Inhibition of Tumor Growth in Mice by Endostatin Derived from Abdominal Transplanted Encapsulated Cells

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Abstract Endostatin, a C-terminal fragment of collagen 18a, inhibits the growth of established tumors and metastases in vivo by inhibiting angiogenesis. However, the purification procedures required for largescale production and the attendant cost of these processes, together with the low effectiveness in clinical tests, suggest that alternative delivery methods might be required for efficient therapeutic use of endostatin. In the present study, we transfected Chinese hamster ovary (CHO) cells with a human endostatin gene expression vector and encapsulated the CHO cells in alginate-poly-L-lysine microcapsules. The release of biologically active endostatin was confirmed using the chicken chorioallantoic membrane assay. The encapsulated endostatin-expressing CHO cells can inhibit the growth of primary tumors in a subcutaneous B16 tumor model when injected into the abdominal cavity of mouse. These results widen the clinical application of the microencapsulated cell endostatin delivery system in cancer treatment.

Key words microencapsulation; endostatin; CHO; tumor; gene therapy

Angiogenesis is essential for the growth and metastasis of tumors. Without angiogenesis, most solid tumors would not grow beyond 2 mm in diameter because of inadequate tissue oxygenation and nutrient supply [1]. The growth of tumors can be inhibited by inhibiting the angiogenesis. This has been shown in many animal experiments and clinical trials [2].

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, was first isolated from the conditioned medium of a murine hemangioendothelioma cell line (EOMA) [3]. Recombinant endostatin purified from prokaryotic and eukaryotic expression systems suppresses the growth of primary human and murine tumors and metastases in different mouse tumor models [4,5]. Thus far, no severe side-effects have been described. Furthermore, antangiogenic therapy with endostatin did not induce acquired drug resistance [5]. A phase II clinical study is currently underway in the USA.

However, injected human endostatin is rapidly cleared from circulation [6,8]. To achieve effective therapeutic levels for significant tumor regression, large amounts of recombinant endostatin should be given in both animal experiments and clinical trials, so that the clinical use of endostatin is limited [9–16].

Alginate-poly-L-lysine (PLL) microcapsules have been used extensively for different applications, particularly for the encapsulation of pancreatic islet cells and insulin delivery [17,18]. This method has also been used for the encapsulation of cells that release cytokines, hormones, and other agents for gene therapy [19]. The alginate-PLL membranes allow the free exchange of nutrients and oxygen between the implanted cells and the host while preventing the escape and immunological elimination of encapsulated cells. More importantly, this approach provides a prolonged
sustained delivery of recombinant protein produced by the cells, thus maintaining high levels of the agent.

In 2001, two reports showed that local delivery of endostatin, by implanting genetically engineered encapsulated cells just around the tumor site, can significantly inhibit tumor growth. However, it is not known if the microencapsulated cell delivery system is efficacious when endostatin released from encapsulated cells enters the circulation first, then infiltrates the tumor through the blood stream; endostatin is not stable in blood and is rapidly cleared from circulation [6,8]. Furthermore, local delivery of drugs might not be feasible in the treatment of many tumors, especially for metastasis. These factors limit the use of this local delivery system in future clinical practices, so further investigations are needed.

In the present study, Chinese hamster ovary (CHO) cells engineered to continuously secrete human endostatin were encapsulated with alginate PLL alginate (APA) and transplanted into the peritoneal cavities of mice bearing B16 melanoma subcutaneously. The ability of this system to secrete biologically active endostatin and inhibit the subcutaneous growth of B16 melanoma in the C57 mice was investigated.

Material and Methods

Mice and cell lines

C57 female mice aged 6–8 weeks were obtained from the Animal Centre of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The CHO and B16 cell lines were obtained from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences and were grown in RPMI 1640 medium (Gibco, Grand Island, USA) supplemented with 10% (V/V) newborn calf serum (Sijiqing, Hangzhou, China). Cells were maintained in tissue culture flasks (Falcon, Bedford, USA) and grown in 5% CO₂/95% air at 37 °C in a humidified incubator.

