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Secretion of a Novel Gene Product through Micro-encapsulated Recombinant Cells a Preliminary Report

By : M. Mansyur Romi[1], A. M. Sun[2], and P. L. Chang[3]

[1]Department of Anatomy, Embryology & Anthropology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. [2]Department of Physiology, University of Toronto, Toronto, Canada, [3]Departments of Pediatrics and Biomedical Sciences, McMaster University, Hamilton, Canada.

INTISARI

M. Mansyur Romi, A.M. Sun & P.L. Chang – *Sekresi produk gena baru dari sel rekombinan yang dibungkus mikrokapsul. Laporan pendahuluan*

Galur sel hasil rekayasa genetik yang berada dalam kondisi terisolasi secara imunologis diharapkan dapat diimplantasikan kepada beragam penerima. Dengan demikian tersedia strategi alternatif bagi terapi gena, yang umumnya bergantung kepada modifikasi genetik terhadap sel dari penderita sendiri. Penelitian ini mengamati ekspresi hormon pertumbuhan manusia (HPM) dari hasil rekayasa genetik sel mencit yang ditempatkan di dalam membran imunoprotektif secara in vitro.

Sel mencit Ltk$^-$ ditransfeksi dengan plasmid pNMG yang mengkode gena HPM dan gena resistensi terhadap G418. Sel-sel yang mensekresi HPM dalam jumlah banyak dipilih untuk dibungkus dengan mikrokapsul berdiameter 300nm yang terbuat dari bahan *alginate – polylysine – alginate*. Prosedur pengkapsulan tidak mengganggu kelangsungan hidup sel, terbukti bahwa lebih dari 95% sel masih tetap hidup seusai pengkapsulan. Sepanjang percobaan selama 3 minggu angka sel yang hidup dapat dipertahankan sekitar 90%, jumlah sel di dalam mikrokapsul meningkat lebih dari sepuluh kali lipat, dan HPM di dalam

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media juga meningkat konsentrasinya. Membran mikrokapsul cukup permeabel, dari perhitungan pada awal percobaan lebih dari 80% HPM yang disekresi dapat berdifusi ke dalam media. Dapat disimpulkan bahwa produk gena baru dari galur sel baku hasil rekayasa genetik dapat dihantarkan melewati mikrokapsul dari bahan *alginate - polylysine - alginate* secara in vitro paling tidak selama tiga minggu. Selain itu, prosedur yang dikerjakan cukup memadai bagi kelangsungan hidup sel, lingkungan dalam mikrokapsul dapat mendukung pertumbuhan sel, dan membran kapsul bersifat permeabel bagi produk gena baru.

Key Words : genetically modified cells – plasmid pNMG – human growth hormone – immuno-protection – microcapsule.

INTRODUCTION

Current approaches to human gene therapy focus on insertion of a desired gene into autologous cells such as bone marrow stem cells (Dzierzak *et al.*, 1988), hepatocytes (Ledley *et al.*, 1987; Wilson *et al.*, 1988), endothelial cells (Wilson *et al.*, 1989), lymphocytes (Culver *et al.*, 1991), or fibroblasts (Chang *et al.*, 1986; Palmer *et al.*, 1987). Since human primary cells are difficult to transfect, we propose an alternative strategy which avoids the dependence on an autologous source of target cells. The reason is that an easily transfectable cell line can be engineered to secret a desired gene product. If these recombinant cells are enclosed in permselective microcapsules which protect the cells from contact with the host's immune mediators (Fan *et al.*, 1980, Winn *et al.*, 1989)) and yet allow the exit of the engineered gene product, these cells should be immunologically tolerated and able to provide the previously missing gene after implantation *in vivo*.

Several criteria must be fulfilled before this strategy can be developed. The permselective membranes must be constructed with a process that is physiologically compatible with cell survival. They must also provide a micro-environment compatible with cell growth, be permeable to the exit of the novel gene product but not the entry of immune mediators from the host, and have the property of being biocompatible after implantation. Biomaterials such as alginate-polylysine-alginate microcapsules (Fan *et al.*, 1989) and acrylic hollow fibres (Winn *et al.*, 1989) enclosing allogeneic (Lim *et al.*, 1980) or xenogeneic (O'Shea & Sun, 1986; Weber *et al.*, 1989; Lacy *et al.*, 1991) pancreatic islet cells, or pheochromocytoma cells (Winn *et al.*, 1991) have been shown to offer many of the above desired properties as implantable immuno-protective devices. We now report on our attempt to deliver human growth hormone through genetically-modified mouse cultured fibroblasts after encapsulation with the biomaterial alginate-polylysine-alginate as a model leading to an alternative method for somatic gene therapy.

