Covalent cross-links between membrane components prevent dissemination of microencapsulated malignant cells

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Introduction

Stem cell-derived cells or tissues[1-3], immortalized cell lines[4, 5] and bioengineered cells[6, 7] are considered potentially therapeutic for the treatment of many diseases including those with deficient hormone production, such as insulin in type 1 diabetes, erythropoietin in anemia[8], factors VIII and IX in hemophilia[9] and growth hormone in dwarfism[10]. However, these three types of cell present risks of malignant transformation. Stem cells, particularly embryonic stem cells and embryonic germ cells, have the ability to improperly differentiate into tumors in animals[2, 11-13]. All efforts should be made to minimize potentially severe consequences.

Microencapsulation of cells or tissues, such as islets of Langerhans, in semipermeable alginate-poly-L-lysine-alginate (APA) membranes has been investigated as a means of protecting transplanted cells from the host immune system and avoiding the requirement for immunosuppression[14]. For this purpose, therapeutic cells are immobilized in calcium alginate beads that are sequentially incubated in solutions of polycation, such as poly-L-lysine (PLL), to form a semipermeable membrane, and in diluted alginate to improve the microcapsule biocompatibility. The strength of such microcapsules is dependent upon electrostatic interactions between alginate (negatively charged) and polycation (positively charged). Since these charged polymers are in competition with any charged molecules in their environment, the stability of standard microcapsules is limited[15]. We previously developed a method for microencapsulating living cells in semipermeable membranes with covalent cross-links between the molecules of the microcapsule semipermeable layer and between such molecules and the microcapsule core bead and outer coating molecules using an heterobifunctionnal cross-linker (ANB-NOS)[16, 17]. Thereby the membranes of such covalently cross-linked microcapsules are considerably strengthened. We have shown *in vitro* that the method is not harmful for living cells.

We hypothese that encapsulation in microcapsules with covalent cross-links can prevents the dissemination of malignant cells and protect the host against a potential attack from the transplanted graft.

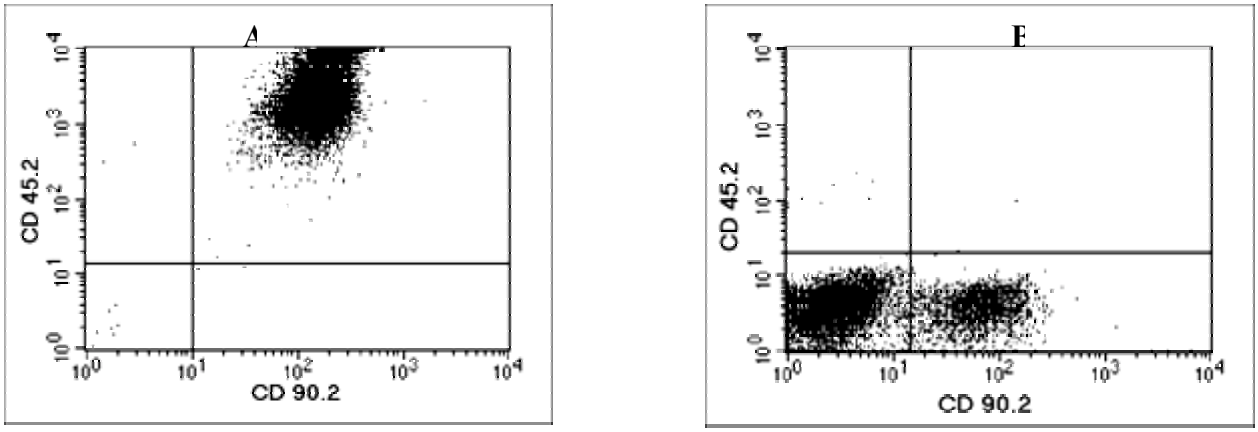
Materials and Methods

To prove this concept, the following animal model was chosen: very malignant EL-4 thymoma cells were transplanted intraperitoneally into B6.SJL mice. EL-4 cells are derived from C57BL/6 mice and their phenotype differs from the recipient phenotype by only one antigen (CD 45.2 vs CD 45.1, respectively). Using fluorescence activated flow cytometry (FACS), such an almost syngeneic model allowed for the identification of the cells derived from implanted cells compared to the recipient cells, while avoiding allogeneic reactions. In the first set of experiments, this model was characterized. Increasing amounts of EL-4 cells were injected into mice peritoneum (from 80 to 250 000 EL-4 cells) and mice survival was followed to determine the minimal dose of EL-4 cells able to induce malignant dissemination. Then, widespread of these EL-4 cells was investigated in several organs such as spleen, liver, bone marrow, lymph nodes, kidneys and peritoneal liquid to be

