Subsieve-size agarose capsules enclosing ifosfamide-activating cells: a strategy toward chemotherapeutic targeting to tumors

Shinji Sakai,[1] Kenji Kawabata,[1] Shinji Tanaka,[3] Norifumi Harimoto,[2] Ichiro Hashimoto,[1] Changjun Mu,[1] Brian Salmons,[4] Hiroyuki Ijima,[1] and Koei Kawakami[1]

[1]Department of Chemical Engineering, Faculty of Engineering, and [2]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [3]Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, Tokyo, Japan; and [4]Austrianova Biotechnology GmbH, Vienna, Austria

Abstract

Localized activation of the prodrug ifosfamide in or close to tumors by implanting encapsulated ifosfamide-activating cells is an efficacious strategy for tumor therapy. The aim of this study was to evaluate the feasibility of subsieve-size agarose capsules for enclosing the cells in this application. Compared with many conventional microcapsules, subsieve-size agarose capsules are about one-tenth the size and have both higher mechanical stability and allow better molecular exchangeability than other systems. Cells that have been genetically modified to express cytochrome *P*450 2B1 enzyme were encapsulated in subsieve-size agarose capsules of ~90 μm in diameter and implanted into preformed tumors in nude mice. Living cells were detected for >1 month after encapsulation *in vitro* and showed enzymatic activity (i.e., they were able to activate ifosfamide). More significant regression of preformed tumors was observed in the recipients implanted with cell-enclosing capsules compared with those implanted with empty capsules. These results suggest that the strategy of using subsieve-size agarose capsules enclosing cytochrome *P*450 2B1–expressing cells is feasible for tumor therapy by chemotherapeutic targeting in combination with ifosfamide administration. [Mol Cancer Ther 2005;4(11):1786–90]

Received 7/5/05; revised 8/11/05; accepted 8/30/05.

Grant support: Ministry of Education, Science, Sports and Culture of Japan Grant-in-Aid for Young Scientist (B) 17760627.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Shinji Sakai, Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Phone: 81-92-642-4109; Fax: 81-92-642-4109. E-mail: sakai@chem-eng.kyushu-u.ac.jp

doi:10.1158/1535-7163.MCT-05-0227

Introduction

The concept of cell transplantation after encapsulating in spherical-shaped vehicles was proposed to protect transplanted cells implanted for therapeutic treatments from attack by the host immune system without the need for the administration of immunosuppressive agents (1). After the first successful report by Lim and Sun in 1980 (2), an increasing number of investigations have focused their interest on the cell-encapsulating devices for the treatment of wide range of diseases, such as hormone deficiencies and neurodegenerative disorders (3). One of the attractive applications is the use of such devices for tumor therapy. The effectiveness of this kind of strategy has been revealed in a phase I/II clinical trial (4, 5) as well as in animal experiments (6–8). The microcapsules of >500 μm in diameter have been traditionally applied to mammalian cell encapsulation (9). As far as we know, except for the encapsulation technology developed by our group, all cell-enclosing capsules with narrow distribution in size have had a diameter of >200 μm. This is probably due to the progress of mammalian cell encapsulation technology based on the techniques developed for enclosing pancreatic islets with diameters of 50 to 300 μm. Recently, we developed a novel process that is suitable for encapsulating single cells (10–30 μm in diameter) and revealed that the process scarcely hindered the viability of mammalian cells (10, 11). The resultant capsules have a diameter of <100 μm such that they are of subsieve size with a narrow size distribution. Such a reduction in capsule size results in an enhancement of both molecular exchangeability and mechanical stability (11). Higher molecular exchangeability is attractive for a rapid release of therapeutic substances having a short half-life into body fluids surrounding the enclosed cells as well as enhancement of oxygenation and nutrient supply to the enclosed cells. In this study, we examined the feasibility of using subsieve-size agarose capsules for a localized chemotherapy by providing an alternative site of the activation of a prodrug, ifosfamide, which normally occurs in the liver, into or near tumors. Ifosfamide is a prodrug that is specifically metabolized into two major active compounds, phosphoramide mustard and acrolein, by cytochrome *P*450 2B1 (CYP2B1) in the liver (12). Due to a very short half-life of the activated compounds in plasma (13), ifosfamide has to be given in relatively high doses despite severe side effects associated with such doses, such as leukopenia with granulocytopenia. One approach for reducing the side effects without lowering response rates is establishing a second site of enzyme conversion near or in the tumor by bringing cells transfected to express CYP2B1 close to the tumor (6). The effectiveness of this approach was revealed by placing the