Plasmid construction

The plasmid containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus and expressing human endostatin (hES), pIRES-hES, was constructed as follows. The cDNA of human endostatin (a gift from Dr. Min WANG, Center for Cardiovascular Research, University of Rochester Medical Center, New York, USA) was inserted into the NotI and EcoRI sites of pIRES-pac (puromycin N-acetyltransferase, constructed in our laboratory) to generate human endostatin expression plasmid pIRES-hES, in which the coding sequences for a mouse immunoglobulin κ chain (lgk) signal peptide and influenza virus hemagglutinin A (HA) epitope were located upstream of the endostatin gene.

CHO cell transfection

CHO cells with 80% confluence were suspended in electroporation solution (272 mM sucrose, 1 mM MgCl₂, 7 mM potassium phosphate, pH 7.4) to a density of approximately 1×10⁷ cells/ml. Cells (800 μl) were added to the cuvette plus 5 μg linear DNA (dissolved in 20 μl of sterile water). The cuvette was put on ice for 15 min before and after electroporation. After electroporation (set voltage, 400 V; capacitance, 25 μF), the cells were transferred into 9 ml of complete medium in a dish and cultured for 72 h. On day 3, puromycin was added into the complete medium at a concentration of 8 μg/ml. On day 10, drug resistant cell colonies were formed and picked out for further culturing. The endostatin secreted into the culture medium was assayed with enzyme immunoassay (EIA). CHO cells transfected with empty vector pIRES-pac were used as the negative control.

Microencapsulation of CHO-ph11 and CHO-pac cells

The method of cell encapsulation used has been described in detail elsewhere [20,21] but was modified. Briefly, droplets of cells dispersed in 1.5% sodium alginate (2×10⁸ cells/ml alginate) were released into a 0.1 M CaCl₂ solution. Before and after treatment with 0.05% PLL (molecular weight of 65 kDa; Sigma, St. Louis, USA), the capsules were washed three times with saline. The capsules were then suspended in 0.15% sodium alginate for 5 min, washed three times with saline again, and allowed to react with 0.05 M sodium citrate (pH 7.4). After washing three times with saline, the alginate beads (500–700 μm in diameter) were cultured in 175 cm² culture flasks containing 50 ml growth medium and kept in a standard tissue culture incubator at 37 °C, 100% humidity, 95% air/5% CO₂.

Detection of endostatin expression by ELISA

The concentration of endostatin in cell culture medium of transfected CHO cells and the corresponding encapsulated cells were quantified by human endostatin Accurate EIA from Oncogene (Boston, USA), which is specific for human endostatin.

Chorioallantoic membrane assay

Angiogenesis assay in the chicken chorioallantoic mem-
brane (CAM) was carried out as previously described [22]. Fertilized chick eggs were pre-incubated for 5 d at 37 °C, 60% humidity. A hole was drilled over the air sac at the end of the egg and an avascular zone was identified on the CAM. The CAM was treated with 100 µl of conditioned medium collected from microencapsulated CHO/pIRES-pac cells, microencapsulated CHO/pIRES-hES cells or empty microcapsules. The windows were sealed with tape and the eggs were incubated for six more days. Then the eggs were opened and their CAMs were inspected.

Animal studies

Female C57 mice, 6−8 weeks old, were inoculated subcutaneously with B16 melanoma cells (5×10⁵ cells/injection). Then animals were randomly divided into four groups of six animals each. The first group received a single peritoneal injection of 1 ml microcapsules containing CHO/pIRES-pac cells (2×10⁶ cells) within 2 ml saline solution; the second group received a single peritoneal injection of 1 ml microcapsules containing CHO/pIRES-hES cells within 2 ml saline solution; the third group received a single peritoneal injection of 1 ml empty microcapsules within 2 ml saline solution; the fourth group received a single peritoneal injection of 2 ml saline solution. After 14 d, the animals received the second peritoneal injection of above samples. Subcutaneous tumor growth was measured transcutaneously with a caliber every day from day 10 to day 22, and the tumor volume was then calculated with formula (volume=0.5×length×width²) [23]. Statistically significant differences in tumor growth among the groups were analyzed by ANOVA. P<0.05 was considered to be statistically significant.

