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UV photoactivation of 7-dehydrocholesterol on titanium implants enhances osteoblast differentiation and decreases *Rankl* gene expression

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ABSTRACT

Vitamin D plays a central role in bone regeneration, and its insufficiency has been reported to have profound negative effects on implant osseointegration. The present study aimed to test the in vitro biological effect of titanium (Ti) implants coated with UV-activated 7-dehydrocholesterol (7-DHC), the precursor of vitamin D, on cytotoxicity and osteoblast differentiation. Fourier transform infrared spectroscopy confirmed the changes in chemical structure of 7-DHC after UV exposure. High-pressure liquid chromatography analysis determined a 16.5 ± 0.9% conversion of 7-DHC to previtamin D_3 after 15 min of UV exposure, and a 34.2 ± 4.8% of the preD_3 produced was finally converted to 25-hydroxyvitamin D_3 (25-D_3) by the osteoblastic cells. No cytotoxic effect was found for Ti implants treated with 7-DHC and UV-irradiated. Moreover, Ti implants treated with 7-DHC and UV-irradiated for 15 min showed increased 25-D_3 production, together with increased ALP activity and calcium content. Interestingly, *Rankl* gene expression was significantly reduced in osteoblasts cultured on 7-DHC-coated Ti surfaces when UV-irradiated for 15 and 30 min to 33.56 ± 15.28% and 28.21 ± 4.40%, respectively, compared with the control. In conclusion, these findings demonstrate that UV-activated 7-DHC is a biocompatible coating of Ti implants, which allows the osteoblastic cells to produce themselves active vitamin D, with demonstrated positive effects on osteoblast differentiation in vitro.

1. Introduction

Calcitriol (1,25-dihydroxyvitamin D_3, 1,25-D_3), the biologically active form of vitamin D_3, is produced by a hydroxylation cascade, which is preceded by photochemical activation. When provitamin D_3 (7-dehydrocholesterol, 7-DHC) is exposed to ultraviolet (UV) B irradiation, it is then converted to precholecalciferol (previtamin D_3). Afterwards, this molecule is transformed into cholecalciferol (D_3) and twice hydroxylated until 1,25-D_3 is formed and released to the circulation. This biological pathway starts in the skin, where 7-DHC is UV-activated, while the rest of the process continues in liver and kidney tissues through hydroxylation reactions [1]. 7-DHC is not normally detectable in tissues and fluids of human beings except in skin, and its synthesis is decreased in the skin with aging [2–4]. Moreover, extrarenal synthesis of 1,25-D_3 is possible and feasible in other tissues or cells, such as the skin [5–7], liver [8], lymph nodes [9], activated monocytes and macrophages [10,11] and dendritic cells [12].

Local production of 1,25-D_3 in bone cells from 25-D_3 was first reported by Howard et al. in 1981 [13]. They found that primary cultures of human bone cells incubated with 25-D_3 synthesized both 1,25-D_3 and 24,25-D_3, with specific activities similar in magnitude to those of the enzymes found in kidney cells [13]. Ichikawa et al. [14] confirmed 1,25-D_3 conversion from 25-D_3 in mouse bone cells and reported the expression of the vitamin D_3 25-hydroxylase CYP27A1 mRNA in mouse osteoblasts. Recently, 25-hydroxyvitamin D_3–1-alpha-hydroxylase CYP27B1 mRNA expression in bone cells has been identified [15,16], and the role in osteoblast differentiation and mineralization of locally produced 1,25-D_3 has been evidenced [17–19].

It was demonstrated recently that the vitamin D precursor 7-DHC can be used locally to produce active vitamin D by osteoblastic cells when 7-DHC is coating a tissue culture plastic (TCP) surface and is UV-irradiated before the cell culture [20]. Hence, UV-activated 7-DHC may be a feasible approach to be used in therapeutics focused on bone regeneration, e.g., functionalizing bioactive titanium (Ti) surfaces to enhance osseointegration in compromised patients. Recently, vitamin D has been suggested to play a central role in bone regeneration, and its insufficiency has been reported to have profound negative effects on implant osseointegration [21,22]. Also a high prevalence of vitamin D deficiency has been found across all age groups in different populations studied worldwide, which results from inadequate dietary intake, together with insufficient exposure to sunlight [23–25].

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Ti is the material most commonly used for bone implants, as it has outstanding physical and biological properties, such as low density, high mechanical strength and good corrosion resistance [22]. Current dental implant research aims to produce innovative surfaces able to promote a more favorable biological response to the implant material at the bone–implant interface and to accelerate osseointegration. Provitamin D_3 coating on Ti surfaces is suggested here to have a stimulatory effect on bone cells and accelerate bone regeneration as result of active vitamin D synthesis. This coating could show several advantages over using other hydroxylated forms of vitamin D, e.g., giving the vitamin D precursor 7-DHC directly to the cells would reduce the risk of vitamin D toxicity in the target cells, as its affinity for the vitamin D receptor is lower than the affinity of 1,25-D_3 [26]. Furthermore, 7-DHC is easily available and cheaper than the other forms of vitamin D_3, so this approach would entail a lower cost.

The aim of the study was to develop and optimize a bioactive coating of Ti implants with UV-activated 7-DHC. For that purpose, different UV time exposures were analyzed, and the one that achieved the highest 25-D3 synthesis and increased osteoblast differentiation in vitro was selected. Fourier transform infrared spectroscopy (FTIR) analysis and high-pressure liquid chromatography (HPLC) were used to characterize and quantify the conversion of 7-DHC to preD_3. Cytotoxicity, alkaline phosphatase (ALP) activity, calcium (Ca) content, 25-D_3 production, gene expression of bone markers and enzymes involved in vitamin D_3 synthesis were analyzed using MC3T3-E1 cells, as in an vitro model.

