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Preparation and in vitro studies of microencapsulated cells releasing human tissue inhibitor of metalloproteinase-2

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Abstract: Objective: To prepare microencapsulated cells releasing human tissue inhibitor of metalloproteinase-2 (TIMP-2), and investigate their biological characteristics in vitro. Methods: Chinese hamster ovary (CHO) cells were stably transfected with a human TIMP-2 expression vector, encapsulated in barium alginate microcapsules and cultured in vitro. Morphological appearance of the microcapsules was observed under a light microscope. Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Enzyme linked immunosorbent assay (ELISA) and reverse zymography were used to confirm the release of biologically active TIMP-2 from the microcapsules. Cryopreservation study of the microencapsulated cells was carried out using dimethyl sulfoxide (DMSO) as preservative agent. Results: The microcapsules appeared like a sphere with diameter of 300–600 μm . The surface of the capsule wall was clearly smooth. The microencapsulated cells survived well and kept proliferating over the 6 weeks observed. No significant difference in TIMP-2 secretion was found between encapsulated and unencapsulated cells. Reverse zymography confirmed the bioactivity of MMP (matrix metalloproteinase) inhibition of TIMP-2. The cryopreservation process did not damage the microcapsule morphology nor the viability of the cells inside. Conclusion: Microencapsulated engineered CHO cells survive at least 6 weeks after preparation in vitro, and secrete bioactive TIMP-2 freely from the microcapsules.

Key words: Microencapsulation, Recombinant cells, Human tissue inhibitor of metalloproteinase-2, Cell culture

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INTRODUCTION

Cell encapsulation in biocompatible and semipermeable polymeric membranes is an effective method for immunoprotection, regardless of the type of recipient (allograft, xenograft, etc.). The semipermeable nature of the membrane prevents high molecular weight molecules, antibodies and other immunologic moieties from coming into contact with the encapsulated cells and destroying them as foreign invaders, but permits the entry of nutrients and oxygen and the exit of therapeutic protein products. Encapsulated cells can maintain their viability while allowing secretion of desired therapeutic agents, either continuously or in response to specific physiologic stimulations (Orive *et al.*, 2004).

TIMP-2 (tissue inhibitor of metalloproteinase-2) is a natural matrix metalloproteinase (MMP) inhibitor that prevents the degradation of extracellular matrix proteins. It suppresses the hydrolytic activity of all activated members of the MMP family and in particular that of MT1-MMP, MMP-2, and MMP-9, which has been particularly implicated in tumor progression and angiogenesis (Stetler-Stevenson, 1999). The anti-neoplastic activity of TIMP-2 in tumor models had been demonstrated in several studies using different experimental approaches. It was reported that transfection of tumor cells with TIMP-2 cDNA results in the reduction of tumor growth, angiogenesis, and metastasis (Montgomery *et al.*, 1994; Valente *et al.*, 1998), and that retrovirus-mediated TIMP-2 gene transfer in vivo showed similar result (Imren *et al.*, 1996).

In the present study, CHO (Chinese hamster

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ovary) cells were stably transfected with a human TIMP-2 expression vector and were encapsulated in barium alginate microcapsules. The ability of this system to maintain cell viability and to secrete biologically active TIMP-2 in vitro was investigated.

MATERIALS AND METHODS

TIMP-2 plasmid construction

The targeting plasmid pcDNA3-hTIMP-2 was constructed by inserting TIMP-2 cDNA into *EcoR* I and *Hind* III restriction sites of multiple cloning region on pcDNA3 vector (Invitrogen, USA), harboring a cytomegalovirus (CMV) promoter and a gene for neomycin resistance. The recombinant plasmids were identified by DNA sequencing (Bioasia, Shanghai, China).

CHO cell transfection

CHO cells obtained from Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences were maintained in RPMI 1640 medium (GIBCO BRL, USA) supplemented with 10% (*V/V*) newborn calf serum (NBCS, Sijiqing, Hangzhou, China), and stably transfected with pcDNA3-hTIMP-2 plasmid DNA using Lipofectamine 2000 (Invitrogen, USA). Control cells were transfected with the empty pcDNA3 vector. G-418-resistant colonies were collected and subsequently analyzed for TIMP-2 expression by Western Blot analysis.