microcapsules enclosing the cells in the artery near the tumor using microcatheter in a clinical trial (4, 5). Compared with the conventional microcapsules of several hundred micrometers in diameter, subsieve-size capsules may be instilled with a low risk of occlusion of blood vessels due to their approximately one-tenth in size as well as reducing the surgical trauma resulting from the reduction in size of the capsule injection device. In this study, we encapsulated the cells transfected to express CYP2B1 in subsieve-size agarose capsules. Agarose is a natural polysaccharide extracted from the cellular walls of agarophyte seaweed and exhibits temperature-sensitive water solubility. Agarose hydrogel has been widely studied for biomedical applications toward transplantation with cells, such as tissue scaffolds (14) and cell encapsulation vehicles due to a high biocompatibility (15). As a first step, the viability and enzymatic function were evaluated *in vitro*. Subsequently, the CYP2B1 cells enclosed in the subsieve-size capsules were transplanted in nude mice with preformed tumors for evaluating the effect on tumor regression in combination with ifosfamide administration.

Materials and Methods

Materials

Agarose with a low gelling temperature (26–30°C at 1.5%) was purchased from Cambrex Bio Science (Rockland, ME). Liquid paraffin was obtained form Kanto Kagaku (Tokyo, Japan). Lecithin from soybean was purchased form Wako (Osaka, Japan). Feline kidney cells engineered to produce the liver active enzyme CYP2B1 (6), an ifosfamide-activating cytochrome, were grown in DMEM (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 400 mg/dL glucose, 75 mg/L penicillin, and 50 mg/L streptomycin. They were encapsulated in subsieve-size agarose capsules. The human tongue squamous carcinoma cell line SAS was used to establish tumors in nude mice. The cells were grown in RPMI 1640 containing the same quantities of fetal bovine serum, glucose, penicillin, and streptomycin with those in the medium for CYP2B1 cells. Both cells were cultured in a humidified atmosphere at 37°C with 5% CO_2. Male nude mice (BALB/cA Jcl-*nu*, 8 weeks old) were used for a preformed tumor regression study using the CYP2B1 cells enclosed in subsieve-size agarose capsules.

Preparation of Capsules

Cell-enclosing subsieve-size agarose capsules of 88 ± 10 μm in diameter were prepared using the droplet generator reported previously (10, 11). Briefly, 4 wt % agarose in Krebs-Ringer-HEPES buffer solution (37°C) containing CYP2B1 cells at 1.7×10^7/mL or 6.0×10^7/mL for *in vitro* and *in vivo* studies, respectively, were extruded into a coflowing immiscible stream of liquid paraffin containing 3 wt % lecithin from soybean from a stainless needle of 300 μm inner diameter and 480 μm outer diameter. The liquid paraffin kept at 37°C was allowed to flow in the same direction with the cell-suspending agarose solution. The resultant suspension was cooled at 4°C for 10 minutes for gelling the agarose solution. The gelled capsules were collected from the suspension using a mesh of 45 μm pore diameter. The collected capsules were washed with Krebs-Ringer-HEPES buffer solution to remove the liquid paraffin from their surface.

Metabolic Viability Determination

Cellular viability of enclosed CYP2B1 cells was determined using a colorimetric assay kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). The resultant absorbance at 450 nm is proportional to the number of intact mitochondria. Results were expressed as mean ± SD for four replicates.

Cell Occupation Area Determination

The percentage of cell occupation area in capsules was determined using image analysis software (Scion Image, Scion Co., Frederick, MD) for individual digital microphotograph containing >30 capsules. Results were expressed as mean ± SD for four replicates.

