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Nitric oxide production by endothelial cells derived from blood progenitors cultured on NaOH-treated polycaprolactone films: A biofunctionality study

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Abstract

Poly(ε-caprolactone) (PCL) is a biodegradable polyester whose biocompatibility has been widely demonstrated both in vivo and in vitro. In the last few years, our group has confirmed that NaOH-treated PCL films can serve as a suitable biomaterial for vascular tissue engineering by supporting the culture of primary vascular cells and, more recently, endothelial-like EC_2 cells derived from endothelial progenitor cells (EPC). In the present study, NO production in basal conditions and after stimulation with different agents has been evaluated and related to the reactive oxygen species (ROS) content and the intracellular calcium levels on EC_2 cells cultured on NaOH-treated PCL films. The results obtained demonstrate that EC_2 seeded on NaOH-treated PCL films enhance the basal NO levels and show a faster, more intense response to physiological stimuli such as VEGF, bradykinin and thrombin than vein endothelial cells (ECv). This result could be indicative of a better capacity of EC_2 cells to maintain their endothelial functionality when seeded on polymers. On the other hand, the culture of both EC_2 and ECv cells on NaOH-treated PCL films induces a significant increase in both ROS content and intracellular calcium that is balanced out through the stimulation of NO production in these cells. In conclusion, these results demonstrate the ability of NaOH-treated PCL films to support endothelial cell production of nitric oxide and reinforce the idea of considering the endothelial-like EC_2 cells derived from blood progenitors as an adequate source of endothelial cells to functionalize vascular grafts. Furthermore, NaOH-treated PCL films could be considered as a promising cellular NO production-inducing biomaterial for vascular tissue engineering applications.

Keywords: Endothelial progenitor cell; Poly(ε-caprolactone); Nitric oxide; Reactive oxygen species; Intracellular calcium

1. Introduction

Poly(ε-caprolactone) (PCL) is a biodegradable polyester whose biocompatibility has been widely demonstrated both in vivo and in vitro [1–5]. Nevertheless, its considerable hydrophobicity has hindered its potential for biomedical applications. In the last few years, our group has confirmed that the treatment with NaOH improves significantly the hydrophilicity of the PCL films [6]. These results have allowed NaOH-treated PCL films to be considered as a suitable biomaterial for vascular tissue engineering by supporting the culture of primary vascular cells [6,7] and, more recently, of endothelial-like EC_2 cells derived from endothelial progenitor cells (EPC) [8]. EPC are considered as a promising alternative to primary endothelial cells for the development of vascular grafts [9]. These cells meet important requirements for tissue engineering applications,

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namely, they have therapeutic potential, a highly proliferative and antithrombogenic behaviour [10], and can be obtained easily from peripheral blood [11,12].

Preliminary studies on the production of nitric oxide (NO) revealed a significant increase when endothelial cells derived from EPC were cultured on PCL films [8]. NO is a signalling molecule produced in endothelial cells by the endothelial isoform of nitric oxide synthase (eNOS), a calcium–calmodulin-sensitive enzyme. In the cardiovascular system, NO triggers a cascade of events leading to smooth muscle relaxation and a subsequent decrease in blood pressure. Furthermore, it prevents blood coagulation and thrombus formation [13]. NO is also involved in the immune response and serves as a potent neurotransmitter at the neuron synapses. In normal physiological conditions, NO has an anti-inflammatory effect, but it could also be a pro-inflammatory mediator in abnormal situations [14]. On the other hand, NO serves as a central regulator of oxidant reactions and diverse free radical-related disease processes [15]. NO production or exogenous administration of NO-donating molecules can play a protective role by inhibiting oxidant-related mechanisms. Thus, the severity of the ischemic injury depends on the balance between NO and free oxygen radicals [16]. In recent years, researchers in tissue engineering have worked hard to develop new materials which could enhance or inhibit NO production because of its relevance to physiological functions. A nitric oxide-releasing polyurethane–polyethylene glycol copolymer [17] and an NO-releasing poly(vinyl chloride) polymer [18] have been recently described as biomaterials which enhance endothelialization and decrease platelet adhesion.