able to follow the disease evolution. Afterward, to test our hypothesis, EL-4 cells encapsulated within covalently cross-linked or standard microcapsules or not encapsulated were implanted into mice peritoneum. Widespread of EL-4 cells were compared in specific organs and the average survival of mice was followed. Finally, the effect of the new microencapsulation procedure using photoactivatable cross-linker was evaluated on *in vivo* islet cell survival and function. Isolated rat islets were encapsulated within covalently cross-linked or standard microcapsules or not encapsulated and transplanted into diabetic scid-STZ mice. Blood glucose was followed as an indicator of the graft survival and function.

Results and Discussion

The results of the animal model characterization showed that it was possible to differentiate implanted EL-4 cells (CD90.2+ CD45.2-) from B6.SJL mice recipient (CD90.2+ CD45.2+) using flow cytometry experiments (Figure 1). This allowed localization and follow-up of cells derived from implanted EL-4 cells into the recipient. The pattern of tissue infiltration by EL-4 showed that with the maximal number of EL-4 cells, all mice died at an average of 22.3 ± 1.0 days post-implantation. Decreasing the number of implanted EL-4 cells slightly delayed the animal death. Nevertheless, all mice transplanted with ≥20,000 non encapsulated EL-4 cells died within a period of 26.5 ± 0.9 days. Out of 8 mice transplanted with 10,000 EL-4 cells, 5 survived. All mice transplanted with ≤2,000 nonencapsulated EL-4 cells survived (Table 1). Moreover, EL-4 cells were principally located in lymph nodes, epididymal fat pads and free into the peritoneal liquid (Table 2). Mice implanted with nonencapsulated EL-4 cells died at a mean time of 22.3 ± 1.0 days after implantation (Figure 2). When 250,000 EL-4 cells encapsulated within standard microcapsules were implanted, the average survival time of recipients was prolonged to 35.2 ± 2.2 days (Figure 2). The key finding was that all mice that were implanted with 250,000 EL-4 cells encapsulated within covalently cross-linked microcapsules were still alive with no sign of disease at 150 days post-implantation. In addition, the analysis of the recipient organs 28 days after EL-4 implantation (Figure 3) showed that EL-4 cells were confined within cross-linked microcapsules since no EL-4 cells were detected in contrast to what was observed with standard microcapsules (41.5% in peritoneal fluid, 41.1% in epididymal fat pads and 9.1% in lymph nodes). The islet ttransplantation study demonstrated that microencapsulation in covalently cross-linked membranes has no harmful effect on *in vivo* islet cell survival and function. In recipients of islets that were encapsulated in either standard or covalently cross-linked microcapsules (Figure 4), the average blood glucose levels decreased rapidly to normal levels (~5 mmol/L) and remained normal six months after transplantation. No mice that were transplanted with nonencapsulated islets normalized their blood glucose.



<u>Figure 1:</u> Specificity of cell marker antibodies. Fluorescence activated flow cytometry analysis of cells stained with antibodies specific for CD 45.2 and CD 90.2.

A. Spleen cells from B6.SJL mice. B. EL-4 cells cultured *in vitro*.

Number of implanted EL-4 cells	Survival: % of mice (N)	Length of survival (days ± SEM)
80	100 (4/4)	-
400	100 (4/4)	-
2,000	100 (4/4)	-
10,000	62.5 (5/8)	29.0 ± 0.0
20,000	0 (0/4)	26.5 ± 0.9
30,000	0 (0/4)	24.0 ± 0.4
40,000	0 (0/4)	22.5 ± 0.5
50,000	0 (0/4)	22.8 ± 0.7
250,000	0 (0/12)	22.3 ± 1.0

Table 1: Dose-response to peritoneal implantations of nonencapsulated EL-4 cells.