CYP2B1 Function of Enclosed Cells

The expression of biologically active CYP2B1 in the cells enclosed in subsieve-size agarose capsules was determined using a biochemical assay, which is specific for the enzyme (16). Briefly, cell-enclosing subsieve-size capsules were incubated in DMEM containing 2 μmol/L 3-methylcholanthrene and 10% fetal bovine serum for 36 hours. After washing several times with PBS, the capsules were suspended in serum-free DMEM containing 10 μmol/L 7-pentoxyresorufin (Sigma) and 10 μmol/L dicumarol (Sigma) at 2.7×10^4 capsules/mL and then incubated in a 12-well dish for 30 minutes at 37°C. The quantity of produced resorufin in supernatant was measured with a fluorometer at 530 nm excitation and 590 nm emission. A standard curve was produced using different amount of purified resorufin (Sigma). Results were expressed as mean ± SD for four replicates.

Treatment of Tumors

The human tumor cell line SAS was used for the establishment of preformed tumors in nude mice as a model for evaluating treatment with encapsulated cells followed by chemotherapy. Each mouse was injected 5×10^6 SAS cells suspended in 0.5 mL saline s.c. into their left flanks using a 26-gauge syringe. Tumors were allowed to grow for 9 days and the greatest and least diameters of the tumors were measured for calculating their volumes using the following formula: volume = $(\text{mean diameter})^3 \times \pi / 6$. Twenty animals with tumors of >350 mm^3 were divided into two groups consisting of 10 animals each. The recipients of one group received CYP2B1 cells enclosed in subsieve-size agarose capsules (containing a total of 4×10^6 cells per recipient) and the recipients of the other group served as a control and were implanted the same number of empty subsieve-size capsules. The capsules were injected into each tumor after suspension in 0.5 mL saline using a 26-gauge syringe. Twelve hours after the implantation, ifosfamide dissolved in saline was injected into the peritoneal cavities of the animals at 100 mg/kg body weight. The injection was done every other day for the first 6 days and then every fourth day until day 24.

All of the protocols using animals were according to the recommendations of Kyushu University entitled Guide for the Care and Use of Laboratory Animals.

Results

Metabolic Viability and CYP2B1 Activity of Enclosed Cells

To determine the viability of the enclosed cells in the subsieve-size capsules, the metabolic activity based on the activity of dehydrogenase enzymes within intact mitochondria in living cells was studied during 42 days of culture (Fig. 1). The capsule-based viability increased over the first 7 days, and the value on day 7 was 2.9-fold larger than that on day 1. Despite the increase in metabolic activity, almost all the CYP2B1 cells enclosed in capsules maintained their initial configuration over the first 7 days of cultivation (Fig. 2A and B). Indeed, there was no significant difference between the cell occupation areas in capsules on day 1 (22.1 $\pm$ 3.6% per capsule) and day 7 (22.4 $\pm$ 6.8% per capsule; P = 0.95, ANOVA). Then, the capsule-based viability gradually decreased over the following culture period. The absorbance on day 42 of culture was below the detection limit. Over the cultivation period, the subsieve-size agarose capsules were not filled by enclosed cells (Fig. 2C) and successfully inhibited the escape of the enclosed cells from the capsules into the medium.

To determine whether the enclosed CYP2B1 cells maintain genetically expressed enzymatic activity, the expression of active CYP2B1 was studied on days 1 and 14 of cultivation. On day 14, the capsule-based quantity of resorufin production from 7-pentoxyresorufin per capsule was 3.6-fold higher than on day 1 (Fig. 3). Compared with the cell viability–based quantities, the cells on day 14 had a 2.8-fold higher CYP2B1 enzymatic activity than those on day 1.

Preformed Tumor Regression after Implanting Capsules

To investigate the local CYP2B1 expression function of the CYP2B1 cells enclosed in agarose subsieve-size capsules on ifosfamide treatment of preformed tumors, time-course changes in volumes of the preformed tumors were measured for the two groups of the recipients: implanted cell-enclosing capsules (group 1) and empty capsules (group 2; Fig. 4). A more significant reduction in tumor volume was observed in the mice that received the cell-enclosing subsieve-size capsules. On day 6, the tumor size of 7 of 10 mice was >80% of the size of those just before capsule implantation (>0.8V_0). In contrast, 1 mouse had a tumor of >0.8V_0 and 7 of 10 mice had tumors of <0.6V_0 in group 1 on day 6. The degree of tumor size reduction became more significant over the following treatment period. In group 1, tumors were not observed in 7 mice in appearance (Fig. 5) and tumors of <0.2V_0 were observed in 2 mice on day 26. In the control group, tumors disappeared in 3 mice in appearance and

