Development Of Structures For Fibroblast Culture Based On Bacterial Cellulose

F.K. Andrade, S. M G. Moreira, L. Domingues, F. M. P. Gama
IBB – Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
E-mail: fabiakarine@deb.uminho.pt

The successful development of tissue engineering scaffolds usually requires proper substrates for cell survival and differentiation. The attachment of the tissue cells to these biomedical materials can be improved by using adhesion molecules. The adhesion molecules are generally extracellular matrix substances, such as fibronectin, vitronectin, or laminin, that regulate the adhesion, migration and growth of cells by binding to integrin receptors located on the outer cellular membranes. In many cases, Arg-Gly-Asp (RGD) was found to be the major functional amino acid sequence responsible for cellular adhesion. In the present study, we have explored a biomaterial based on bacterial cellulose (BC). BC is secreted by *Gluconacetobacter xylinus* (=*Acetobacter xylinum*) and has unique properties including high water holding capacity, high crystallinity, a fine fiber network, and high tensile strength. A method for producing chimerics proteins, RGD-CBM with functions similar to fibronectin, which contains a cellulose-binding domain (CBM), was developed. A CBM from the cellulosoma of the bacteria *Clostridium thermocellum*, was used. The genes encoding these CBM-containing chimeric proteins were cloned, and the proteins expressed. Bacterial cellulose, "coated" with these RGD-containing proteins, was then used in preliminary adhesion/biocompatibility tests, using a mouse embryo fibroblasts culture.

Materials and Methods

Production of bacterial cellulose. *G. xylinus* was grown in Hestrin-Schramm medium, pH 5.0 and incubated statically at 30 ^{o}C, for 7 days. BC pellicles were purified by 4% SDS treatment at 70 ^{o}C, for 12 h and then 4% NaOH at 70 ^{o}C, for 90 min. Samples were autoclaved and stored in PBS at 4ºC, prior to use.

Production and purification of recombinant proteins. The recombinants coding sequences were cloned in pET21a (Novagen) using *E. coli* XL1 Blue as cloning strain. High-level expression studies and large scale protein production were carried out in *E. coli* BL21 (DE3) grown at 37 ^{o}C in LB medium supplemented with ampicillin at 100µg/ml. Cultures were induced with IPTG at 1mM. The RGD/CBM, CBM, RGD/CBM/RGD, GRGDY/CBM/GRGDY and GRGDY/CBM proteins were expressed with a C-terminal hexahistidine (His6) Tag allowing the purification of the proteins from the supernatant by affinity chromatography.

Interaction of recombinant proteins with the cells. In order to demonstrate the interaction of the recombinant RGD with cell membrane, the recombinant proteins were chemically conjugated with FITC. The fluorescent proteins were added to the wells of 96-well polystyrene plates and the fibroblasts were added in 200µl of DMEM medium with/without serum). The plates were incubated for 1 hour at 37 ^{o}C, in atmosphere of 5% CO_2 and 95% humidified air. The cells were observed through fluorescence microscopy.

Effect of the recombinant proteins on the adhesion and spreading of fibroblasts The mitochondrial activity of the cultured cells is determined by colorimetric assay, which is related to cell viability. The MTT assay was performed as follows: the produced proteins were added to the wells of 96-well polystyrene plates, which were either left drying at 37º C for 4 days or adsorbing to the plate at 4ºC, overnight. Unbound protein (from the non-dried protein plates) was washed out with PBS. In a second test, the adhesion proteins were added to the wells of 24-well polystyrene plates, which were either left untreated or coated with bacterial cellulose sheets. The plates were incubated overnight at 4 ºC. Unbound protein was removed and a total of 2 x 10^4 cells fibroblast 3T3 (in DMEM medium without serum) were added to each well. The plates were incubated at 37 ^{o}C, in atmosphere of 5% CO_2 and 95% humidified air. One hour after the addition of the cells, the wells were washed with PBS and DMEM with serum (10%) was added. The MTT assay and microscope observations of the attachment and spreading of 3T3 fibroblasts was carried out at 1h, 5h, 24h and 48h after the addition of the cells.

Results

The fluorescence microscopy analysis of fibroblasts, incubated with the recombinant proteins conjugated with FITC, showed that, when the assays were performed with medium serum free, only the cells treated with RGD/CBM and GRGDY/CBM became fluorescent. These results demonstrate the interaction of the RGD with the integrins on the cell membranes. Indeed, when medium containing serum was used no fluorescent cells were observed. The recombinant proteins containing the adhesion peptide were able to promote adhesion, spreading and proliferation of the cells. The proteins containing the sequence RGD showed a stronger effect than the GRGDY sequence on fibroblast cells. The plates coated with the adsorbed recombinant proteins seem to promote the adhesion of the cells more efficiently then the ones coated with dried protein. Preliminary studies on the adhesion of fibroblast cultures on cellulose sheets functionalized with the recombinant proteins showed positive effects involving the adhesion and proliferation of fibroblasts.