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Electrical stimulation of adipose-derived mesenchymal stem cells in conductive scaffolds and the roles of voltage-gated ion channels

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ABSTRACT

Since electrical stimulation (ES) can significantly accelerate bone healing, a conductive scaffold that can deliver ES locally at the defect site is desirable for bone defect therapy. Herein, an electrically conductive scaffold was prepared via incorporation of polypyrrole (PPY) in a polycaprolactone (PCL) template scaffold. *In vitro* tests with mouse osteoblasts indicate that the PPY/PCL scaffold has good biocompatibility, and is suitable for use as an ES substrate. When human adipose-derived mesenchymal stem cells (AD-MSCs) were cultured in the PPY/PCL scaffold and subjected to 200 μA of direct current for 4 h per day for 21 days, the amount of calcium deposited was 100% higher than that without ES. When these cells were subjected to ES together with blockers of voltage-gated calcium (Ca_v^{2+}), sodium (Na_v^+), potassium (K_v^+), or chloride (Cl_v^-) channels, the ES-induced enhancement of AD-MSCs' functions was reduced with Na_v^+, K_v^+, or Cl_v^- blockers and completely nullified with Ca_v^{2+} blocker. These results indicate that ion fluxes through these channels activated by ES induce different cascades of reactions in the cells, which subsequently regulate AD-MSCs' functions, and Ca_v^{2+} plays a more critical role than the other three channels. Our results further the current understanding of the mechanisms by which ES regulates stem cells' behavior, and also showed that the conductive PPY/PCL scaffold with application of ES has good potential in bone defect therapy.

Statement of Significance

In this work, an electrically conductive and biocompatible scaffold was prepared by incorporating polypyrrole in a polycaprolactone template scaffold. Application of 200 μA direct current for 4 h per day to human adipose derived-mesenchymal stem cells cultured on this scaffold promoted migration of these cells into the inner region of the scaffold and enhanced their osteogenic differentiation. The roles of voltage-gated ion channels (Ca_v^{2+}, Na_v^+, K_v^+ and Cl_v^-) in osteogenic differentiation stimulated by the electric current were investigated. The results from these experiments further the current understanding of the mechanisms by which electrical stimulation regulates stem cells' behavior, and also show that the polypyr role–polycaprolactone scaffold with application of electrical stimulation has good potential in bone defect therapy.

1. Introduction

Over two million bone graft transplantations are conducted worldwide annually to address bone defects arising from major trauma, infection, and other causes [1]. However, the commonly used procedures, autografting, allografting and xenografting, suffer from a number of disadvantages, such as lack of supply of tissue for grafting, donor site morbidity, and immune rejection (for allografting and xenografting) [2]. As a result of these shortcomings, research interest has shifted toward tissue-engineered bone constructs comprising suitable scaffolds and viable osteogenic cells [3–5].

Local stimulation of cells in scaffolds is beneficial for bone tissue engineering, and different strategies have been investigated for this purpose. The most common technique is incorporation of bioactive agents such as growth factors and extracellular matrix components in the scaffold [5]. However, the incorporated

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bio-agents are usually expensive, and may result in adverse side effects if uncontrolled release occurs in the human body [6]. Besides incorporation of bio-agents, physical cues such as electrical stimulation (ES) has also been used to regulate the functions of cells in scaffolds [7,8]. Since the discovery of bioelectric property of bone in 1950s, ES has been used clinically to treat bone fractures and enhance spinal fusions [9]. Although the stimulatory effects of ES on bone cells have been demonstrated, the combined use of ES and scaffolds for localized and guided bone regeneration is still a major challenge since the mechanisms by which ES interacts with cells remain far from being understood [10].

Due to the lack of understanding of these mechanisms, ES-based studies have been plagued with uncontrolled variations in experimental design, and inconsistent or controversial results [7,11,12]. This significantly hinders further development of ES techniques for clinical applications. It has been proposed that ES can elevate cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in osteoblasts, which subsequently regulates osteoblast functions via calmodulin pathways [13]. Such elevation in $[Ca^{2+}]_i$ was believed to be a result of the activation of voltage-gated Ca^{2+} channel (Ca_V^{2+}) by external ES, which increases the influx of Ca^{2+} into osteoblasts [14].

Compared with osteoblasts, mesenchymal stem cells (MSCs) are much more frequently used in bone tissue engineering due to its self-renewal and multipotent differentiation ability [15]. MSCs can be isolated from different tissue sources, and the most commonly-used type is bone marrow-derived MSCs (BM-MSCs). Nevertheless, adipose-derived mesenchymal stem cells (AD-MSCs) have attracted much attention in recent years since the harvesting procedure is less invasive, and these cells have better proliferation and differentiation ability as compared to BM-MSCs [16]. Enhanced osteogenic differentiation of AD-MSCs by ES has been reported [17,18]. However, to the best of our knowledge, no studies have been conducted to reveal the mechanisms for this phenomenon. Based on the proposed role of Ca_V^{2+} in the interaction between ES and osteoblasts (as mentioned above), it is reasonable to hypothesize that external ES may also activate Ca_V^{2+} in AD-MSCs, and subsequently affect the growth and differentiation of AD-MSCs. AD-MSCs also have other types of voltage-gated ion channels (VGICs) such as voltage-gated Na^+ channels (Na_V^+), voltage-gated K^+ channels (K_V^+) and voltage-gated Cl^- channels (Cl_V^-) [19]. Thus, it is possible that these channels may also be activated by external ES, and the fluxes of ions through the activated VGICs subsequently modulate AD-MSCs' functions.