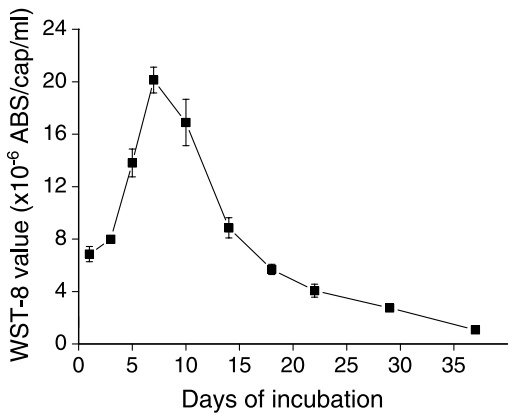

Figure 1. Viability of CYP2B1 cells enclosed in subsieve-size agarose capsules determined using a colorimetric assay kit. *Points,* mean (n = 4); *bars,* SD.

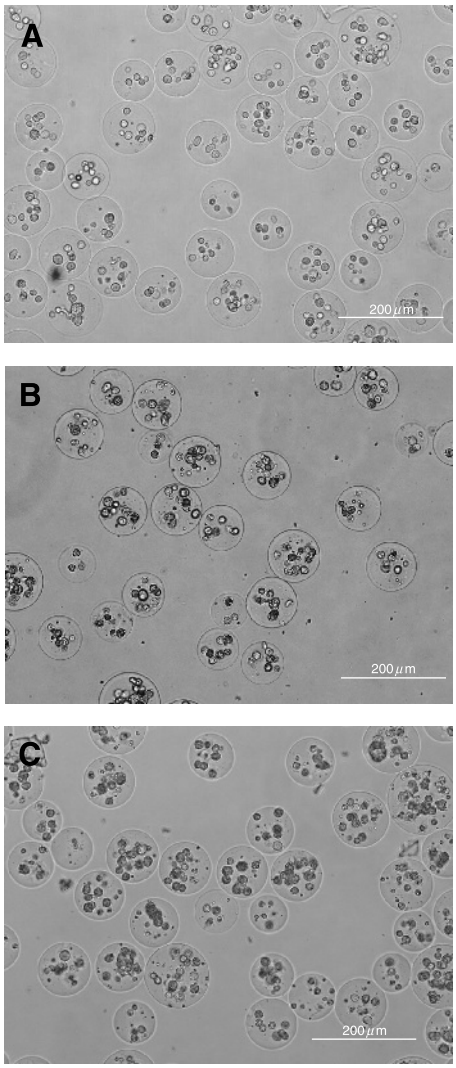

Figure 2. Photographs of subsieve-size agarose capsules enclosing CYP2B1 cells after 1 d (**A**), 7 d (**B**), and 29 d (**C**) of incubation *in vitro*. The cell occupation areas in capsules were 22.1 $\pm$ 3.6%, 22.4 $\pm$ 6.8%, and 20.7 $\pm$ 2.2% (mean $\pm$ SD, n = 4) on days 1, 7, and 29, respectively. No significant difference was observed between the values (P = 0.96, ANOVA).

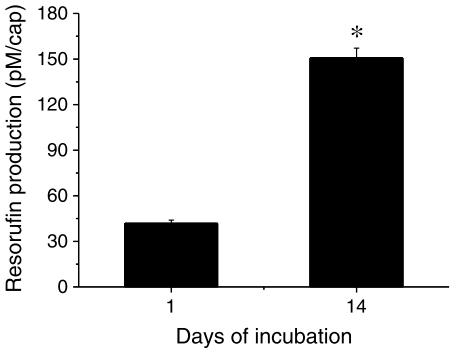

Figure 3. Resorufin production activity of CYP2B1 cells enclosed in subsieve-size capsules after 1 and 14 d of cultivation *in vitro*. *Columns,* mean ($n = 4$); *bars,* SD. *, $P < 0.05$, two-tailed Student's unpaired t test.