MATERIALS AND METHODS

Recombinant fibroblasts

Mouse Ltk$^-$ cells, a spontaneously immortalized mouse cell line, were cultured in MEM (Minimum essential medium) media supplemented with L-glutamine (2mM), new born calf serum (10%), penicillin (100 U/ml) and streptomycin (100 ug/ml) under the

usual conditions. These cells were transfected with calcium-phosphate precipitated (Graham & Bacchetti, 1983) pNMG encoding the human growth hormone (hGH) gene with a mouse metallothionen promoter MMT1 at the 5'-end and SV40 polyadenylation signal at the 3'-end. This plasmid also encodes the phosphotransferase gene permitting selection of transfectants with G418. The construction of the plasmid has been described (Chang *et al.*, 1990).

Encapsulation procedure

The cells were harvested with trypsin and encapsulated in alginate-polylysine-alginate microcapsules as previously described (Sun, 1988). Briefly, the cells were resuspended in 1.5% sodium alginate (a seaweed extract composed of the polysaccharide polyman-nuronic and polyglucuronic acid). Droplets of 300-600 nm in diameter were extruded with an airjet into a Ca^{++} bath allowing the gellation of the alginate. The surface layer was cross-linked with a polycation polylysine (MW range 12,500-32,500) and was then finally coated with alginate on the outside again. The core of non-crosslinked polysaccharide was dissolved with sodium citrate, leaving the cells floating free within the capsule space. The encapsulated cells were kept under the usual culture condition and photographed under phase-contrast (10 x objective) microscopy.

Assay for human growth hormone (hGH)

The hGH was monitored by either a radioimmunoassay according to Schalch & Parker (Schaclch & Parker, 1964) or an ELISA technique (UBI-Magiwel Hgh kit from United Biotech Inc., CA.) in which samples were dispensed into microwell coated with anti-hGH antibodies and incubated with enzyme conjugate for 60 minutes at room temperature. After incubation, the wells were rinsed with running tap water. Chromogen substrate was dispensed into each well and incubated for 30 min at a dark room temperature. The reaction was stopped with 1N H_2SO_4 and OD_{450nm} was read with a microwell spectrophotometer reader (Multiskan PLUS MK II).

Assay for viability and cell growth

At timed intervals after encapsulation, an aliquot of the microcapsules was removed and crushed with a glass coverslip. The proportion of viable cells was assessed with trypan blue exclusion test. The average cell number per capsule was counted with a haemocytometer from a known number of microcapsules crushed with a disposable pestle (Baxter 749520-0000).

Assay for hGH secretion rate from encapsulated cells

Microcapsules containing encapsulated cells were washed 5x with fresh media, resuspended in a known volume of media, and incubated under the usual tissue culture conditions. At hourly intervals up to 3 hours of incubation, an aliquot of the media was removed for hGH determination to obtain the hourly secretion rate. At the end of the experiment, the microcapsules were crushed to release the enclosed cells which were assessed for viability and cell counts.

RESULTS

Mouse Ltk$^-$ cells, after transfection with calcium phosphate precipitated plasmid DNA encoding hGH, were enriched for transfectants by G418 selection. Clones resistant to G418 were screened for hGH production and a high secreting clone (C4, secreting hGH at a rate of about 34 ng/10^6 cells/h) was used for subsequent encapsulation.

Survival of the recombinant cells after the encapsulation procedure was monitored by counting at timed intervals the average number of cells present within each microcapsule (FIGURE 1) and monitoring the viability of the enclosed cells (FIGURE 3). Immediately after encapsulation, the cell number per capsule was about 70, which over the subsequent week, increased to 150 on day 3 and reached about 600 by day 7. By day 10 post-encapsulation, cell number could no longer be accurately counted because of the large number of cells and clumping of cells that started to occur as the cells proliferated further. The increase in cell mass, however, was evident, as indicated under phase-contrast microscopy (FIGURE 2) monitored from day 7 to day 21.

