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Bacterial Cellulose Modified Using Recombinant Proteins to Improve Neuronal and Mesenchymal Cell Adhesion

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A wide variety of biomaterials and bioactive molecules have been applied as scaffolds in neuronal tissue engineering. However, creating devices that enhance the regeneration of nervous system injuries is still a challenge, due the difficulty in providing an appropriate environment for cell growth and differentiation and active stimulation of nerve regeneration. In recent years, bacterial cellulose (BC) has emerged as a promising biomaterial for biomedical applications because of its properties such as high crystallinity, an ultrafine fiber network, high tensile strength, and biocompatibility. The small signaling peptides found in the proteins of extracellular matrix are described in the literature as promoters of adhesion and proliferation for several cell lineages on different surfaces. In this work, the peptide IKVAV was fused to a carbohydrate-binding module (CBM3) and used to modify BC surfaces, with the goal of promoting neuronal and mesenchymal stem cell (MSC) adhesion. The recombinant proteins IKVAV-CBM3 and (19)IKVAV-CBM3 were successfully expressed in E. coli, purified through affinity chromatography, and stably adsorbed to the BC membranes. The effect of these recombinant proteins, as well as RGD-CBM3, on cell adhesion was evaluated by MTS colorimetric assay. The results showed that the (19)IKVAV-CBM3 was able to significantly improve the adhesion of both neuronal and mesenchymal cells and had no effect on the other cell lineages tested. The MSC neurotrophin expression in cells grown on BC membranes modified with the recombinant proteins was also analyzed.
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Keywords: bacterial cellulose, recombinant protein production, CBM3, cell adhesion, MSCs neurotrophin expression

Introduction

Nerve tissue engineering is a rapidly expanding area of research providing a new and promising approach to nerve repair and regeneration.[1] A key challenge in nerve regeneration resides in the identification of biomaterials able to provide a continuous path for regeneration, promoting the infiltration of cells and the secretion of inductive factors for axonal elongation.[1] In addition, an understanding of neuronal mechanisms and cell behavior in contact with different biomaterials is essential for implementation of advanced prostheses.[2]

A wide variety of biomaterials and bioactive molecules have been exploited in the field of tissue engineering.[3–9] A great number of cell adhesion motifs have been identified and used in biopolymer structures to mediate cell attachment, such as RGD (Arg-Gly-Asp) and IKVAV (Ile-Lys-Val-Ala-Val), which are bioactive cell adhesion motifs found in extracellular matrix (ECM) proteins such as fibronectin and laminin.[10–17] Among the biomaterials, biological scaffolds, composed of natural polymers combined with ECM molecules, have been shown to facilitate the constructive remodeling of several tissues by the establishment of an appropriate environment essential for the regulation of cell processes.[18,19] In recent years, bacterial cellulose (BC) has emerged as a promising biomaterial in tissue engineering. BC is a linear glucose polymer secreted by *Gluconacetobacter xylinus* composed of a nanofiber network, with appealing properties that include high crystallinity, wettability, high tensile strength, and moldability in situ, and can be produced relatively simply.[20] Despite having chemical properties identical to those of plant cellulose, BC is produced in a pure form, free of other polymers and its macromolecular properties and structure are also different.[21,22] These characteristics, in combination with its biocompatibility, make BC an ideal material for tissue engineering constructs.

Previous studies reported the surface modification of biomaterials by immobilization of proteins as a strategy to control and guide, with high selectivity, the interactions between cells and materials.[10,23,24] One approach to achieve this goal involves the incorporation of small cell-binding peptides into biomaterials via chemical or physical modification.[25] As an alternative to peptide chemical grafts, the use of recombinant proteins containing carbohydrate binding domains (CBMs) fused to the bioactive peptides represents an attractive way to specifically adsorb these peptides onto cellulose surface.[11,26] The CBM3 from the cellulosomal-scaffolding protein A of *Clostridium thermocellum* has high affinity for cellulose, particularly crystalline cellulose.[27]

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Table 1. Primers Used for Cloning the DNA Sequences Encoding the Peptides fused To CBM3

Construct	Primers
Forward (19)IKVAV-Linker-CBM3	5′ CTA **GCT AGC** TGT TCA AGG GCT AGG AAG CAG GCT GCT TCA ATA AAG GTA GCT GTA TCA GCT GAT AGG ACA CCG ACC AAG GGA G 3′
Forward IKVAV-Linker-CBM3	5′ CTA **GCT AGC** ATA AAG GTA GCT GTA ACA CCG ACC AAG GGA G 3′
Reverse (for all)	5′ CAC **CTC GAG** TTC TTT ACC CCA TAC AAG AAC 3′