Microencapsulation

For microencapsulation, the TIMP-2 expressing cells were harvested with trypsin and then resuspended in 1.5% (*w/V*) sterile filtered sodium alginate (Sigma-Aldrich, USA) at a concentration of 1×10^6 cells/ml. The suspension was sprayed through a 22-gauge needle located inside an air jet-head droplet-forming apparatus into a solution of 20 mmol/L barium chloride, where it gelled. The microcapsules were washed three times in saline solution after retrieval, and then cultured at 37 °C and 5% (*V/V*) CO₂ in RPMI 1640 medium containing 10% (*V/V*) NBCS. Morphological investigation of microencapsulated cells was carried out by phase-contrast light microscopy to examine the shape and dimension of alginate microcapsules, and cell morphology.

Viability of microencapsulated cells

The viability of the encapsulated cells was measured by MTT assay using 10 capsules per well of a 96-well microplate. Twenty μ l MTT solution (Sigma-Aldrich, USA, 5 mg/ml) and 200 μ l fresh complete medium were added to each well and incubated at 37 °C for 6 h, after which time the MTT/medium solution was removed by aspiration. One-hundred fifty μ l DMSO was added to dissolve the formazan product with manual agitation. Absorbance of each well was then measured in a spectrophotometer using a wavelength of 570 nm, with reference wavelength set at 630 nm.

ELISA of TIMP-2

Monolayer and microencapsulated CHO-TIMP-2 cells were suspended in RPMI 1640 supplemented with 10% (*V/V*) NBCS at density of 1×10^5 cells/well and incubated at 37 °C, 5% (*V/V*) CO₂. The media were collected after 24 h and assayed for TIMP-2 using ELISA assay (RapidBio, USA).

Reverse zymography

Serum-free conditioned media of monolayer and encapsulated cells were mixed with nonreducing sample loading buffer and applied to 12% (*w/V*) polyacrylamide gels copolymerized with 2.5 mg/ml gelatin (Sigma-Aldrich, USA) and 160 ng/ml human MMP-2 (BIOMOL, USA). After electrophoresis, gels were rinsed in 2.5% (*V/V*) Triton X-100 3 times for 2 h to remove sodium dodecyl sulfate (SDS), followed by incubation overnight at 37 °C in 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L CaCl₂, 200 mmol/L NaCl, and 0.02% NaN₃. The gels were then stained with 0.5% (*w/V*) Coomassie blue in 25% (*V/V*) methanol and 10% (*V/V*) acetic acid and destained in the same solution lacking Coomassie blue. Functional TIMP-2 appeared as a dark band at 21 000, corresponding to the area where gelatin degradation by the gelatinase added to the gel is prevented by the inhibitor.

Cryopreservation of microencapsulated cells

Microencapsulated cells were collected and suspended in complete culture medium supplemented with 10% (*V/V*) DMSO. The microcapsule suspension was collected in cryovials and placed at 4 °C for 1 h, -80 °C for 24 h and then transferred in liquid nitrogen for storage. After one week of storage, the

microencapsulated cells were thawed in a 40 °C bath within 2 min at the rate of 150 °C/min, washed 3 times with complete culture medium, and then incubated at 37 °C, 5% (V/V) CO₂ in RPMI 1640 supplemented with 10% (V/V) NBCS.

Statistical analysis

Values were presented as means±standard deviation (SD) when appropriate. Comparison of means was performed by two-tailed student *t* test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Morphology of microencapsulated cells

Microcapsules displayed a consistent appearance of a sphere with diameter of 300–600 µm. The surface of the capsule wall was clearly smooth. The number of cells contained in each capsule was approximately $(0.5\sim1)\times10^3$. Cells were evenly distributed within the beads on the day of encapsulation. After two to three weeks in culture, spheroids developed gradually within the beads (Fig.1).

