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Rapid Communication

Effect of 830 nm Laser Phototherapy on Osteoblasts Grown *In Vitro* on Biosilicate® Scaffolds

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Abstract

Objective: The purpose of this study was (i) to develop a method for successfully seeding osteoblasts onto a glass-ceramic scaffold designed for use in clinical settings, and (ii) to determine whether the application of laser phototherapy at 830 nm would result in osteoblast proliferation on the glass-ceramic scaffold. ***Background:*** The use of bioscaffolds is considered a promising strategy for a number of clinical applications where tissue healing is sub-optimal. As *in vitro* osteoblast growth is a slow process, laser phototherapy could be used to stimulate osteoblast proliferation on bioscaffolds. ***Methods:*** A methodology was developed to seed an osteoblastic (MC3T3) cell line onto a novel glass-ceramic scaffold. Seeded scaffolds were irradiated with a single exposure of 830 nm laser at 10 J/cm^2 (at diode). Non-irradiated seeded scaffolds acted as negative controls. Cell proliferation was assessed seven days after irradiation. ***Results:*** Osteoblastic MC3T3 cells were successfully grown on discs composed of a glass-ceramic composite. Laser irradiation produced a 13% decrease in MC3T3 cell proliferation on glass-ceramic discs (mean $\pm$ SD $= 0.192 \pm 0.002$) compared with control (non-irradiated) discs (mean $\pm$ SD $= 0.22 \pm 0.002$). ***Conclusions:*** Despite successful seeding of bioscaffolds with osteoblasts, laser phototherapy resulted in a reduction in cell growth compared to non-irradiated controls. Future research combining laser phototherapy and glass-ceramic scaffolds should take into account possible interactions of the laser with matrix compounds.

Introduction

Laser phototherapy at specific wavelengths and energy densities has been established in numerous studies as having the capacity to cause cell proliferation *in vitro*. Previously, we demonstrated that osteoblast cell proliferation and alkaline phosphatase activity increased significantly after 830 nm laser phototherapy at 10 J/cm^2.[1] This outcome suggests that laser phototherapy may be beneficial in a range of settings including novel applications such as promoting osteocyte growth on bioscaffolds for use in clinical settings for the treatment of fracture, delayed bony union, non-union or mal-union. This study set out to: (i) develop a method for successfully seeding osteoblasts onto biosilicate scaffolds designed for use in clinical settings; and, (ii) determine whether the application of laser phototherapy at 830 nm (continuous; 10 J/cm^2 at diode) would result in cell proliferation on the glass-ceramic scaffold.

Materials and Methods

Culturing and passaging of osteoblastic cells

The neonatal, murine (*Mus musculus*), calvarial, osteoblastic MC3T3 cell line was employed in this study. Cells were grown in sterile Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen, Mount Waverley, Australia) supplemented with heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), and 200 IU/ml penicillin + 200 μg/ml streptomycin (Invitrogen) All tissue culture assays and passages were performed under strict aseptic conditions in a biological safety cabinet. The cell line was grown in sterile, vented, 25 cm^2 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) in a humidified incubator (Model MCO-15AC, Sanyo, Breda, The Netherlands) at 37°C in 5% carbon

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dioxide (CO_2), 95% air, and passaged every 4–5 days using standard tissue culture techniques.

Glass-ceramic scaffold

A novel fully-crystallized bioactive glass-ceramic of the quaternary P_2O_5-Na_2O-CaO-SiO_2 system (Biosilicate®, patent application WO 2004/074199) was utilized in this study. This compound was chosen as full crystallization of the material may lead to enhanced mechanical properties of the bulk material or less sharp and abrasive particles when the material is milled to obtain a powder. In disc form, this glass-ceramic has supported stable protein content and alkaline phosphatase activity, and exerts a stimulatory effect on bone cell metabolism supporting significantly larger areas of calcified matrix for up to 17 days.[2] Previous results indicate that full crystallization of bioactive glasses in a range of compositions of the P_2O_5-Na_2O-CaO-SiO_2 system may promote enhancement of *in vitro* bone-like tissue formation in an osteogenic cell culture system.