Besides increasing the cell–material interaction, an ideal scaffold should maintain the cells in viable and functional condition; in addition, cells should be capable of secreting growth factors that enhance tissue regeneration. Neural tissue engineering strategies focusing on the development of scaffolds that artificially generate favorable cellular microenvironments, promoting regeneration, particularly in conjunction with stem cells, have generated promising results.[28] The use of stem cells in tissue engineering constructs is a promising strategy, because these cells can express a variety of growth factors important for tissue regeneration and cell differentiation. The transplantation of stem cells can provide a neuroprotective environment by secreting cytokines and neurotrophic factors.[29] For example, neurotrophins such as nerve growth factor (NGF) are a family of proteins that induce the survival, development, and function of neurons.[30,31] Clinical improvement was reported when MSCs were administered into animal models of various neurologic disorders, and the therapeutic effect was related to the secretion of neurotrophic factors such as NGF and brain-derived neurotrophic factor (BDNF), along with other factors secreted by these cells.[29]

The main purpose of this work was to produce recombinant proteins containing a bioactive peptide fused to the CBM3 to functionalize BC surfaces and improve biocompatibility. Neuronal and MSCs adhesion and viability were evaluated on these modified surfaces. The MSC neurotrophin expression by cells growth on BC membranes modified with the recombinant proteins was also verified, to identify potential factors contributing to a microenvironment that promotes neuronal regeneration.

Materials and Methods

Production of BC

The pellicles of BC were produced by the *G. xylinus* (ATCC 53582) cultured in Hestrin & Schramm medium, into 24-wells polystyrene plates (800 μL per well), for 4 days at 30°C, in static culture. The membranes were purified with 2% sodium dodecyl sulfate (SDS) overnight, then washed with distilled water until the complete removal of SDS, and immersed in a 4% NaOH solution, shaking for 90 min at 60°C. After adjusting the medium to neutral pH, the pellicles were autoclaved in phosphate buffered saline (PBS) and stored at 4°C.

Cloning, expression, and purification of recombinant proteins

In this work, we produced two recombinant proteins consisting of different peptides fused to CBM3, including the native glycanase linker: IKVAV-Linker-CBM3 and (19)IKVAV-Linker-CBM3 (CSRARKQAASIKVAVSADR-Linker-CBM3) corresponding to the extended amino acid sequence based on the proteolytic laminin fragment PA-22 containing the sequence IKVAV.[32–36] The linker sequence contains 40 amino acids. The cloning, expression, and purification of recombinant proteins were developed following the protocol described by Andrade et al.[11] Briefly, coding sequences were obtained by PCR using the pET21a-CBM3 vector and the primers shown in Table 1, including *NheI* and *XhoI* restriction sites (in bold). The PCRs conditions used were: preheating at 95°C for 2 min, 40 cycles at 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s, followed by an elongation cycle at 72°C for 10 min. The PCR products were analyzed by agarose gel, purified (Quiagen), digested with *NheI* and *XhoI* restriction enzymes, and cloned into the expression vector pET21a (Novagen), previously digested with the same restriction enzymes. This vector includes a C-terminal His6-tag in the recombinant proteins to allow the purification by immobilized metal ion affinity chromatography (IMAC) using a 5 mL nickel His-Trap column (GE Healthcare). The *E. coli* XL1 Blue (Stratagene) was used as cloning strain, and the integrity of cloned PCR products was verified by DNA sequencing.[37]