Histological analysis

The C57 mice bearing B16 melanoma tumors with different treatments were given ether inhalation anesthesia to excise subcutaneous tumors. Tumors were fixed in 4% polyformaldehyde, embedded in paraffin and stained with hematoxylin-eosin according to standard procedure.

Results

In vitro expression and release of endostatin from encapsulated CHO cells

The cDNA of human endostatin was fused in its N-terminus with a synthetic intron (IVS8), the coding sequence for a secretion signal from the Igκ chain and an epitope tag derived from influenza virus HA (Igκ-HA). This fragment of IVS8-Igκ-HA-endostatin was inserted into the multiclonal site of plasmid pIRES-pac containing a cytomegalovirus enhancer/promoter and the IRES of the encephalomyocarditis virus, to generate human endostatin expression plasmid pIRES-hES. A diagram of the expression plasmid pIRES-hES is shown in Fig. 1.

CHO cells with 80% confluence were transfected with linear pIRES-hES by electroporation, and the transfected cells were selected by puromycin treatment. The cell colonies with potentially higher expression of endostatin were picked out under the higher concentration of puromycin treatment. Eighteen drug resistant cell colonies were selected for further endostatin expression measurement. Among these, number 11 was the clone with the highest endostatin expression. The clone was then assigned the name CHO-ph11. The CHO cells transfected with empty vector pIRES-pac were used as the negative control (CHO-pac). To make encapsulated cells, the CHO-ph11 cells or CHO-pac cells dispersed in 1.5% sodium alginate (2×10⁶ cells/ml alginate) were injected in droplet form into a 0.1 M CaCl₂ solution and treated with 0.05% PLL. After gel formation, the alginate beads were washed three times in saline. The microcapsules have an average diameter of 0.6 mm (ranging from 500 to 700 nm) (Fig. 2). To determine the concentration of endostatin released from encapsulated CHO-ph11 cells, 1 ml microcapsules containing approximately 2×10⁶ CHO-ph11 cells were cultured in

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4 ml medium. After a 48 h culture in vitro, the medium was collected, and an ELISA was carried out on medium from the cultured CHO-ph11 and CHO-pac capsules. The concentration of endostatin secreted by $2 \times 10^6$ cultured encapsulated CHO-ph11 cells after 48 h was over 80 ng/ml.

### Angiogenesis in the CAM is inhibited by conditional culture medium of CHO-ph11

To analyze the effects of endostatin released from encapsulated CHO-ph11 cells on angiogenesis, the chicken CAMs of 5−6 d were treated with 100 µl culture supernatant of encapsulated CHO-ph11, or encapsulated CHO-pac, or 100 µl saline. After incubation for another 5 d, the eggshells were opened, and it was found that the vessels in the CAM treated with culture supernatant of encapsulated CHO-ph11 were sparser and more tenuous compared with the vessels in the CAM treated with culture supernatant of encapsulated CHO-pac or saline (Fig. 3).

### Endostatin released from encapsulated CHO-ph11 inhibited B16 melanoma subcutaneous growth in mice

The inhibition effect of the encapsulated CHO-ph11 on B16 melanoma growth in C57 mice was proven by the experiment data summarized in Fig. 4. Our results
suggested that endostatin released from encapsulated CHO-ph11 could significantly inhibit the subcutaneous growth of B16 melanoma cells in vivo. The tumor growth was reduced by over 80% in animals (Fig. 4) and fewer vessels were found when examined pathologically with hematoxylin-eosin stain (Fig. 5).

Discussion

At present, malignant tumors are mainly treated with surgery, radiotherapy or chemotherapy. However, they tend to recur at their primary sites and metastasize to distant sites after these treatments, thus have a poor prognosis. Therefore, new methods are needed for the treatment of malignant tumors. Antiangiogenic therapy has become an important alternative. Endostatin is one of the most potential antiangiogenic drugs that can directly induce apoptosis in tumor cells [24,25]. However, results from clinical trials are not optimistic. The higher level of drug concentration and short half-life in vivo limit the clinical application of recombinant endostatin protein. So it is necessary to find a way for effective endostatin delivery.