Viability of microencapsulated cells

Microencapsulated CHO-TIMP-2 cells cultured in complete growth medium were evaluated for cell viability after 1 d, 1 week, and 6 weeks using MTT viability assay. After 1 d, 1 week, and 6 weeks in culture formazan absorbance was respectively, 0.2383 ± 0.021 , 0.272 ± 0.01 , and 0.3567 ± 0.032 , the formazan absorbance after 6 weeks being significantly ($P<0.05$) higher than that at 1 d and 1 week (Fig.2).

In vitro expression and release of TIMP-2

Western Blot analysis of media from CHO-TIMP-2 cells showed expression of TIMP-2, no TIMP-2 protein was detected in media collected from the control, empty vector-transfected cells and CHO cells (Fig.3). After microencapsulation, an ELISA was performed on media collected from the cultured monolayer and encapsulated CHO-TIMP-2 cells. The average amount of TIMP-2 secreted by 1×10^5 cultured encapsulated CHO-TIMP-2 cells over 24 h was 19.5368 ± 0.4164 ng. Similarly, cultured monolayer

CHO-TIMP-2 cells secreted TIMP-2 at a level of 20.2892 ± 0.716 ng per 1×10^5 cells. No significant difference was found ($P>0.05$).

Reverse zymography

Secreted TIMP-2 was functional, as assessed by reverse zymography. Assay of serum-free conditioned media of monolayer and encapsulated CHO-TIMP-2 cells showed a dark band at 21 000, corresponding to the area where gelatin degradation by the gelatinase is prevented by the inhibitor. No such band was detected in conditioned medium of CHO cells (Fig.4).

Cryopreservation of microencapsulated cells

The morphology and viability of microencapsulated CHO-TIMP-2 cells were evaluated after cryopreservation in liquid nitrogen and consequent thawing. The integrity of the microcapsules was well maintained, and no difference in cell morphology was noted between pre- and postcryopreservation microencapsulated cells by microscopic observation (Fig.5). Formazan absorbance was 0.2763 ± 0.018 using MTT based viability assay.

DISCUSSION

Since the early 1980s, when Lim and Sun (1980) developed a technique for microencapsulation of pancreatic islets in an alginate matrix covered with a layer of poly-lysine to protect the graft from cellular or humoral assault, microencapsulation has become one of the most important techniques for immobilizing cells for biomedical applications. Many researchers proposed that the transplantation of microencapsulated universal cell lines or tissue would be useful for the delivery of bioactive molecules in vivo. Transplantation of cells or tissues, such as islets of Langerhans, hepatocytes, parathyroid cells, pituitary cells, and thymic epithelial cells, which are enclosed in a semipermeable membrane had been proposed as an alternative to hormone and enzyme replacement therapy (Orive *et al.*, 2003).

A number of delivery vehicles have been developed to do this. In particular, alginate-poly(L) lysine-alginate (APA) microcapsules had been extensively used for this application, while much effort

Fig.1 Morphology of microencapsulated cells at different time of culture under a light microscope ($\times 10$) (a) 24 h; (b) 6 weeks

Fig.2 Result of MTT assay of microencapsulated cells at different time of culture ($^*P < 0.05$)

Fig.5 Morphology of microencapsulated cells after cryopreservation ($\times 10$)

had also been focused on identifying alternative encapsulation systems to the classical APA microcapsules (Uludag *et al.*, 2000). A simple technique that consists of one step encapsulation with a highly purified alginate crosslinked with barium, without a traditional permselective layer such as PLL (poly-L-

Fig.3 Western Blot analysis of recombinant TIMP-2
a: CHO-TIMP-2 cells; b: Empty vector-transfected cells; c: CHO cells

Fig.4 Reverse zymography of secreted TIMP-2
a: CHO cells; b: CHO-TIMP-2 cells; c: Microencapsulated CHO-TIMP-2 cells

lysine) can produce microcapsules working very well, showing even higher stability and biocompatibility. Porcine neonatal pancreatic cell clusters encapsulated with simple barium alginate can differentiate into β -cells and reverse high blood glucose levels in immunocompetent mice without immunosuppression for >20 weeks (Omer *et al.*, 2003). By means of air-jet technique, we obtained barium alginate microcapsules having consistently the appearance of a sphere, inside which the encapsulated cells survived well at least 6 weeks after preparation *in vitro*, confirming the permeability of barium alginate microcapsules that allows the exchange of nutrients, oxygen and waste products.