Production and purification of recombinant proteins

Recombinant proteins were produced using *E. coli* BL21 (DE3) cells transformed with the expression vectors containing the different coding sequences, pET21a-CSRARKQAA-SIKVAVSADR-Linker-CBM3, pET21a-IKVAV-Linker-CBM3, and pET21a-KHIFSDDSSE-Linker-CBM3, grown at 37°C in LB medium supplemented with ampicillin (100 μg/mL). The RGD-Linker-CBM3 recombinant protein, cloned by Andrade et al.,[20] was also produced and used in the cell cultivation studies. Cultures were induced with isoPropyl β-D-1-thiogalactopyranoside (IPTG, Invitrogen) at 1 mM. Five hours after induction, the cells were separated from the culture medium by centrifugation (13,000*g*, 10 min), resuspended in buffer A (20 mM Tris, 20 mM NaCl, 5 mM CaCl2, pH 7.4), and then lysed by sonication. The soluble and insoluble fractions were separated by centrifugation (15,000*g*, 4°C, 30 min). The purification was made by affinity chromatography, using a HisTrap™ HP (GE Healthcare). Imidazole was added to the cell lysate (40 mM final concentration), and the pH was adjusted to 7.4 before its application on the nickel column. After purification, proteins were dialyzed against the buffer A, sterilized by filtration (0.22 μm), and stored at −20°C before use. Recombinant proteins were analyzed by 12% SDS-PAGE (SDS–polyacrylamide gel electrophoresis)[38] stained with Coomassie blue.

Adsorption assay

The wells of a 24-well polystyrene plate were covered with BC pellicles; the recombinant proteins were added to the wells (0.25 mg protein per well) and were left to adsorb at 4°C, overnight. The nonadsorbed proteins were collected, and the membranes washed three times with Buffer A to remove the nonadsorbed protein. Then, the membranes were washed three times with Buffer A containing 1% SDS to remove the adsorbed protein and collected. The initial

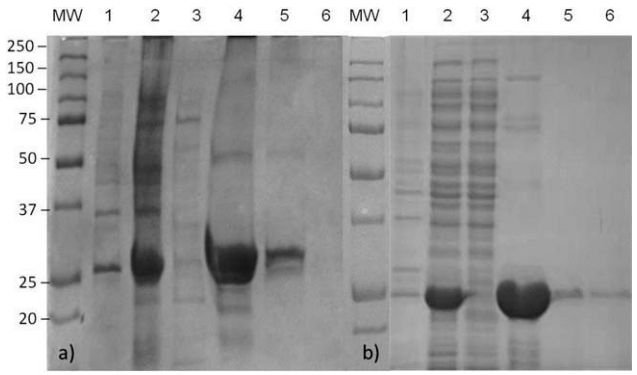

Figure 1. SDS-PAGE analysis of expressed and purified recombinant proteins.

MW-Molecular weight marker (Biorad); (a) (19)IKVAV-CBM3 and (b) IKVAV-CBM3. 1, Pellet; 2, supernatant; 3, flow fraction; 4, purified protein fraction 1; 5, purified protein fraction 2; 6, cleaning solution.

protein solution, the nonadsorbed proteins (supernatant fraction), and the adsorbed protein fraction were analyzed by SDS-PAGE.

Cell culture

SH-SY5Y human neuroblasts, N1E-115 rat neuroblasts, rat Pheochromocytoma (PC12), and rat Mesenchymal stem cells (MSCs) were maintained under standard tissue culture conditions, at 37°C in humidified atmosphere (5% CO_2 and 95% air). SH-SY5Y cells were cultured in a complete medium containing 1:1 Dulbecco's Modified Eagle Medium (DMEM; Gibco) and Ham Nutrient Mixture (Ham F-12; Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin; N1E-115 were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. PC12 were cultured in RPMI with 10% and 15% of FBS (inactivated), respectively, and 1% penicillin/streptomycin. Rat MSCs (rMSCs) were isolated from femur and tibias of adult Wistar rats as previously described[39] and cultured in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. The culture medium was replaced every 2–3 days.

Cell adhesion and viability on recombinant proteins coated surfaces

Cell adhesion was determined by mitochondrial activity through a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay, performed as follows: the proteins were added to a 24-well polystyrene plate (0.25 mg protein per well) covered with the BC pellicles. Plates were incubated overnight at 4°C. The unbound proteins were removed, and the BC pellicles washed with PBS. Cells were then seeded in serum-free medium (excepted for SH-SY5Y cells) at a density of 6 × 104 cells/well on BC pellicles. After 2 h, the medium containing nonadhered cells was removed; BC pellicles were washed with PBS and transferred to new wells; then, 300 μL of complete medium and 60 μL of MTS reagent were added. The plates were incubated for 2 h with MTS reagent, and then 100 μL of each well were transferred to a new 96-well plate and read on a Micro Elisa reader (Biotech Synergy HT), with a wavelength of 490 nm. The control used was the BC membranes treated only with buffer A. The cell adhesion experiments were run in triplicate at two separate times.