Since it was first developed by Lim and Sun in the early 1980s [17], cell microencapsulation has been extensively used for the transplantation of different kinds of cells and tissues [27–30]. Recently, recombinant cells secreting gene products with therapeutic purposes have been encapsulated in APA microcapsules [31]. Microencapsulation of recombinant cells enables such cells to be implanted into any host to deliver the desired gene product without triggering graft rejection. The advantage of this method is obvious: it does not require the modification of the host’s genome; it provides amply material for quality assessment before implantation; it is a safe method of in vivo delivery [29,30]; and it also avoids the cumbersome purification process of gene products.

In 2001, Read et al. and Joki et al. showed that encapsulated cells secreting endostatin could significantly inhibit the growth of tumors in mice and rats [6,7]. However, in these studies, encapsulated cells secreting endostatin were transplanted around the tumor site. This method tremendously limits the encapsulated cell endostatin delivery system for future clinical use, because local delivery of drugs might not be feasible in the treatment of many tumors, especially for metastasis. It is not clear whether this microencapsulated cell endostatin delivery system is efficacious when the cells were not transplanted around tumor site, for example, in peritoneal cavities of mice.

In this study, we constructed an engineered CHO cell line, CHO-ph11, which stably expresses human endostatin. This cell line was microencapsulated and transplanted into the peritoneal cavities of mice bearing subcutaneous B16 melanoma. Our results clearly proved that the intraperitoneally embedded CHO-ph11 cell line could significantly inhibit the subcutaneous growth of melanoma in mice. It is very important to note that, although we did not measure the real concentration of endostatin in mice after encapsulated cells were transplanted because of technological limitations, we can estimate the amount of endostatin in a mouse is approximately 40 ng per day from the data produced by in vitro measurement. This is at least 10,000-fold lower than normal recombinant endostatin protein treatment (20 mg/kg per day) [26]. So, we further proved this method could be given systemically for the treatment of cancer, which will surely widen its clinical applications in cancer treatment.

Those cells qualified for the delivery of therapeutic recombinant gene products with microencapsulation technology should have several important properties [32]: robustness and ability to proliferate, to permit gene transfection and cell expansion in vitro; the capacity to secrete and release the transgene product; and stable transgene expression following encapsulation. A more important property is that these cells should be able to differentiate terminally, which prevents overcrowding of cells within the microcapsules and excludes the probability of tumorigenicity.
However, cells fulfilling all of these requirements are rare. In the present study, we chose CHO because it is the most commonly used cell line for expression of foreign proteins for medical purposes, its safety is well proved, and the expansion and manipulation of foreign gene transfection in vitro is easy. More importantly, CHO, derived from Chinese hamster, is a xenogeneic cell line to mice and human, so, even if the microcapsules containing the cells are broken in the peritoneal cavity, they will be rejected quickly and will not develop tumor in vivo.

Microcapsules can isolate recombinant cells from the immune system of the host, preventing the rejection of cells. However, the long-term survival of microencapsulated cells is still a problem. This might not relate to the origin of cells because microencapsulated autologous or heterozygous cells have similar survival rates in rats [33]. This could partly be explained by the purity of alginate. Alginate is a crude product extracted from alga, contaminated with several inflammation-provoking components. Another important factor is thought to be insufficient nutrition as a consequence of insufficient blood supply to the encapsulated cells. Mannuronic acid and guluronic acid of alginate were found to be associated with more severe fibrotic overgrowth [34,35], which limited the supply of nutrition as well as the release of transgene product. In our study, encapsulated cells retrieved from the peritoneal cavity were also found to be wrapped by fibrotic tissue and collagen to a certain extent.

However, long term or permanent survival of encapsulated cells within the host in the treatment of malignant tumors might not be as essential as in the treatment of some hereditary diseases. In the present study, we also found that, although the survival term of encapsulated recombinant cells secreting endostatin is limited, the subcutaneous growth of B16 melanoma was inhibited significantly. Furthermore, encapsulated recombinant cells can be cryopreserved for long periods, and taken out when necessary for repeating the treatment, as with traditional medicines [36].

In conclusion, the results show that APA microcapsules containing cells secreting hES, given twice intraperitoneally, can significantly suppress the subcutaneous growth of B16 melanoma cells. This will widen the clinical application of the microencapsulated cell endostatin delivery system in cancer treatment.

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