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Enhancement of fibroblastic proliferation on chitosan surfaces by immobilized epidermal growth factor

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Abstract

Chitosan membranes were modified with mouse epidermal growth factor (EGF) by a photochemical technique. Photochemical immobilization was performed via a two-step process, in which EGF was first reacted with a heterobifunctional cross-linker sulfo-SANPAH (sulfosuccinimidyl 6(4′-azido-2′-nitrophenyl-amino)hexanoate) and then immobilized on the chitosan membrane by UV irradiation. The success of immobilization process was checked by Fourier transform infrared attenuated total reflection spectroscopy and X-ray photoelectron spectroscopy. Atomic force microscopy was used to evaluate the surface topography. The mitogenic effect of the EGF-modified chitosan membrane was investigated using mouse fibroblasts (L929 cell line), and cell proliferation was investigated by MTT and crystal violet assays. The results obtained from cell culture experiments showed that immobilized EGF stimulated fibroblast growth on chitosan membranes, and a considerable difference in cell proliferation was detected on EGF-modified chitosan membranes.

Keywords: Chitosan membrane; EGF; Photochemical technique; Cell proliferation; Mouse fibroblasts

1. Introduction

Growth factors are protein fractions that induce a change in cellular function by transducing proliferation or differentiation signals and play a comprehensive role in the modulation of tissue growth and development [1]. Mitogenic activity is one of the principal mechanisms of growth factor action by which the rate of cell turnover is increased. It is proposed that mitosis is stimulated by growth factors through secondary intracellular messengers that can upregulate DNA synthesis, resulting in mitosis [1]. The growth factor molecules interact with the cognate receptor on the cell surface to form a complex, and the complex aggregates on the cell surface before being internalized into the cell. The internalized complex is then decomposed in the lysosomes [2]. As a result of this rapid internalization process the continuous effect of growth factor on cell metabolism is decreased. It has been discovered that the events on the cell surface are sufficient to transduce the signal to the cellular nucleus. Hence biomaterials with immobilized growth factors have the potential to regulate cellular functions [2–4]. Ito et al. investigated the regulation of cellular functions by immobilized growth factors on various synthetic materials [5–8]. They found that immobilized insulin enhanced cell growth, and they observed higher mitogenic activity for fibroblasts on insulin-immobilized poly(acrylic acid) (PAA) and polystyrene (PS) films. The uptake of insulin by the cells was inhibited after immobilization and the activity prolonged when compared with native insulin. Similar results were obtained by using immobilized epidermal growth factor (EGF) on a poly(methyl methacrylate) (PMMA) surface where DNA synthesis of Chinese hamster ovary cells (CHO) was stimulated in the presence of EGF [9].

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In this study, EGF, which is a 6 kDa mitogenic polypeptide, was covalently immobilized on chitosan membranes by using photochemical immobilization. EGF is a single polypeptide consisting of 53 amino acid residues, and is known to induce precocious eyelid opening and incisor eruption resulting from epidermal growth and keratinization [10,11]. It is present in many cell types including fibroblasts, vascular endothelial cells and epithelial cells, and various body fluids such as blood, urine and saliva [12]. EGF is a strong mitogen and stimulates mitosis through the regulation of intracellular calcium and pH [1].

Chitosan, which is a polycationic biopolymer obtained by alkaline deacetylation of chitin, is receiving a great deal of attention for tissue engineering applications due to its cheap and environmental-friendly production, good bacteriostatic effects and high biocompatibility [13–17]. As its biological, physical and chemical properties can be controlled and engineered under mild conditions, chitosan has been an appealing choice for various applications in tissue engineering, such as wound-healing management [18,19]. A very promising approach to achieve stimulated cell response on chitosan for tissue engineering applications involves the immobilization of bioactive molecules. To date, cell-adhesive peptides have been immobilized on chitosan membranes or scaffolds [20,21] by using various techniques such as imide-bond-forming reactions [21] to stimulate cell adhesion. In recent studies, the immobilization of biological molecules has been realized by using the photochemical immobilization technique, based on phenyl-azido chemistry [22–24]. In those applications, biological molecules were first attached to water-soluble functional moieties to form phenyl-azido derivatized molecules which were then grafted to material substrates by ultraviolet (UV) irradiation. In particular, Chung et al. recently applied the photochemical immobilization technique to graft cell-adhesive peptides on to chitosan membranes [25].