Live and dead assay

The viability of the cells cultured on BC membranes coated with the recombinant proteins for 13 days was determined through the live/dead assay. The LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) provides two-color fluorescence cell viability assay based on the determination of live and dead cells with two probes that measure intracellular esterase activity and plasma membrane integrity. A total of 100 μL of a solution of calcein and ethidium homodimer-1 in sterile PBS were added to the wells and incubated for 30 to 45 min at 37°C and 5% carbon dioxide atmosphere. The BC membranes were visualized on a fluorescence microscope.

Enzyme-linked immunosorbant assay

To determine the levels of NGF neurotrophin secreted by rMSCs in the medium, a commercial ELISA kit (NGF Emax® ImmunoAssay System, Promega) was used, according to the manufacturer's instructions. The cells were cultured in DMEM 2% FBS on BC membranes treated with recombinant proteins. BC without recombinant proteins and polystyrene plate were used as assays controls. The supernatant was removed at 3, 6, and 13 days and kept under −80°C, and fresh medium was added to the wells. NGF standards in the range 3.9–250 pg/mL were used to generate a linear calibration curve used to estimate the neurotrophin concentrations. Samples were run in duplicate. The interassay variability was less than 8.5% for 3 days, 22% for 6 days, and 12% for 13 days.

Statistical analysis

All results are presented as mean ± standard deviation, determined using the GraphPad software. Multiple comparisons were performed by nonparametric ANOVA analysis followed by Bonferroni's secondary test for significance between experimental conditions and control. Significant differences between BC with protein coating and control conditions are given $*P < 0.05$.

Results

In this study, recombinant proteins were expressed using an *E. coli* expression system and were purified to functionalize BC membranes, improving the adhesion of neuronal and mesenchymal cells. The peptides used, described in the literature as promoters of adhesion and proliferation of different cell lineages, were fused to a carbohydrate-binding module, which performs as a biosticker, promptly adsorbing to BC and bearing functional fused peptides.

The (19)IKVAV-CBM3 and IKVAV-CBM3 proteins were successfully expressed in the soluble fraction of *E. coli* and purified through affinity chromatography. Figure 1 shows the SDS-PAGE analysis of the samples obtained after the purification procedure.

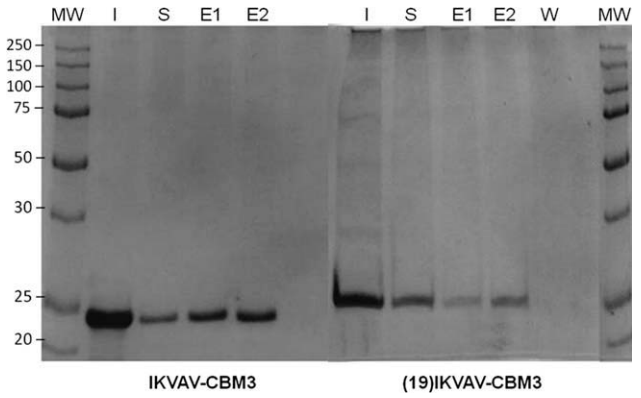

Figure 2. SDS-PAGE analysis of recombinant protein adsorption on BC membranes.

MW-Molecular weight marker (Biorad); I, initial recombinant protein (0.5 mg/mL); S, supernatant containing the nonadsorbed protein; E, elution fraction of recombinant proteins in buffer containing SDS; W, washing fraction without SDS.

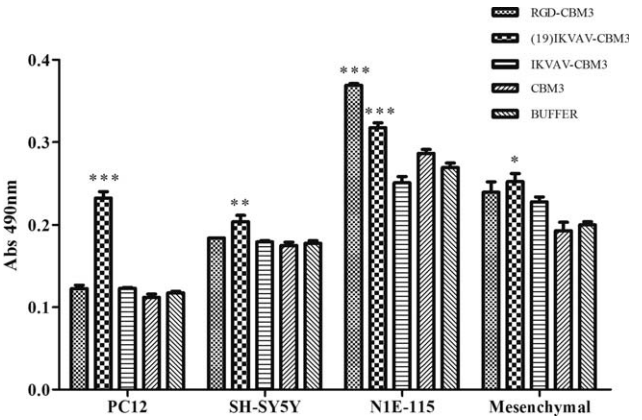

Figure 3. MTS assays of PC12, SH-SY5Y, N1E-115, mesenchymal cells.