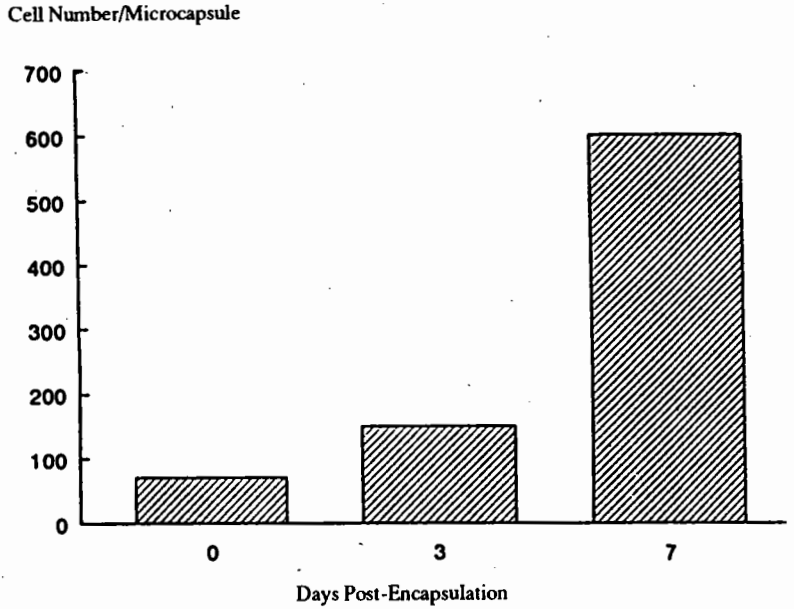

FIGURE 1. – Proliferation of hGH-secreting mouse Ltk$^-$ cells in alginate-polylysine-alginate microcapsules.

At timed intervals after the cells were encapsulated, aliquots of the capsules were removed, and known number of microcapsules were crushed to release the encapsulated cells for counting. Data were averaged from duplicate to quaduplicate determinations.

Throughout the first three weeks after encapsulation, the viability of the cells in the capsule was maintained at about 90%, starting from immediately after the encapsulation procedure (FIGURE 3). In addition, the secreted hGH was recovered in the culture medium at increasing amounts, reaching a concentration of about 9 ng/ml media by day 21. When the rate of secretion was monitored on day 3 post-encapsulation, it was found

that at least 83% of the hGH secreted by the encapsulated cells was recovered in the media, based on the observed rate of hGH secretion of 28.1 ng of hGH secreted per h by 10^6 viable cells inside the capsules, compared to the secretion rate of un-encapsulated cells at 34 ng/10^6 cells/h. These estimates were based on the average from three experiments.

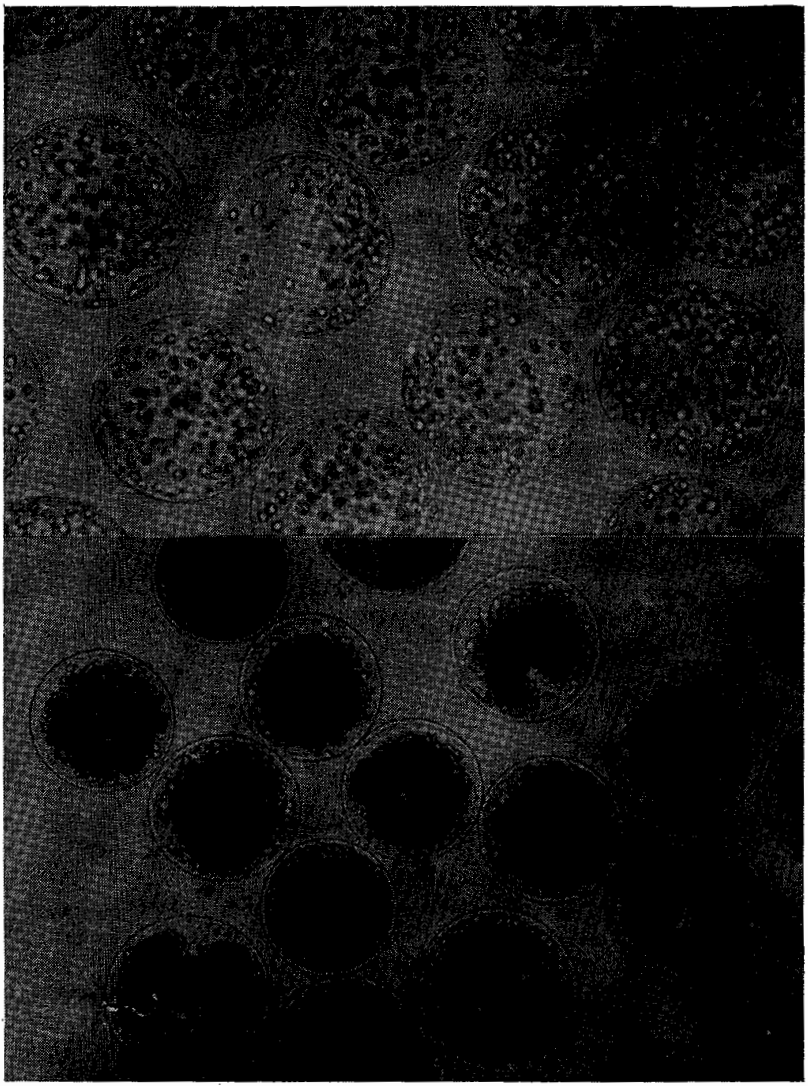

FIGURE 2. – Appearance of hGH-secreting mouse Ltk$^-$ cells in microcapsules.