Microencapsulation of recombinant cell lines is viewed as a new delivery system for gene therapy. In this approach cell lines are engineered to secrete a desired gene product. Enclosing these cells in immunoprotective devices would allow such nonautologous

cells to be implanted into any host to deliver the desired gene product without triggering graft rejection. The advantages of this nonautologous method of gene delivery are: it does not require modification of the host's genome, thus providing additional measures of safety and cost saving; it provides ample material for quality assessment before implantation, a safety feature not available to most other forms of in vivo delivery (Chang *et al.*, 1999; Tai and Sun, 1993). Read *et al.* (2001) described a technique for the treatment of malignant brain tumors based on local delivery of endostatin from genetically engineered cells encapsulated in sodium alginate. Endostatin-transfected cells encapsulated in alginate maintained endostatin secretion for at least four months after intracerebral implantation in rats. During the implantation period 70% of the encapsulated cells remained viable, as opposed to 85% in vitro-cultured capsules. Rats that received transplants of BT4C glioma cells, together with endostatin-producing capsules survived 84% longer than the controls. Joki *et al.* (2001) also reported the effectiveness of encapsulated endostatin-secreting cells for the treatment of human glioblastoma xenografts. Microencapsulated recombinant cells secreting angiostatin (Cirone *et al.*, 2003), and microencapsulated iNOS-expressing cells (Xu *et al.*, 2001) also demonstrated good efficacy in tumor suppression.

MMPs play key roles in the responses of cells to their microenvironment. By effecting proteolytic degradation or activation of cell surface and extracellular matrix (ECM) proteins they can modulate both cell-cell and cell-ECM interactions, which influence cell differentiation, migration, proliferation and survival. The proteolytic activity of MMPs is regulated by a number of physiological inhibitors. Specific inhibition is mediated by the TIMPs. A tightly regulated balance between proteases and their inhibitors must exist at any developmental and physiological stage. A disruption in this balance is observed in many pathological conditions, including different stages of tumor progression (Hojilla *et al.*, 2003). The anticancer efficiency of TIMP-2 has been confirmed in murine models. Li *et al.* (2001) reported that preinfection of tumor cells with TIMP-2 delivered by recombinant adenovirus (AdTIMP-2) resulted in a significant inhibition of tumor establishment, and a single local injection of AdTIMP-2 into preestab-

lished tumors significantly reduced tumor growth rates and tumor-associated angiogenesis. In addition, AdTIMP-2-treated mice showed significantly prolonged survival. Nonviral method for delivering TIMP-2 gene also effectively inhibited tumor growth and tumor-associated vascularization in vivo (Vincent *et al.*, 2003). Moreover, a nude mouse model of colorectal liver metastasis was used to overexpress TIMP-2 in the liver prior to, or following, tumor challenge by metastatic LS174T cells in vivo. Transduction of ~50% of hepatocytes resulted in 95% reduction in metastasis compared with controls, while TIMP-2 gene transfer into livers with preexisting metastatic spread resulted in a 77% reduction in tumor cell growth (Brand *et al.*, 2000).

We aimed at preparing TIMP-2-releasing microencapsulated recombinant cells for anticancer study, and our present data show that microencapsulation procedure does not affect secretion and bioactivity of the secreted TIMP-2.

CONCLUSION

This study shows that recombinant CHO-TIMP-2 cells survive well in barium alginate microcapsules produced by means of an air-jet technique. The microcapsules also have sufficiently large pore size to allow for the diffusion of bioactive TIMP-2. The results of our study provide a basis for anticancer experiments on microencapsulated cells releasing TIMP-2.

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