The cells were seeded and able to adhere for 2 h on BC membranes coated with recombinant proteins. The control was BC pellicle treated with Buffer A without recombinant proteins. Significant differences between BC with protein coating and control conditions are given $*P < 0.05$.

Adsorption assay

The modification of BC surface was achieved through adsorption of CBM3. This interaction is stable and desorption occurred only in the presence of buffer containing 1% SDS, as shown in Figure 2.

Cell adhesion and viability

Figure 3 shows the MTS results. The recombinant protein (19)IKVAV-CBM3 significantly increased the adhesion of all cell lineages tested, an effect that was dependent on the cell type. This protein improved by almost 100% the adhesion of PC12 cells. The RGD-CBM3 protein also improved the adhesion of N1E-115 and mesenchymal cells, revealing a cell specific behavior. On the other hand, the IKVAV-CBM3 only presented a marginal effect on mesenchymal cell adhesion. The presence of serum in the culture medium represented a relevant factor in cell attachment. The SH-SY5Y cell adhesion occurred only in medium-containing serum, whereas the adhesion of other cell types was significantly increased in serum-free medium.

Figure 4 shows fluorescence images of PC12 and mesenchymal cells on BC membranes coated with the recombinant protein (19)IKVAV-CBM3, after 13 days in culture. The results showed that both cell types remained adhered and alive (green stained) on the BC, with practically no dead cells (stained in red), but still cells presented a rounded morphology. It can be seen that, in control wells, fewer cells are attached, mainly in PC12 culture. These results are in agreement with adhesion results, where (19)IKVAV-CBM3 improved strongly the adhesion of PC12 cells, improving mesenchymal cell adhesion too, when compared with control.

Neurotrophin expression

To investigate the NGF neurotrophin expression of MSCs on BC coated with the recombinant proteins, an ELISA kit was used to quantify the NGF released to the culture medium. The results showed that NGF is produced by the MSCs and released to the culture medium, after 3 and 6 days (Figure 5). In agreement with the higher cell adhesion observed on BC coated with the recombinant proteins, the RGD-CBM3 and (19)IKVAV-CBM3 groups produced a higher amount of NGF in the supernatant, when compared with CBM3 and buffer controls. As expected, cells on polystyrene showed a higher amount of NGF in supernatant, also caused by the number of adhered and proliferating cells on this material (data not shown). Moreover, the quantity of NGF in the supernatant was superior at the 13th day. At day 6, a slight decrease of the NGF in supernatant when compared with day 3 was observed.

Discussion

One of the main challenges of tissue engineering technologies is the production of adequate scaffolds for the growing of cells and tissues.[40] Improvement of cell adhesion may be achieved by the immobilization of ECM adhesion proteins or of its signaling motifs, onto the biomaterial surface.[24] Attempting to improve cell attachment and to elicit specific cell responses, we produced different recombinant proteins with the bioactive peptides IKVAV, (19)IKVAV, and RGD. Different cell lineages were used to evaluate the efficacy of these bioactive peptides fused to a CBM3 on the functionalization of BC membranes for its application as a scaffold in neuronal tissue engineering.

It is known that the use of short peptides containing the signaling motifs instead of the whole adhesive proteins (laminin, fibronectin) have advantages including the ease and reproducibility of synthesizing peptides, when compared with isolating ECM molecules from a natural source.[41] However, this approach has limitations, as the biological activity of short peptide sequences is often substantially lower when compared with the complete protein, owing at least partially to the absence of complementary domains that are involved in cell receptor binding.[42,43] In fact, in this work, the recombinant protein (19)IKVAV-CBM3 significantly increased the adhesion of all cell lineages tested, the effect being dependent on the cell type. The MTS results showed an improvement of almost 100% in cell adhesion for PC12 cells and 30% of mesenchymal stem cells. The RGD-CBM3 protein also improved the adhesion of N1E-115 and