In the present study, the biofunctionality of EC_2 endothelial cells cultured on NaOH-treated PCL films has been evaluated by measuring the NO production in basal conditions and after stimulation with vascular endothelial growth factor, bradykinin and thrombin. Synthesis and/or release of NO by endothelial cells are calcium-dependent processes and free radical production is correlated to cytosolic calcium increase. For these reasons, the intracellular content of both reactive oxygen species and calcium has been also analyzed using EC_2 cells cultured on NaOH-treated PCL films.

2. Materials and methods

2.1. Membrane preparation and characterization

The PCL films were prepared by hot pressing under 20 tons at 100 °C for 2 min as previously described [19]. PCL was directly used as purchased (Sigma–Aldrich Corporation, St. Louis, MO, USA; $M_w = 65{,}000\ g\ mol^{-1}$), placed between two steel plates covered with an aluminium foil and pressed. The PCL films obtained were characterized as previously described [6]. In order to improve the surface hydrophilicity, some films were immersed in a 2N NaOH solution for 2 h (PCL–NaOH or NaOH-treated PCL films) and then cut in circular pieces and sterilized for 30 min by ultraviolet irradiation in a laminar flow chamber. Tissue culture plastic (TCP) was used as a control surface for all the experiments except for confocal microscopy studies, for which glass coverslips were used.

2.2. Isolation and culture of endothelial cells from pig vena cava on NaOH-treated PCL films

Primary vascular endothelial cells (ECv) were isolated from pig inferior vena cava as previously described [6]. Briefly, a segment of vein (5 cm of length) was clamped in one of its ends and filled with 5 ml of 0.1% collagenase IA (Sigma–Aldrich Corporation, St. Louis, MO, USA) in sterile phosphate-buffered saline (PBS) containing 1.6 mM $CaCl_2$. The other end was then clamped, and the vein fragment was incubated for 15 min at 37 °C. After incubation, the collagenase solution containing EC was collected and the luminal surface was washed with 5 ml of Dulbecco's modified Eagle's medium (Sigma–Aldrich Corporation, St. Louis, MO, USA). The EC suspension was centrifuged at 208*g* for 10 min. The cell pellet was seeded in culture flasks under CO_2 (5%) atmosphere and at 37 °C with endothelial growth medium-2 (EGM-2) (Lonza, Walkersville, MD, USA). For the experiments, ECv were cultured into 6-well culture plates with or without NaOH-treated PCL films at a density of 2×10^4 cells cm^{-2} in EGM-2. The experiments described were performed in accordance with the CEE (86/609) and Ministerio de Agricultura (Spain, BOE 223/1988, 265/1990) guidelines for care and use of laboratory animals.

2.3. Obtention and culture of mature endothelial cells derived from peripheral progenitor cells on NaOH-treated PCL films

Mature endothelial cells (EC_2) were obtained from EPC as previously described [8,20]. Briefly, whole pig blood was diluted (1:1) in PBS with 0.1% bovine serum albumin (BSA) and 0.6% sodium citrate. Mononuclear cells (MNC) were isolated using a density gradient formed with Histopaque-1077 solution (Sigma–Aldrich Corporation, St. Louis, MO, USA) in Accuspin™ tubes (Sigma–Aldrich Corporation, St. Louis, MO, USA). The samples were centrifuged at 800*g* for 30 min at room temperature. The MNC layer was carefully collected and seeded in EGM-2, without hydrocortisone, in F75 polystyrene culture flasks (Corning, NY, USA) at a density of $2–3 \times 10^5$ cells cm^{-2} under a CO_2 (5%) atmosphere and at 37 °C. The culture medium was replaced at 96 h and then every 48 h until confluence. Confluent cultures of EPC were maintained in EGM-2 until cells acquired the cobblestone morphology characteristic of mature endothelial cells (7 weeks). For the experiments, and after confirmation of their endothelial phenotype, EC_2 cells (late differentiation stage) were cultured in 6-well culture

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