.

Culture Days	CONTROL	TGF-β1	OSTEOG.	OSTEOG. TGF-β1
2	-	-	-	-
7	-	-	-	-
14	-	-	+	+
21	-	-	+	+
28	-	-	+	+

(-) Absence of mineralized deposits of calcium phosphate
(+) Presence of mineralized deposits of calcium phosphate

Discussion

Tissue engineering has emerged as a promising alternative to bone tissue repair strategies[9,15-19]. The development of skeletal tissues in vitro and in vivo depends on the successful application of phenotype-specific cells or bioactive growth factors integrated into matrices or understructures. Repair strategies based on tissue engineering have shown efficacy in applications of bone tissue regeneration, demonstrating that the repair of defects with a critical volume loss may be treated by a combination of mesenchymal stem cells seeded in tridimensional understructures[23].

Cell sources for restoration of the biological function of the bone tissue may or may not originate from the bone tissue itself. Native bone-forming cells such as osteoblasts are difficult to isolate and expand in vitro[24]. Therefore, an alternative source of cells that function like osteoblasts is of great interest. In general, this cell population should be non-immunogenic, rapidly expanded, and acquired through a minimally invasive procedure. Although bone marrow cells have been used for this purpose, in tissue engineering these cells require a specialized culture and a purification procedure that may result in a cell population that is smaller than desired[23]. Non-osteogenic cells, such as dermal fibroblasts, are a potential cell source because they are easily obtainable from autologous tissues and can rapidly expand in vitro[13,20-22,24].

The addition of ascorbic acid, β-glycerophosphate, and dexamethasone seems to be necessary for osteoblastic differentiation of human dermal fibroblasts. Hee *et al.*[17] demonstrated that these three supplements are more effective at inducing expression of osteogenic markers when used simultaneously. Although the employment of a culture medium

without FBS provides a favorable environment for cellular differentiation of bone lineage cells, as shown for HDF culture aimed at cellular differentiation[5], the employment of 7% FBS in our study did not affect the results.

The expression of proteins of the extracellular matrix known to be osteoblast-specific, such as alkaline phosphatase and osteocalcin, were used to determine the degree of cellular differentiation. The CONTROL and TGF-β1 groups did not show detectable levels of these proteins, indicating that the simple presence of TGF-β1 did not induce osteoblastic differentiation in the dermal fibroblasts. In both OSTEOG and OSTEOG/TGF-β1 groups, the expression of ALP and OC was dectable and significant higher in comparison with the controls groups ($p<0.05$). The levels of ALP and OC were significantly higher in the OSTEOG/TGF-β1 group than in OSTEOG group ($p<0.05$), which demonstrated that the presence of TGF- β1 in a osteogenic culture medium stimulates a higher production of osteoblastic markers by dermal fibroblasts. In all osteogenic groups there was a progressive decrease in the ALP activity and increase of OC levels, this behavior is in agreement with that expected for these proteins since they have the same behavior in normal physiology. This result reinforces the idea that non osteogenic cells can take another behavior when stimulated by culture medium and showing similar patterns found in the typically osteogenic cells

One of the distinctive characteristics of an osteogenic phenotype is the ability to form mineralized calcium phosphate deposits. The collagen sponge did not impose any difficulty on the development of these deposits, in contrast to the difficulties observed by Hee *et al*[17] in the utilization of understructures based on beta-tricalcium phosphate[5]. The control and TGF-β1 groups did not produce mineralized deposits, while the OSTEOG and OSTEOG/TGF-β1 groups showed mineralized deposits beginning on day 14, increasing until day 28, but there was no difference in coloration among the study groups..

Additional studies are necessary to determine whether the rates of increase of the protein markers for osteoblastic activity and of differentiated dermal fibroblasts are sufficient to induce bone tissue repair in vivo.

Conclusion

The addition of transforming growth factor beta 1 to the osteogenic culture medium increased the activity of alkaline phosphatase and the amount of osteocalcin, but TGF-β1 did not alter the presence of mineralized calcium phosphate deposits.