2 mice had tumors of $<0.2V_0$ on that day. Over the study period, only 1 mouse in group 1 showed no reduction in tumor size. The size of the tumor of that mouse was $1.6V_0$ on day 26. In group 2, tumor regression was not observed in 3 mice, $1.0V_0$, $1.2V_0$, and $1.6V_0$, after 26 days of treatment with ifosfamide.

Discussion

This work was motivated by the need to develop a safer and more efficient cell delivery device placed in or near tumors using catheter as a novel supply source of antitumoral compounds converted from prodrug ifosfamide for cancer therapy. To accomplish this objective, we investigated the use of subsieve-size capsules that we developed recently (10, 11). Recently, Lohr et al. (4, 5) revealed the clinical benefit of a localized chemotherapy using microencapsulated cells genetically engineered to express CYP2B1 for advanced pancreatic tumor in a phase I/II clinical trial with systemically given low-dose ifosfamide. They reported that

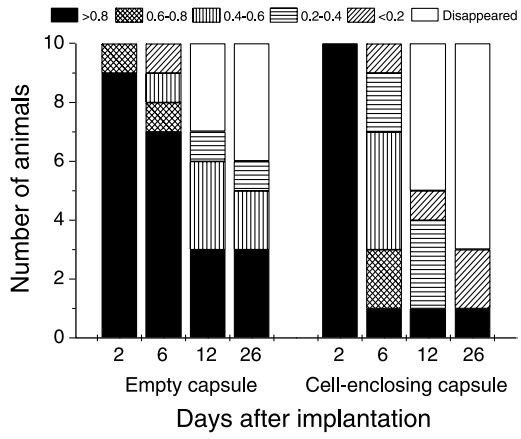

Figure 4. Preformed tumor size changes of the recipients transplanted with empty and CYP2B1 cell-enclosing subsieve-size agarose capsules. Ten nude mice each were used as recipients of empty and cell-enclosing capsules. Tumor size just before capsule implantation was set to 1 and each block represents the proportional size of the original tumor.

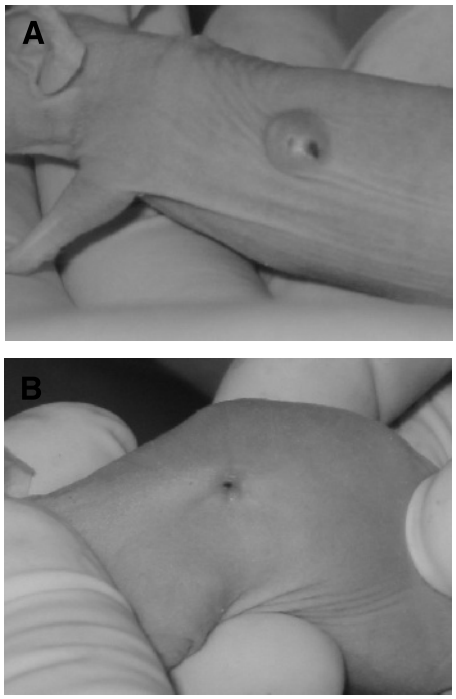

Figure 5. Subcutaneously xenotransplanted human tumors just after injection of the CYP2B1 cells enclosed in subsieve-size agarose capsules (**A**) and 26 d after treatment with ifosfamide (**B**).

there were no side effects from the chemotherapy in the patients and described that it is the strategy to maximize efficiency and minimize toxicity (17). Smaller capsules may be better for this kind of approach because they are easier to administer. We showed previously that there is better molecular exchange between the enclosed cells and environmental fluids from subsieve-size capsule as well as better mechanical stability (11). In addition, we revealed that the capsule generation process scarcely hindered the viability of enclosed mammalian cells as measured of immediately after encapsulation (10, 11).