2. Materials and methods

2.1. Implants and treatments

Ti disks with diameter 6.25 mm and height 2 mm were machined from cp Ti rods (grade 2) and subsequently ground, polished and cleaned as described elsewhere [27]. For the surface modification of Ti implants, a stock solution of 2 mM 7-dehydrocholesterol (7-DHC; Sigma, St. Louis, MO, USA) were prepared in absolute ethanol and filtered with a 0.22 μm pore size filter before use.

To coat the implant surfaces, 10 μl of 7-DHC dilution (to have 0.2 nmol per Ti disk) or only ethanol were left on the surfaces and allowed to air-dry for 15 min in a sterile flow bench. UV-irradiation of implants was performed with a UV lamp emitting light at a wavelength of 302 nm with an intensity of ~6 mW cm^{-2}. In a previous pilot study, it was shown that 0.2 nmol was the optimal amount of 7-DHC to be used in polystyrene TCP (30.7 mm^2 of well surface area) under UV irradiation for the production of active vitamin D in MC3T3-E1 osteoblasts [20]. The same amount of 7-DHC was applied on the surface of Ti disks in the present study, as the culture plates containing the Ti disks had the same surface area as those previously used.

Thus, different groups were prepared: (1) non-irradiated samples, 7-DHC (0.2 nmol per Ti disk), D_3 and 25-D_3 (2×10^{-2} nmol per Ti disk) and ethanol (used as control for the non-irradiated group, EtOH); and (2) UV-irradiated samples, 7-DHC (0.2 nmol per Ti disk) and EtOH (used as control for the 7-DHC-irradiated group).

2.2. FTIR analysis of 7-DHC and D_3 coating on Ti surfaces

FTIR spectroscopy in reflective mode (DRIFT; Spectrum 100, Perkin Elmer, USA) was used to analyze the effect of UV irradiation on vitamin D conversion after 0, 15, 30 and 60 min of UV irradiation. Ti disks coated with 7-DHC or D_3 were UV-irradiated as described above. An equally irradiated but untreated Ti disk was used as a background for the FTIR measurements. The spectra obtained by FTIR spectroscopy were analyzed for typical absorbances connected to changes in chemical structure of 7-DHC and D_3 after UV exposure of the surface coatings. The spectra were smoothed and baseline corrected with the Spectrum program (version 6.3.2.0151, PerkinElmer, Inc., Waltham, USA). Typical peak areas were fitted and quantified with CasaXPS (version 2.3.15, Casa Software Ltd.) for comparison.

2.3. Quantitative determination of the conversion efficiency of 7-DHC to preD_3 by HPLC

The amounts of 7-DHC and preD_3 present in the 0.2 nmol 7-DHC surface coating after 15 min of UV irradiation were quantified by HPLC. Results were compared with non-irradiated 7-DHC-coated surfaces. Pure ethanol was used as control. All solvents used were HPLC or analytical grade. Methanol (HPLC gradient grade), acetonitrile and tetrahydrofurane (both HPLC grade) were purchased from Fisher Scientific (Thermo Fisher Scientific, MA, USA). High-purity deionized Milli-Q water was obtained from a Millipore system (Millipore Corporation, Billerica, MA, USA). Absolute ethanol was purchased from Scharlab (Barcelona, Spain). Individual stock standard solutions of 7-DHC (250 μg ml^{-1}) and D_3 (10 mg ml^{-1}) were prepared in methanol and stored at −20 °C. Standard solutions of lower concentrations were obtained by diluting stock solutions with methanol.

The coating of each surface was extracted by adding 100 μl of methanol/acetonitrile/tetrahydrofurane/water (67:16:2:15, v:v) to each well and shaking the plate for 2 min at 20 rpm. The content of three replicate wells was mixed to give a sample of ~300 μl. An aliquot of 100 μl of the sample was injected into the HPLC system. Two replicate samples were prepared and analyzed for each group.

The analysis were carried out using a Waters liquid chromatographic system (Milford, MA, USA), equipped with a refrigerated automatic injector WISP700 and a 600 pump system, connected to a Waters 996 photodiode array detector. The Empower software was used for instrument control and data analysis. Detection was carried out at 282 nm.

A Nova Pak C18 column (Waters) was used to separate sample components before detection. The column temperature was set to 30 °C. Two solvents were used in gradient elution mode as the mobile phase: A, methanol/acetonitrile/tetrahydrofurane/water (67:16:2:15, v:v); and B, methanol/acetonitrile/tetrahydrofurane (75:20:5, v:v). Solvents A and B were vacuum-filtered through a nylon membrane (0.45 μm pore diameter) and degassed before use. The mobile phase flow rate was 1 ml min^{-1}. The binary gradient used was as follows: from 5% B to 90% B in 3 min, held for 9.5 min at 90% B, from 90% B to 5% B in 1 min and equilibrated between injections at the initial conditions for 5 min (total run time = 15 + 5 min equilibration between injections).

Quantification was performed by integration of the peak area of the corresponding analyte and interpolation of the peak area in 7-DHC or D_3 standard curves.

2.4. Cell culture

The mouse osteoblastic cell line MC3T3-E1 (DSMZ, Braunschweig, Germany) was chosen as an in vitro model. Cells were routinely cultured in α-MEM (PAA Laboratories GmbH, Pasching, Austria), which contains ascorbic acid (45 μg ml^{-1}) and sodium dihydrogen phosphate (140 mg l^{-1}), and supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and antibiotics (50 IU penicillin ml^{-1} and 50 μg streptomycin ml^{-1}) (Sigma, St. Louis, MO, USA) under standard cell culture conditions (at 37 °C in a humidified atmosphere of 5% CO_2). Under these conditions, these cells are able to differentiate and