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Effects of tricalcium silicate cements on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells in vitro

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ABSTRACT

Tricalcium silicate cements have been successfully employed in the biomedical field as bioactive bone and dentin substitutes, with widely acclaimed osteoactive properties. This research analyzed the effects of different tricalcium silicate cement formulations on the temporal osteoactivity profile of human bone marrow-derived mesenchymal stem cells (hMW-MSCs). These cells were exposed to four commercially available tricalcium silicate cement formulations in osteogenic differentiation medium. After 1, 3, 7 and 10 days, quantitative real-time polymerase chain reaction and Western blotting were performed to detect expression of the target osteogenic markers ALP, RUNX2, OSX, OPN, MSX2 and OCN. After 3, 7, 14 and 21 days, alkaline phosphatase assay was performed to detect changes in intracellular enzyme level. An Alizarin Red S assay was performed after 28 days to detect extracellular matrix mineralization. In the presence of tricalcium silicate cements, target osteogenic markers were downregulated at the mRNA and protein levels at all time points. Intracellular alkaline phosphatase enzyme levels and extracellular mineralization of the experimental groups were not significantly different from the untreated control. Quantitative polymerase chain reaction results showed increases in downregulation of *RUNX2*, *OSX*, *MSX2* and *OCN* with increasing time of exposure to the tricalcium silicate cements, while *ALP* showed peak downregulation at day 7. For Western blotting, OSX, OPN, MSX2 and OCN showed increased downregulation with increased exposure time to the tested cements. Alkaline phosphatase enzyme levels generally declined after day 7. Based on these results, it is concluded that tricalcium silicate cements do not induce osteogenic differentiation of hBM-MSCs in vitro.

1. Introduction

Bioactivity is one of the most coveted properties of tricalcium silicate cements (TSCs), and is responsible for the continuously expanding number of clinical applications of these cements in biomedical tissue engineering. By definition, bioactivity is the ability of a biomaterial to induce a specific biological response [1]. Tricalcium silicate cements are hydraulic cements the major components of which, calcium di- and trisilicates, react with water to form calcium silicate hydrate and calcium hydroxide [2]. The water that is required for these reactions may be derived in vivo from interstitial tissue fluid in the vicinity of the area of application of the TSCs.

Of particular interest to clinicians and researchers is the ability of TSCs to induce osteogenic responses when they are applied to bone defects (i.e. osteoactivity). The literature is replete with studies addressing the osteoactivity of TSCs, with the overall results being supportive of their osteogenic potential [3–11]. However, a closer inspection of this body of literature raises important concerns. For instance, the aforementioned studies employed cell lines from different species to study the osteogenic properties of TSCs. While consensus on the cytotoxicity of a biomaterial may be reached because cytotoxicity ranking is preserved among different cell lines [12–14], the same does not apply to cell differentiation studies, since different cell lines react differently to the same stimulus [4,12]. Intraspecies variation is another salient issue; for

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example, murine mesenchymal stem cells (MSCs) differ not only from human MSCs, but also between strains in their genetic marker expressions and culture behavior [15–17]. Another source of confusion arises from the frequent use of fully or partially differentiated cell lines with established secretory functions and hard tissue forming potential for analysis of the osteogenic differentiation potential of a biomaterial. Results from these studies only show how well-differentiated cells perform their normal physiological secretory functions in the presence of the biomaterial of interest; they do not provide information on how that biomaterial affects the differentiation of those cells from their immature counterparts.

Bone marrow is a promising source of MSCs for a broad range of cellular therapies in regenerative medicine. Human bone marrow-derived MSCs (hBM-MSCs) provide an excellent model for studying the in vitro osteoactivity of TSCs. They are among the most thoroughly characterized cell lines in the field of regenerative medicine [18–21]. These cells are pluripotent, undifferentiated adult stem cells with multilineage differentiation potential [22–24]. They can be harvested from the bone marrow relatively easily [25], and have the ability to differentiate into mature, specialized cells with chondrogenic, osteogenic and adipogenic lineages [22–24]. To the best of our knowledge, the osteoactive effects of TSCs on hBM-MSCs have not been studied previously. To address this gap in our knowledge and to better understand the bioactivity of TSCs, the objective of the present study was to evaluate the effects of several commercial TSC formulations on osteogenic differentiation of hBM-MSCs. Two null hypotheses were tested: (i) TSC exposure has no osteogenic effects on hBM-MSCs; and (ii) there are no differences among the different commercial TSC formulations in their prospective influences on osteogenic differentiation of hBM-MSCs.

2. Materials and methods

2.1. Materials

The main constituents and primary phases of the tested TSCs are summarized in Table 1. White (WMTA) and gray (GMTA) ProRoot MTA (Dentsply Tulsa Dental Specialties, Tulsa, OK) were mixed with distilled water. White (WMTAP) and Grey (GMTAP) MTA Plus® (Avalon Biomed Inc., Bradenton, FL) were mixed with the proprietary hydrogel supplied by the manufacturer. These TSCs were mixed in a liquid to powder ratio of 0.3 according to their respective manufacturer's instructions. The mixed materials were placed in pre-sterilized Teflon molds (5 mm diameter and 3 mm thick), covered with pre-sterilized Mylar sheets and allowed to set in a 100% humidity chamber for 24 h.