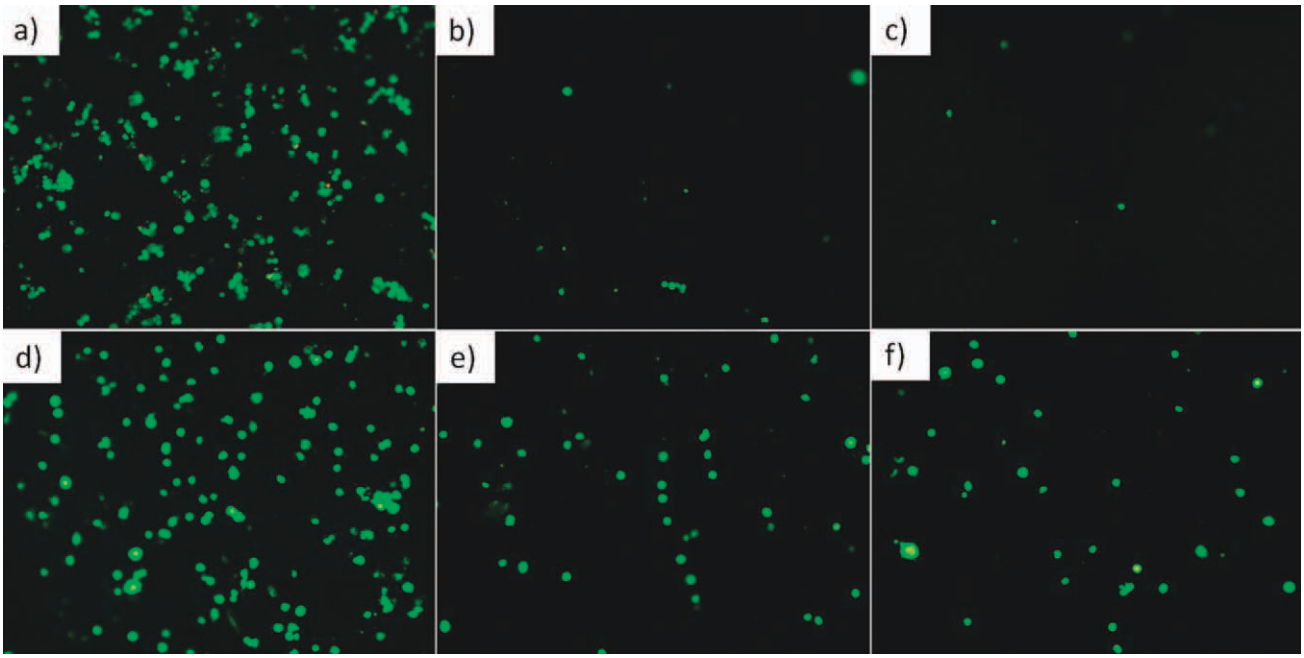

Figure 4. Images showing the live/dead assay of PC12 and mesenchymal cells seeded on BC membranes.

(×100 original magnification) (a,b,c) PC12 cells (d,e,f) mesenchymal cells cultured for 13 days on BC coated with (19)IKVAV-CBM3 (a and d); CBM3(b and e); Buffer (c and f).

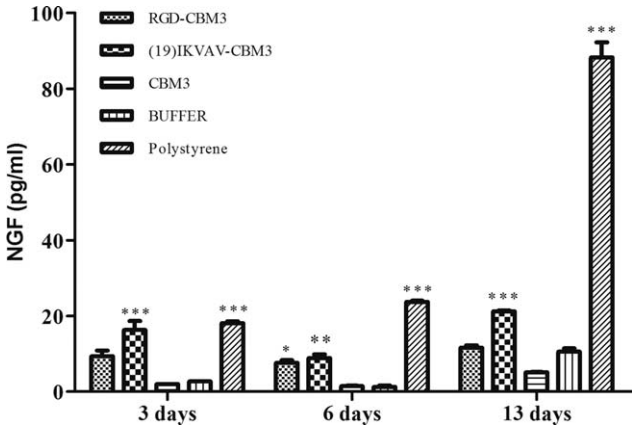

Figure 5. ELISA results of NGF expression by rMSCs on BC coated with recombinant proteins and polystyrene.