The encapsulated cells were photographed under phase contrast with a 10× objective on day 7 (D7) and day 21 (D21) after encapsulation. The density of cells inside microcapsules was increased, indicating the growth of cells.

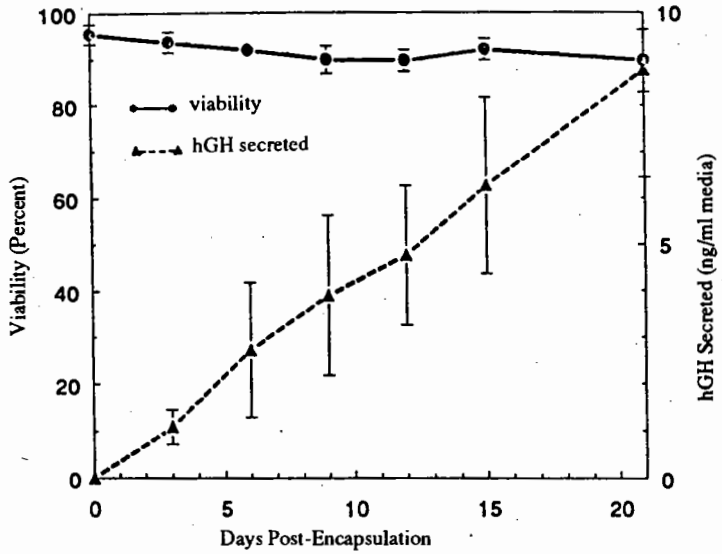

FIGURE 3. – The viability and hGH secretion of encapsulated cells.

Aliquots of the microcapsules were removed at timed intervals after encapsulation and crushed with glass coverslips to release the cells for trypan blue enclusion test for viability. The data were averaged from 4-5 experiments ± SD.

The cumulative amount of hGH was estimated by removing an aliquot of the culture media at the indicated times when the culture media was also replenished by exchanging half of the media with fresh media. The hGH concentration was determined before and after the exchange, all determinations at least in duplicate. The data represent the average from two separate experiments ± range.

DISCUSSION

The procedure of creating permselective microcapsules made of the sea-weed alginate is a mild and physiologically compatible method to compartmentalize cultured cells. The viability of the cells immediately after the microcapsule fabrication step was excellent, maintaining over 95% of viable cells as assessed with trypan blue exclusion (FIGURE 3). Furthermore, the mouse Ltk$^-$ cells, which normally grew as attached cells on culture dishes, were able to adapt to grow inside these microcapsules, showing an almost tenfold increase in cell number in 7 days. The ability of culture fibroblasts to proliferate inside the capsules appeared to be cell line-dependent. We have encapsulated other anchorage-dependent fibroblasts which did not grow as well as these transformed mouse cells. Although the continued proliferation of these mouse cells could not be monitored with cell counting as the cells became too numerous and clumped later on, it was obvious that even by the third week after encapsulation, the cell number was still increasing, as monitored through phase-contrast microscopy (FIGURE 2). The excellent viability of about 90% was maintained throughout this entire period of observation (FIGURE 3).

The success of this method to provide a nutritionally supportive environment for cell survival is the first requirement for using these microcapsules for somatic gene

therapy. The next important question is the adequacy of the microcapsules to provide free passage to the secretory recombinant gene product. As shown in FIGURE 3, the hGH secreted by these transfected encapsulated mouse cells was accumulating in the media at increasing concentration up to day 21 post-encapsulation. Since this estimation did not take into account the possible proteolytic degradation of hGH that may have occurred in the media through the three weeks of observation, the actual amount of hGH recoverable in the meda was likely to be even higher. When monitored soon after encapsulation, over 80% of the secreted hGH was able to diffuse out of the capsule which remained intact throughout this period of observation, as monitored through phase-contrast microscopy. Therefore, not only are the encapsulated cells able to survive, their secreted gene product was able to exit from the microcapsule compartment.

In conclusion, the current results clearly demonstrated the feasibility of using microencapsulated engineered cell lines to provide a novel gene product *in vitro*. The basic requirements for this strategy to be efective clinically is that the gene product must be secreted from the cells and that therapeutic effects can be achieved in the patients through delivery of the gene product to the systemic circulation. However, before this approach can be further developed, it will be necessary to address two important issues: the long-term performance of these microcapsules and encapsulated recombinant cells *in vitro* as well as the feasibility of this approach to deliver new gene product *in vivo*. If it can be demonstrated that such encapsulated cells continue to deliver the desired gene product over an extended period, the possibility of using an allogeneic standard cell line to provide recombinant gene products *in vivo* should have wide application not only for therapeutic purposes, but also in veterinary medicine for the provision of engineered vaccine or growth modulating substances *in vivo*.

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