Table 1
Comparison of gray and white varieties of ProRoot MTA and MTA Plus®.

Characteristics	White ProRoot MTA	Gray ProRoot MTA	White MTA Plus®	Gray MTA Plus®
Liquid	Water		Water-based gel with water-soluble thickening agents and polymers[a]	
Powder:liquid ratio (by weight)	3:1		Variable from 1:1 to 4:1 depending on indication	
Primary Phases	$3CaO{\cdot}SiO_2$ $2CaO{\cdot}SiO_2$ Bi_2O_3[b] $3CaO{\cdot}Al_2O_3$ $CaSO_4$	$3CaO{\cdot}SiO_2$ $2CaO{\cdot}SiO_2$ Bi_2O_3[b] $3CaO{\cdot}Al_2O_3$ $CaSO_4$ $Ca_2(Al,Fe)_2O_5$[c]	$3CaO{\cdot}SiO_2$ $2CaO{\cdot}SiO_2$ Bi_2O_3[b] $3CaO{\cdot}Al_2O_3$ $CaSO_4$	$3CaO{\cdot}SiO_2$ $2CaO{\cdot}SiO_2$ Bi_2O_3[b] $3CaO{\cdot}Al_2O_3$ $CaSO_4$ $Ca_2(Al,Fe)_2O_5$[c]

[a] Contents are GRAS (generally regarded as safe).
[b] Bismuth oxide added for radiopacity.
[c] Calcium aluminoferrite is only present in the gray variety of both cements. It is involved in the hydraulic phase and is more radiopaque than calcium silicate hydrate.

To avoid the initial cytotoxic effects of TSCs as demonstrated in a previous study [26], a previously published aging/elution protocol based on the cements' specific cytotoxic profiles was adopted to render the cement discs non-cytotoxic prior to initiation of the experiment [26,27]. According to this protocol, cement discs were sterilized by UV light for 4 h, aseptically placed in their respective sterile Transwell® inserts (3.0 micron porous filter, Corning Inc., MA) and immersed in complete culture medium (CCM) for 2 weeks at 37 °C in 100% humidity. The CCM consisted of alpha minimum essential medium and 20% fetal bovine serum (Atlanta Biologicals, GA), supplemented with 2 mmol l^{-1} L-glutamine (Lonza, Wakersville, MD) and 100 U ml^{-1} penicillin G/streptomycin sulfate (Invitrogen Corp, Carlsbad, CA). The culture medium was changed every 3 days to eliminate cytotoxic eluents that may interfere with their bioactive properties.

2.2. Cell culture

Previously characterized hBM-MSCs with a CD73+/CD105+/CD45− immunophenotype [28] were used in the present study. The cells were received from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White, through a grant from NCRR of the National Institute of Health (Grant #P40RR017447). This cell line has a subpopulation of small, rapidly proliferating cells with high multidifferentiation potential that have been identified as rapidly self-renewing cells, among larger, mature MSCs with limited differentiation potential and slow proliferation rate [29]. The multilineage potential of this cell line has been confirmed in previous studies [18]. For the current experiment, cells from passages 4–6 were plated in CCM at 1×10^4 cells cm^{-2}. After 24 h, or after the cells reached ~70–80% confluence, Transwells® containing the pre-treated cement discs were placed in their respective wells, and the medium was changed to osteogenic differentiation medium (ODM). The latter consisted of the original CCM, supplemented with 50 mg ml^{-1} ascorbic acid, 10 mmol l^{-1} β-glycerophosphate and 100 nmol l^{-1} dexamethasone (Sigma–Aldrich, St. Louis, MO). The ODM was changed every 3 days.

2.3. RNA isolation and quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the relative expression of target markers of osteogenic differentiation among the test groups. The procedures were performed by isolating total RNA from hBM-MSCs after 1, 3, 7 and 10 days of exposure to the test materials. Total RNA was isolated and purified using QIAshredder and RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's recommended protocol for RNA purification from animal cells. The purity and quantity of the resultant RNA were assessed from 2 μl samples by using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE). Equal amounts of total RNA (0.1 mg RNA ml^{-1}) were then reverse-transcribed into single-stranded complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a thermal cycler with the recommended settings (25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min). The resultant cDNA was stored at −20 °C until commencement of the qRT-PCR procedures.

For qRT-PCR, alkaline phosphatase (*ALP*), osteocalcin (*OCN*), osteopontin (*OPN*), Runt-related transcription factor 2 (*RUNX2*), osterix (*OSX*) and muscle segment homeobox 2 (*MSX2*) were selected as target markers for osteogenic differentiation. Untreated