As a first stage in the following investigations, such as the studies using large animals and clinical trials, we evaluated genetically expressed enzymatic function and viability of the enclosed cells in the subsieve-size agarose capsules during cultivation. The measurement for enzymatic function revealed that the cells enclosed in the subsieve-size agarose capsules retained expression of the CYP2B1 enzyme (Fig. 3). The cell viability–based productivity of resorufin by the active enzyme within enclosed cells on day 14 was 2.8-fold higher than those on day 1. It is interpreted as a result of an alteration of the enclosed cells in adapting to the new microenvironment. Such a change in secretion productivity of individual cells according to the surrounding microenvironment has been observed for both unenclosed cells (18) and enclosed cells in agarose microcapsules (19).

Although the mechanism of decreasing viability from 7 days after encapsulation (Fig. 1) is still unclear, the tendency that the viability increases over the early cultivation period

followed by subsequent gradual decrease is not specific to this study. It is in agreement with studies reported by Orive et al. (20) for several kinds of mammalian cells enclosed in the microcapsules made from alginate-agarose composite and with a diameter of 350 to 450 μm. Compared with our capsules, the microcapsule contains alginate as an additional component. Studies done to analyze the effect of alginate on changes in viability of the cells enclosed in alginate-agarose subsieve-size capsules (data not shown) showed a tendency of the cells to lose viability in agarose subsieve-size capsules and this was independent on the size of the cell-enclosing capsule (i.e., it is not due to the reduction in capsule diameter to subsieve size). Despite increasing viability in the early cultivation period, almost all of the enclosed cells maintained their initial appearance and there was no significant difference in the cell occupation areas in capsules (Fig. 2). This may be a consequence of limited space; thus, cell proliferation is only possible in the small spaces that were originally filled with individual cells at the actual time of agarose gelation. The growth profiles of the cells embedded in the gel are dependent on their origin (20, 21); however, the solid stress induced by the surrounding rigid agarose gel is a possible explanation of the unchanged configuration of the cells enclosed in agarose capsules prepared from 4% agarose solution over the cultivation period. Helmlinger et al. (22) reported that the clonal efficiency of the tumor cells enclosed in 1.8% agarose gel was only 5% despite that in a 1.0% gel it was >90%. In fact, we observed the formation of cell clusters and an improved viability of the cells enclosed in subsieve-size agarose capsules prepared from 2% agarose solution (data not shown). However, a large number of cells left from the capsules into the medium. Although decreasing the solid stress induced by the surrounding gel may prolong the therapeutic functional period of cell-enclosing capsules as a result of cell growth within them, escape of cells into the surrounding environment is not optimal, from a safety point of view. In the case of transplanting a proliferative cell line *in vivo*, suppressing an excess proliferation of enclosed cells is vital for avoiding the risk of tumorigenesis (23).

The effect of local activation of ifosfamide using the CYP2B1 cells enclosed in agarose subsieve-size capsule on tumor regression was next investigated to examine the potency of the device for tumor therapy. The CYP2B1 cell used in this study were used previously to show feasibility of local chemotherapy of tumors with ifosfamide using cellulose sulfate microcapsules of ~500 μm in diameter (6). Based on these results, we investigated two groups of mice implanted with either empty or CYP2B1 cell-enclosing capsules. Our results indicate that the CYP2B1 cells enclosed in the subsieve-size agarose capsules successfully converted the ifosfamide to toxic metabolites at the site where they were instilled. The number of the recipient mice showing a significant reduction in tumor volume or tumor disappearance was 3-fold greater in the group that received the cell-enclosing capsules than those receiving empty capsules. The tumor regression of the recipients in the latter group is due to endogenous conversion of ifosfamide by the liver (6). Importantly, the feasibility of using subsieve-size agarose capsules enclosing the cells genetically modified to express the CYP2B1 was shown in this study.