Seeding of glass-ceramic scaffold with mammalian cells

Prior to cell seeding, sterile, glass-ceramic scaffolds were thoroughly immersed and incubated in tissue culture growth medium supplemented with heat-inactivated FBS at 37°C for at least 48 hours, in order to pre-wet the scaffold and displace any air within the scaffold fabric.

To seed the scaffolds with cells, a confluent cell monolayer grown in a 25 cm^2 flask (as described above) was trypsinized, washed with fresh tissue culture medium, and centrifuged to pellet the cells. The cell pellet was then resuspended in 2 mL of fresh tissue culture medium. The pre-soaked Biosilicate® scaffold discs were placed in a 12-well tissue culture plate, with one disc per well. Discs were then seeded with cells by carefully dripping a concentrated cell suspension of 5×10^7 cells/mL over the surface of the scaffolds. Seeded scaffolds were then placed into a 37°C, 5% CO_2, 95% air, humidified incubator for approximately 1.5 hours to allow cells to adhere to the scaffold surface. For each experiment, at least four seeded discs were employed (two discs irradiated with laser and two discs as the non-irradiated controls). The non-irradiated controls were placed in wells on the same culture plate as remote as possible from the treated discs. Each of the wells was then filled with 2 ml of fresh tissue culture medium. Cell growth on all scaffolds and the in-house control wells (cells grown in wells with no scaffold) was monitored daily using an inverted light microscope and the culture medium was replenished as required. Cell viability and growth could be observed on the edges of the scaffolds using phase contrast and low light parameters. During initial experimental standardization procedures, seeded discs were also assessed for cell growth by staining with 0.1% Ponceau S (w/v) in 5% acetic acid (Sigma-Aldrich, St. Louis, MO) and compared to unseeded discs incubated in tissue culture medium in the same manner and time frame. Staining of live cells was also visually apparent with the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) and indicated cell growth over the surface of the discs at seven days post-seeding.

Laser irradiation

Twenty-four hours after the scaffold discs were seeded, cells were exposed to laser radiation. The wavelength used was 830 nm continuous [Smart Laser Medilaze, Adlaser Pty Ltd, New South Wales, Australia: 30 mW (−1.5/+4 mW), GaAlAs, 10 mm^2 beam area, 9.5° (−1.5°/+4.5°) beam divergence, 335 s irradiation per spot]. A single dose of radiation was administered at a machine-set energy density of 10 J/cm^2 as per our previous methodology.[1] To apply the laser, the irradiating probe was fixed perpendicularly above each culture well at a prescribed distance of 5 mm. Seven days after irradiation, cell proliferation was assessed. For all experiments, negative (non-irradiated) controls were incorporated and each experiment was repeated in at least triplicate for statistical accuracy.

Cell proliferation assays

Cell proliferation was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. In brief, this assay employs a colorimetric method based on the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] for determining the number of viable cells in proliferation. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. Cell proliferation was calculated from absorbance readings determined using a SpectraMax 250 spectrophotometer (Molecular Devices, Sunnyvale, CA) at an absorbance wavelength of 490 nm. This method has been successfully employed in our previous studies.[1]

Scanning electron microscopy

Standard scanning electron microscopy (SEM) protocols were utilized in this project to better determine the micro-

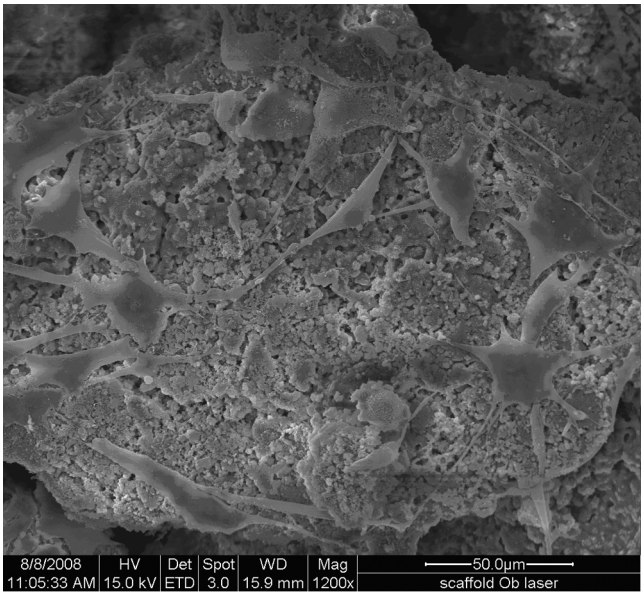

FIG. 1. Osteoblast cells on Biosilicate® scaffold.