ORGANS	EL4 CELLS
Bone marrow	-
Spleen	+
Lymph nodes	++
Peritoneal liquid	+++
Kidney	+
Epididymal fatpad	++
Liver	+

Table 2: EL4 infiltration and accumulation .

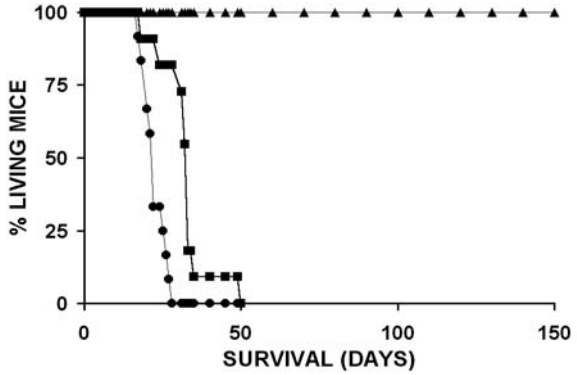


Figure 2: Follow-up of B6.SJL mice survival (%) after EL-4 implantation. Circles: nonencapsulated EL-4 cells (N=12); squares: EL-4 cells microencapsulated in standard membranes (N=11); triangles: EL-4 cells microencapsulated in covalently cross-linked membranes (N=11).

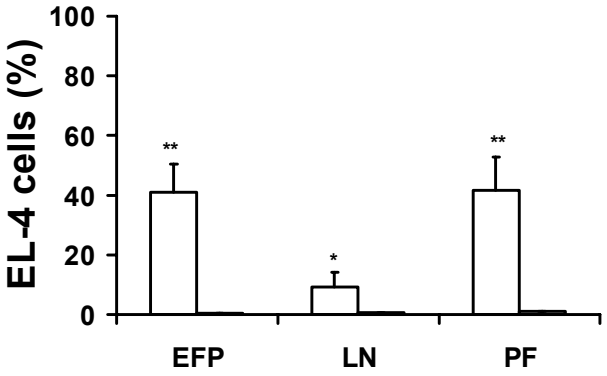


Figure 3: EL-4 thymoma cell dissemination from standard and covalently cross-linked microcapsules 28 days following the implantation in mice. White: standard microcapsules. Black: covalently cross-linked microcapsules.

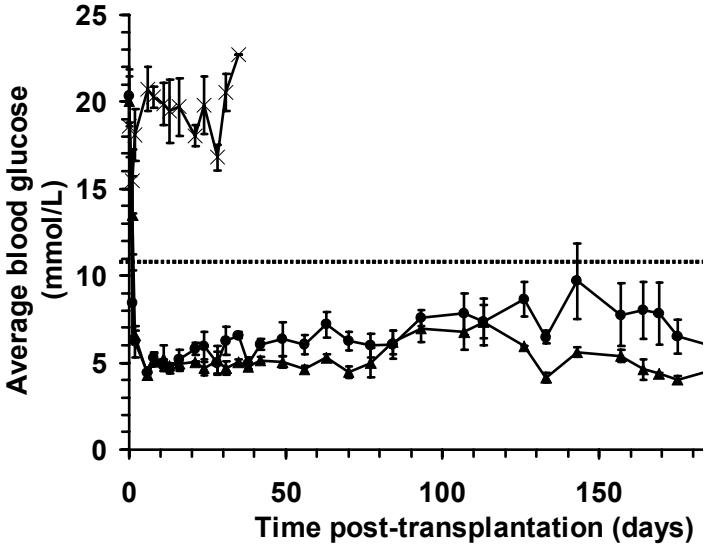


Figure 4: ***In vivo*** **microencapsulated islet survival and function in covalently cross-linked membranes. Average blood glucose (mmol/L) of diabetic mice transplanted with: triangles: nonencapsulated islets (N=3); closed circles: islets encapsulated in standard microcapsules (N=5); open circles: islets encapsulated in microcapsules with covalently cross-liked membranes (N=4).**

Conclusions

Cell encapsulation within covalently cross-linked microcapsules offers a double protection: protection of the graft against the immune system of the host and now protection the host against a possibility of malignant cell dissemination from stem cells or transformed cells.

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