References

1. Chang TMS. Semipermeable microcapsules. Science 1964;146:524–5.
2. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908–10.
3. Hauser O, Prieschl Grassauer E, Salmons B. Encapsulated, genetically modified cells producing *in vivo* therapeutics. Curr Opin Mol Ther 2004;6:412–20.
4. Lohr M, Kroger JC, Hoffmeyer A, et al. Safety, feasibility and clinical benefit of localized chemotherapy using microencapsulated cells for inoperable pancreatic carcinoma in a phase I/II trial. Cancer Ther 2003;1:121–31.
5. Lohr M, Hoffmeyer A, Kroger JC, et al. Microencapsulated cell-mediated treatment of inoperable pancreatic carcinoma. Lancet 2001;357:1591–2.
6. Lohr M, Muller P, Karle P, et al. Targeted chemotherapy by intratumour injection of encapsulated cells engineered to produce CYP2B1, an ifosfamide activating cytochrome *P*450. Gene Ther 1998;5:1070–8.
7. Joki T, Machluf M, Atala A, et al. Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol 2001;19:35–9.
8. Read TA, Sorensen DR, Mahesparan R, et al. Local endostatin treatment of gliomas administered by microencapsulated producer cells. Nat Biotechnol 2001;19:29–34.
9. Uludag H, de Vos P, Tresco PA. Technology of mammalian cell encapsulation. Adv Drug Deliv Rev 2000;42:29–64.
10. Sakai S, Kawabata K, Ono T, Ijima H, Kawakami K. Preparation of mammalian cell-enclosing subsieve-sized capsules (<100 μm) in a coflowing stream. Biotechnol Bioeng 2004;86:168–73.
11. Sakai S, Kawabata K, Ono T, Ijima H, Kawakami K. Development of mammalian cell-enclosing subsieve-size agarose capsules (<100 μm) for cell therapy. Biomaterials 2005;26:4786–92.
12. Dirven HA, Megens L, Oudshoorn MJ, Dingemanse MA, van Ommen B, van Bladeren PJ. Glutathione conjugation of the cytostatic drug ifosfamide and the role of human glutathione *S*-transferases. Chem Res Toxicol 1995;8:979–86.
13. Kurowski V, Wagner T. Comparative pharmacokinetics of ifosfamide, 4-hydroxyifosfamide, chloroacetaldehyde, and 2- and 3-dechloroethylifosfamide in patients on fractionated intravenous ifosfamide therapy. Cancer Chemother Pharmacol 1993;33:36–42.
14. Hu JC, Athanasiou KA. Low-density cultures of bovine chondrocytes: effects of scaffold material and culture system. Biomaterials 2005;26:2001–12.
15. Iwata H, Simada H, Fukuma E, Ibii T, Sato H. Bioartificial pancreas research in Japan. Artif Organs 2004;28:45–52.
16. Donato MT, Gomez Lechon MJ, Castell JVA. Microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. Anal Biochem 1993;213:29–33.
17. Lohr M, McFadyen MC, Murray GI, Melvin WT. Cytochrome *P*450 enzymes and tumor therapy. Mol Cancer Ther 2004;3:1503.
18. Park TG. Perfusion culture of hepatocytes within galactose-derivatized biodegradable poly(lactide-co-glycolide) scaffolds prepared by gas foaming of effervescent salts. J Biomed Mater Res 2002;59:127–35.
19. Cadic C, Dupuy B, Pianet I, Merle M, Margerin C, Bezian JH. *In vitro* culture of hybridoma cells in agarose beads producing antibody secretion for 2 weeks. Biotechnol Bioeng 1992;39:108–12.
20. Orive G, Hernandez RM, Gascon AR, Igartua M, Pedraz JL. Survival of different cell lines in alginate-agarose microcapsules. Eur J Pharm Sci 2003;18:23–30.
21. Sefton MV, May MH, Lahooti S, Babensee JE. Making microencapsulation work: conformal coating, immobilization gels and *in vivo* performance. J Control Release 2000;65:173–86.
22. Helmlinger G, Netti PA, Lichtenbeld HC, Melder RJ, Jain RK. Solid stress inhibits the growth of multicellular tumor spheroids. Nat Biotechnol 1997;15:778–83.
23. Chang PL, van Raamsdonk JM, Hortelano G, Barsoum SC, MacDonald NC, Stockley TL. The *in vivo* delivery of heterologous proteins by microencapsulated recombinant cells. Trends Biotechnol 1999;17:78–83.

Molecular Cancer Therapeutics

Subsieve-size agarose capsules enclosing ifosfamide-activating cells: a strategy toward chemotherapeutic targeting to tumors

Shinji Sakai, Kenji Kawabata, Shinji Tanaka, et al.

Mol Cancer Ther 2005;4:1786-1790.