To verify this hypothesis, a conductive scaffold was prepared by incorporating polypyrrole (PPY), an electrically conducting polymer, into a polycaprolactone (PCL) template scaffold for local delivery of ES to AD-MSCs cultured in the scaffold. Since the PPY/PCL scaffold is conductive, an applied current during the ES experiments would pass mainly through the scaffold instead of the cell culture medium to minimize electrolysis of the medium which may produce cytotoxic agents such as reactive oxygen species [20]. To investigate the possible roles of VGICs during ES, AD-MSCs seeded in the scaffold were treated with different VGIC blockers plus ES, and the results obtained with and without blockers were compared.

2. Materials and methods

2.1. Materials

PCL (Mn of 42,500), pyrrole, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 4,4′-diisothiocyano-2,2′-stil benedisulfonic acid (DIDS), nifedipine (Nif), 4-aminopyridine (4-AP) and 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) were purchased from Sigma–Aldrich, Singapore. Amiloride hydrochloride (Am) was purchased from Merck Millipore, Singapore. Pyrrole was distilled twice before use. Mouse osteoblasts (MC3T3-E1 subclone 14) were obtained from American Type Culture Collection (ATCC, USA). AD-MSCs (specified to be from a 40 years old female) were purchased from Lonza, Singapore. All other chemicals, if not specified, were purchased from Sigma–Aldrich, Singapore.

2.2. Preparation of PPY/PCL scaffold

PCL template scaffolds were prepared by salt leaching in combination with phase separation technique according to a reported procedure with minor modification [21]. The details are given in the Supplementary information. The PCL scaffold (8 mm × 8 mm × 4 mm) was then used as a template to prepare the PPY/PCL scaffold. The PCL template scaffold was first immersed in ethanol for 1 h. It was then taken out and the ethanol on the surface of the scaffold was removed using filter paper. The scaffold was then immediately immersed in 4 mL of a freshly prepared reaction mixture containing $FeCl_3$ and pyrrole at a molar ratio of 2.38:1 in deionized (DI) water. Different weight ratios of pyrrole in the reaction mixture to the PCL template scaffold (0.05, 0.1, 0.15, 0.2, and 0.25) were tested. After reaction for 24 h under continuous shaking at 150 rpm, the scaffolds were washed thoroughly with DI water followed by freeze-drying.

2.3. Scaffold characterization

The scaffold was weighed before and after PPY incorporation to calculate the weight percentage of PPY in the so-obtained PPY/PCL scaffold. Electrical conductivity of the PPY/PCL scaffolds was measured by a four-point probe (Signatone, S-302-4, USA), and electrical resistance was measured using a Fluke 175 True RMS digital multimeter (Fluke, Singapore). Static apparent contact angle of DI water on the scaffold was measured by a telescopic goniometer (Rame-Hart, USA). The morphology and pore size of the scaffolds were characterized by scanning electron microscopy (SEM, JSM-5600LV, JEOL, Japan). Scaffold porosity and water absorption were measured using the methods reported in the literature [22]. X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Kratos AXIS Ultra DLD spectrometer (Kratos, UK) with a monochromatized Al Kα X-ray source (1486.7 eV photons). *In vitro* degradation was carried out by immersion of the scaffolds in phosphate buffered saline (PBS) at 37 °C for up to 10 weeks and compressive modulus of the scaffolds were measured by an Instron 5543 testing machine (Instron, USA). The details of these scaffold characterization experiments are given in the Supplementary information.

2.4. Cell culture

MC3T3-E1 osteoblasts were cultured in Minimum Essential Medium Eagle Alpha medium (Gibco®, Life Technologies, Singapore) supplemented with 10% fetal bovine serum (FBS; ATCC, USA), 100 unit/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Singapore). AD-MSCs were cultured in AD-MSC basal medium supplemented with AD-MSC-GM SQ kit (Lonza, Singapore). Cell cultures were maintained at 37 °C under a humidified atmosphere of 5% CO_2 in air. For osteoblasts, the medium was changed every 2 days, while for AD-MSCs, the medium was refreshed every 3 days as per the instructions provided with the medium. Both osteoblasts and AD-MSCs were passaged when the culture was 80% confluent, and cells at passage number less than 10 were used for the subsequent experiments.