References

1. Vacanti CA, Kim W, Vacanti D, Mooney B, Vacanti JP. Tissue engineered growth of bone and cartilage. Transplant Proc. 1993;25(1Pt2):1019-21.
2. Tholpady SS, Freyman TF, Debiie C, Ogle RC. Tensional forces influence gene expression and sutural state of rat calvariae in vitro. Plast Reconstr Surg. 2007;120:601-11.
3. Vlasselaer P, Borreman B, Gorp U. Interleukin 10 inhibits transforming growth factor-β synthesis required for osteogenic commitment of mouse bone marrow cells. J Cell Biol. 1994;124(4):569-77.
4. Farhadi J, Jaquiery C, Barbero A, Jakob M, Schaeren S, Gerhard P. Differentiation-dependent up-regulation of BMP-2, TGF-β1 e VEGF expression by human bone marrow stromal cells. Plastic Reconstr Surg. 2005;10:1379-86.
5. Chen G, Sato T, Ohgushi H, Ushida T, Tateishi T, Tanaka J. Culturing of skin fibroblasts in a thin PLGA-collagen hybrid mesh. Biomaterials.2004;26:2559-66.
6. Goldstein AS, Juarez TM, Helmke CD, Gutin MC, Mikos AG. Effect of function in biodegradable polymer foam sacaffolds. Biomaterials. 2001;22:1279-88.
7. Urist MR. Bone: formation by autoinduction. Science. 1965 Nov 12;150(3698):893–99.
8. Reddi AH, Huggins C. Biochemical sequences in the transformation of normal fibroblasts in adolescents rats. PNAS. 1972;69(6):1601-5.
9. Gron B, Stoltze K, Andersson A, Dabelsteen E. Oral fibroblasts produce more HGF and KGF than skin fibroblasts in response to co-culture with keratinocytes. APMIS. 2002;110:892-8.
10. Andrades JA, Bo HAN, Becerra J, Sorgente N, Hall FL, Nimmi M. A recombinant human TGF-β1 fusion protein with collagen-binding domain promotes migration, growth and differentiation of bone marrow mesenchymal cells. Exper Cell Res. 1999;250:485-98.
11. Coelho MJ, Fernades MH. Human bone cell cultures in biocompatibility testing. Part I: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteosblastic differentiation. Biomaterials. 2000;21:1087-94a.
12. Jaworski J, Klapperich M. Fibroblast remodeling activity and three dimensional collagen-glycosaminoglicanos. Biomaterials. 2006;27:4212-20.
13. Saraiva GL, Lazaretti-Castro M. Marcadores bioquímicos da remodelação óssea prática clínica. Arq Bras Endocrinol Metab. 2002;46(1):72-8.
14. Hee C, Jonikas MA, Nicoll SB. Influence of three dimensional scaffold on the expression of osteogenic markers by human dermal fibroblasts. Biomaterials. 2006;27:875-84.
15. Coelho MJ, Fernades MH. Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteosblastic differentiation. Biomaterials. 2000;21:1095-102b.
16. Bernhardt A, Lode A, Boxberger S, Pompe W, Gelinsk M. Mineralised collagen-an artificial, extracellular bonw matrix-improves osteogenic differentiation of bone marrow stromal cells. J Mater Sci. 2006;24:134-45.
17. Reyes JM, Fermanian S, Yang F, Zhou S, Herretes S, Murphy DB, Elisseeff JH, Chuck RS. Metabolic changes in mesenchymal stem cells in osteogenic medium measured by autofluorescence spectroscopy. StemCells. 2006;24:1213-7.
18. Walsh S, Jordan GR, Jefferiss C, Stewart K, Beresford JN. Hihg concentrations of dexamethasone suppress the proliferation but the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis. Rheumatology. 2001;40:74-83.

19. Kozhevnikova MN, Mikaelyan AS, Starostin VL. Molecular and genetic regulation of osteogenic differentiation of mesenchymal stromal cells. Cell Biol. 2008;3:223-38.
20. Chang HY, Dudoit S, Bondre C, Brown PO. Diversity, topographic differentiation, and positional memory in human fibroblasts. PNAS. 2002;99(20):12877-82.
21. Beloti MM, Rosa AL. Osteoblast differentiation of human bone marrow cells under continuos and descontinuos treatment with dexamethasone. Braz Dent J. 2005;16(2):156-61.
22. Locklin RM, Richard O, Triffitt JT. Effects of TGF-β and FGF on the differentiation of human bone marrow stromal fibroblasts. Cell Biol Int. 1999;23(3):185-94.
23. Smith RS, Smith TJ, Blieden TM, Phipps RP. Fibroblasts as sentinel cells. Am J Pathol. 1997;151(2):317-22.

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