scopic structure of the glass-ceramic both before and after cell seeding, and to enhance visualization of cell growth, migration patterns, and morphology of cells growing on the three-dimensional scaffold. Scanning electron micrographs were also examined in detail to determine if there were any morphological differences between irradiated cells seeded onto scaffolds, and those grown on scaffolds without irradiation.

Prior to SEM, seeded discs were fixed in a 4% glutaraldehyde in cacodylate buffer solution overnight. The fixed specimens were then washed thoroughly, immersed in osmium tetroxide for 30 minutes, washed in distilled water, and then dehydrated in a graded series of alcohol solutions. Specimens were then critical point-dried, mounted onto SEM stubs and sputter-coated with gold particles using a Bio-Rad SC500 sputter coater (Regents Park, Australia) at 15mAmps for 2 minutes. SEM was performed using an FEI Quanta 200 scanning electron microscope (Hillsboro, OR) at an accelerating voltage of 10 kVolts.

Results

Osteoblastic MC3T3 cells were successfully grown on discs composed of a novel glass-ceramic composite. Cells were of normal osteoblastic morphology and adhered, proliferated and migrated readily across disc surfaces. However, cell proliferation studies revealed that laser irradiation produced a 13% decrease in cell growth on the glass-ceramic discs (mean $\pm$ SD $= 0.192 \pm 0.002$) compared with control (non-irradiated) cells grown on discs (mean $\pm$ SD $= 0.22 \pm 0.002$). Figure 1 shows a scanning electron micrograph of the osteoblastic cells seeded onto the glass ceramic scaffold.

Discussion

The incorporation of cellular cues into bioscaffolds (in this case in the form of osteoblastic cells) could eliminate or reduce the need for additional growth factors and enhance scaffold/tissue compatibility. This short report has described the methods used to successfully seed osteoblasts into Biosilicate® scaffolds. Our results demonstrate that irradiation by laser phototherapy at 830 nm (10 J/cm^2 at diode) of osteoblasts on a glass-ceramic scaffold results in a small inhibition of cell proliferation compared with untreated controls. The effect was noted in each of the repeated trials. The results are in contrast to our previous work, which demonstrated an increase in cell proliferation of laser-irradiated osteoblasts grown as standard monolayers *in vitro*.[1] The reasons for the results of the present study are unclear. However, as the scaffold is composed of a glass-ceramic material, it may be influenced by the focal intensity, power output of the laser light during the treatment duration, or physical characteristics of light as photons are reflected, refracted, or absorbed in the material. Such effects may subsequently inhibit cellular migration and growth on the surface of the glass-ceramic composite. An alternative explanation is that of a crystalline substrate which was noted in all culture wells (laser and controls) at the conclusion of the experiments, suggesting a degree of non-toxic dissolution of the scaffold matrix as a result of the methods used. Further investigations into the physicochemical effects of laser light on a crystalline scaffold for use in bioengineering is warranted (such as DRX and FTIR analysis of the scaffold before and after the laser treatment).

Conclusions

Despite successful seeding of Biosilicate® scaffolds with osteoblasts, laser phototherapy resulted in a reduction in cell proliferation compared to non-irradiated controls. Future research combining laser phototherapy and glass-ceramic bioscaffolds should take into account possible interactions of the laser with matrix compounds and structure.

Author Disclosure Statement

No competing financial interests exist.

References

1. Renno, A.C.M., McDonnell, P.A., Parizotto, N.A., and Laakso, E.L. (2007). The effects of laser irradiation on osteoblast and osteosarcoma cell proliferation and differentiation *in vitro*. Photomed. Laser Surg. 25, 275–280.
2. Moura, J., Teixeira, L.N., Ravagnani, C., et al. (2007). In vitro osteogenesis on a highly bioactive glass-ceramic (Biosilicate). J. Biomed. Mater. Res. A. 82, 545–557.

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