The cells seeded on BC membranes were able to release NGF to the culture medium after 3, 6, and 13 days. Significant differences between BC with protein coating and control conditions are given $*P < 0.05$.

mesenchymal cells, revealing a cell specific behavior. On the other hand, the IKVAV-CBM3 only yielded a slight effect on mesenchymal cell adhesion. It is possible that the use of the smallest recognition sequence IKVAV on the protein construction led to a weaker interaction with the receptors on the cell surface. In this context, some studies showed that, by using an extended peptide containing the IKVAV sequence, such as CSRARKQAASIKVAVSADR, it is possible to increase the cell–protein interaction.[3,15,44–48] Shaw and Soichet[46] compared the cell adhesion on modified surfaces with the laminin-derived cell adhesive peptides CIKVAV and CQAASIKVAV. The surfaces modified with extended peptide sequences CQAASIKVAV demonstrated a greater number of cells attached compared to those modified with the shorter peptide sequences, indicating that the extended peptides do better mimic the native three-dimensional conformation of the peptides in laminin.[46] Andrade et al. also described differences in cell adhesion dependent on amino acids flanking the RGD sequence in recombinant proteins, the RGD-CBM3 and GRGDY-CBM3 supporting differing degrees of fibroblast cell adhesion.[11]

Moreover, the surface where the proteins are adsorbed can influence the exposition of the bioactive site, leading to different patterns of cell attachment.[11,49] Interestingly, we observed no effect on the cell adhesion when the (19)IKVAV-CBM3 protein was tested on polystyrene (data not shown), while the RGD-CBM3 had a higher cell adhesion on polystyrene. This may be explained by the interference of the surface topology and roughness, which may influence the effective density of exposed adhesive biomolecules accessible to the cell receptors as well as the affinity of the receptor-ligand binding.[44,50,51] It is known that in physiological settings, cells interpret signals from the ECM and different cell types interact with different matrix proteins.[52] Therefore, it is not surprising that the intrinsic conditions of the in vitro system used, including cell line, culture medium, presence of serum, roughness and topography of material, and the structure and conformation of peptide, have a strong influence on the pattern of cellular behavior, as observed in this work.

In the absence of cell–matrix interactions, anchorage dependent cells undergo apoptosis.[53–55] Thus, when designing hydrogel niches to serve as synthetic ECM environments, preservation of matrix–cells receptor interactions is critical to promote long-term cell survival and function.[55] To confirm the survival of cells on BC modified with the recombinant protein (19)IKVAV-CBM3, the live and dead assay was performed. The results showed that PC12 and mesenchymal cells remained adhered and viable after 13 days on BC coated with (19)IKVAV-CBM3 protein. However, cells

maintained a rounded morphology, without evidence of cell spreading, in accordance with some results previously described by other authors using different materials, such hydrogels and nanofiber gel.[56,57] Wu et al.[56] showed that the self-assembly peptide IKVAV promoted the cell adhesion and viability of bone MSCs but exerted no influence on the MSC proliferation. Also, IKVAV ligand on poly(ethylene glycol) hydrogels increased MSC viability on nondegradable hydrogel, but not in degradable hydrogel, and on its own was not capable of influencing cell spreading.[58]

It has recently been demonstrated that MSCs, even without any induction, are able to secrete neurotrophins, providing a natural source of these molecules that can be exploited in tissue engineering applications.[39] Our results showed that NGF is produced by the MSCS seeded on BC membranes and released to the culture medium after 3, 6, and 13 days. The cells adhered on BC modified with RGD-CBM3 and (19)IKVAV-CBM3 secreted a higher amount of NGF into the supernatant compared to control, probably due to the larger initial number of cells adhered on BC treated with those proteins. Cells on polystyrene had a higher amount of NGF detected in supernatant not only caused by the upper initial number of adhered cells but also because of cell proliferation on this material.

BC has already been tested on neuronal tissue regeneration with promising results.[59,60] Our data indicate that the BC functionalized with recombinant proteins—bearing cell adhesive peptides—further expands the potential of this biomaterial for neuronal tissue regeneration. The improvement of MSCs adhesion, the support of cell viability, and release of neurotrophins are important to create a suitable environment with adequate stimulus to tissue regeneration. Nevertheless, additional work, such as the comprehensive characterization of the neurotrophins being released by MSCs cultivated on BC, as well as in vivo studies, are required for the full evaluation of this potential.

Conclusions

BC is a promising biomaterial to be used as scaffold in tissue engineering applications but, as in most scaffolds, it is still necessary to increase the interaction of cells with the material to obtain a matrix that maintains the growth, viability, and selectivity of different cell types. The recombinant proteins were successfully expressed in *E. coli* and adsorbed in a stable way onto BC membranes. The recombinant protein (19)IKVAV-CBM3 improved PC12 and mesenchymal cell adhesion on BC membranes and also allowed the release of NGF secreted by MSCs to the culture medium, indicating that modified BC has the potential to be used in neuronal tissue engineering applications.

Acknowledgments

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