In the present paper, we have applied the above-mentioned technique to immobilize EGF on chitosan membranes by inducing reactions between the azido group and the hydroxyl group of the chitosan structure [25]. The mitogenic activity of EGF was combined with chitosan in order to synthesize a bioactive material for stimulated fibroblast growth in tissue engineering applications. Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) were used to characterize the surface of unmodified chitosan and EGF-modified chitosan membranes. Cell culture studies were performed with L929 mouse fibroblasts and the stimulated cell growth was investigated by 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) and crystal violet assays.

2. Materials and methods

2.1. Materials

EGF was purchased from Sigma (Germany). Chitosan derived from crab shell with a minimum degree of deacetylation of 85% was purchased from Sigma (Cat. No. C 3646, Germany). The photochemical cross-linker sulfosuccinimidyl 6(4′-azido-2′-nitrophenyl-amino)hexanoate (sulfo-SANPAH) with a formula weight of 492.4 was obtained from Pierce (USA). Phosphate-buffered saline (PBS) tablets were purchased from Sigma (Germany). Tissue culture clusters, flasks and plates were purchased from Nunc (Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 0.01% trypsin/10 mM EDTA were obtained from Sigma (Germany).

2.2. Immobilization of EGF on chitosan membranes

1.0 g of chitosan was first dissolved in 100 ml of 1% (v/v) acetic acid solution, poured into glass Petri dishes and air dried. After the drying stage, the membrane was immersed in a NaOH aqueous solution (1 N) to neutralize the remaining acetic acid. The immobilization of EGF on chitosan membranes was done by using a photochemical technique. In the first-step, EGF was coupled with the photoreactive molecule sulfo-SANPAH in order to prepare photoreactive EGF molecules. For this purpose, EGF (1 ml, 0.01 mg ml^{-1} in PBS, pH 7.4) and sulfo-SANPAH (1 ml, 1 mg ml^{-1} in PBS, pH 7.4) were reacted in the dark at 4 °C for 8 h. The resulting azidophenyl-derivatized EGF (500 μl) was then poured onto the chitosan membranes. The membranes were removed from the solution after 1 h and air dried at room temperature. The dried membranes were irradiated by UV light for 4 min to induce the photochemical binding of EGF on the chitosan surfaces. The membranes were fully rinsed with distilled water after the immobilization process in order to remove the unreacted reagents, and the absence of released EGF was confirmed by UV absorption spectroscopy at 280 nm.

2.3. Surface characterization of chitosan membranes

2.3.1. FTIR-ATR spectroscopy

FTIR-ATR spectra for the unmodified (CHI) and EGF-modified (CHI-EGF) chitosan membranes were obtained by using a Perkin–Elmer Spectrum One IR spectrometer (USA). The spectra of samples were measured over the wavelength range 400–4000 cm^{-1} and analyzed with a standard software package (Perkin–Elmer, Spectrum One).

2.3.2. X-ray photoelectron spectroscopy

XPS analysis was performed by using a PHI 5600 Multi Technique Spectrometer equipped with dual Al/Mg anode, hemispherical analyzer and electrostatic lens system (Omni Focus III). The electron take-off angle was typically 45°, corresponding to a sampling depth of about 6 nm. The analyzer was operated in FAT mode using Al $K_{\alpha 1,2}$ radiation with pass energies of 187.5 eV for survey scans and 11.75 eV for the detailed scans. The binding energy of 285.0 eV of the main C 1s component (assigned to C–C and C–H bondings) was used as a reference to calibrate the energy position of the various peaks.

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