Bioengineered Implantation of Megalin-Expressing Cells: A Potential Intracorporeal Therapeutic Model for Uremic Toxin Protein Clearance in Renal Failure

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Abstract. Patients who have renal failure and are on dialysis therapy experience serious complications caused by low-molecular-weight uremic toxin proteins normally filtered by glomeruli and metabolized by proximal tubule cells (PTC). Dialysis-related amyloidosis is one such complication induced by systemic deposition of amyloid proteins derived from 12-kD β2-microglobulin (β2-m). Despite the use of high-flux membrane hemodialysis devices and direct absorbent columns, the removal of β2-m is suboptimal, because the effects are transient and insufficient. Megalin is expressed in the apical membranes of PTC and recognized as a multiligand endocytic receptor that binds numerous low-molecular-weight proteins, including β2-m. This study tested the feasibility of an intracorporeal therapeutic model of continuous β2-m removal using megalin-expressing cell implantation. By cell association and degradation assays, rat yolk sac–derived L2 cells were identified to internalize and degrade β2-m via megalin. The cells were effectively implanted within the subcutaneous tissues of nude mice using a type I collagen scaffold and a method inducing local angiogenesis. After nephrectomy and intraperitoneal injection with 125I-β2-m, it was found that the implanted cells took up the labeled ligand, efficiently removing it from the blood. Bioengineered implantation of megalin-expressing cells may represent a new supportive therapy for dialysis patients to compensate for the loss of renal protein metabolism and remove uremic toxin proteins.

The incidence and prevalence of ESRD are increasing worldwide (1). Because kidney transplantation is not sufficient for patients with ESRD, a number of them are under dialysis therapy (hemodialysis or peritoneal dialysis). However, the therapy is still suboptimal, with poor clinical outcomes for patients. One of its crucial limitations is that it does not efficiently compensate for the loss of renal protein metabolism that is normally accomplished by glomerular filtration and reabsorption and degradation by proximal tubule cells (PTC) of low-molecular-weight proteins (LMWP). Consequently, LMWP accumulate in the serum and tissues of dialysis patients, and some of these, such as β2-microglobulin (β2-m), parathyroid hormone (PTH), and advanced glycation end product peptides (2), act as uremic toxins, causing serious complications. Among them, β2-m is the most established 12-kD uremic toxin protein that causes dialysis-related amyloidosis (3). It is characterized by osteoarthropathy and variable organ failure as a result of deposition of β2-m–derived amyloid proteins (4,5). Despite the development of high-flux membrane hemodialysis devices and direct absorbent columns, β2-m accumulation is inevitable in afflicted patients because the therapeutic effects are transient and insufficient (4).

Recently, Humes et al. (6,7) developed an extracorporeal hemoperfusion system that uses porcine PTC grown along the inner surface of hollow fibers. However, this cell-therapy model is still an intermittent type of treatment, and its effects on uremic toxin protein metabolism have not been clarified. Therefore, the development of continuous, intracorporeal, cell (or molecule)-mediated types of therapeutic models is required for future clinical application.

Megalin, a member of the LDL receptor gene family (8), is recognized as a multiligand endocytic receptor (9–11). It is highly expressed at the apical surface of PTC (12,13). Megalin binds numerous LMWP, including transcobalamin-B12 (14), vitamin D–binding protein (15), retinol-binding
protein (16), PTH (17), insulin, β2-m, epidermal growth factor, prolactin, lysozyme, cytochrome c (18), α₁-microglobulin, PAP-1, odorant-binding protein (19), and transthyretin (20). Megalin-deficient mice excrete LMWP in their urine (19), suggesting that it mediates the proximal tubular uptake of such proteins. Thus, megalin could be a candidate molecule for therapeutic application to compensatory LMWP metabolism in ESRD.

Here, we demonstrate for the first time direct evidence that megalin mediates the cellular uptake and metabolism of β2-m. We tested the feasibility of an intracorporeal therapeutic model for the continuous metabolism of β2-m in renal failure by subcutaneous implantation of megalin-expressing cells. Cell implantation was carried out using two recently developed techniques: preimplantation of a three-dimensional extracellular matrix scaffold and a local drug delivery system (21–23). The scaffold consists of a type I collagen sponge and is necessary for the implanted cells to attach and grow on. The DDS was designed for local release of basic fibroblast growth factor (bFGF), to induce angiogenesis in and around the scaffold, thereby promoting adequate blood supply to the implanted cells.

Materials and Methods

Materials

Recombinant human β2-m was purchased from Oriental Yeast Co., Ltd., (Tokyo, Japan). Na 125I (Iodine-125; 3.7 GBq/ml) and Hyperfilm MP were obtained from Amersham Pharmacia Biotech UK Limited (Little Chalfont, Buckinghamshire, England). Iodo-Beads were purchased from Pierce (Rockford, IL). Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) and FCS were obtained from Life Technologies BRL Life Technologies (Rockville, MD). BSA (Fraction V) was from Sigma (St. Louis, MO). Recombinant human bFGF was supplied by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). The gelatin microspheres were sterilized with ethylene oxide gas and impregnated with bFGF as described previously (21). The bFGF-containing gelatin microspheres were suspended in 0.15 ml of PBS and incorporated by injection into a 1 × 1 × 0.5-cm type I collagen sponge sheet sterilized in advance with ethylene oxide gas. The sponge sheet was subcutaneously implanted into the backs of female 5-wk-old BALB/c A Jcl-nu mice (Clea Japan, Inc., Tokyo, Japan). One week later, L2 cells grown in DMEM with 10% FCS were removed from culture plates with trypsin-EDTA (Life Technologies) and suspended in PBS (1 × 10⁷/ml). The cell suspension (0.15 ml) was injected into the collagen sponge sheet in the mice. Only PBS was injected into the sponge sheet in the control mice. All animal experiments in this study were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals or the equivalent.

Immunohistochemistry

Two weeks after either L2 cell implantation or PBS injection for the control, the mice were anesthetized by inhalation of diethyl ether and killed by systemic perfusion from the left ventricle with 4% paraformaldehyde in PBS, and the implanted cell tumors and the scaffolds were resected. The tissues were embedded in paraffin and sectioned at a thickness of 4 μm. The sections were deparaffinized with xylene and rehydrated through graded ethanol and distilled water. The sections were then treated with 0.3% Triton X-100–containing PBS and with 0.03% hydrogen peroxide in methanol. After preincubation with normal goat serum for 30 min, the sections were sequentially incubated with rabbit anti-megalin IgG (37 μg/ml) for 2 h and goat anti-rabbit IgG conjugated to peroxidase-labeled dextran polymer for 1 h. The peroxidase reaction products were colored with 0.5 mg/ml 3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide and counterstained with hematoxylin.

Radioiodination

Recombinant human β2-m (100 μg) was radioiodinated using 1 mCi of Na 125I and one Iodo-Bead according to the manufacturer’s instructions. The specific activity of 125I–β2-m was 7 × 10⁶ cpm/μg.

Cell Association and Degradation Assays

The L2 cell line was established from rat yolk sac as an epithelial-derived tumor from a chemically induced carcinoma (27). The cells were grown (37°, 5% CO2) to confluence (1 × 10⁸ cells/well) in DMEM supplemented with 10% FCS on 12-well tissue culture plates coated with 0.1% gelatin. The cells were washed with DMEM and incubated in DMEM containing 0.1% BSA with 125I–β2-m (1.0 μg/ml, 83 nM) in the absence or presence of competitors. The media containing 125I–β2-m were also incubated on cell-free gelatin-uncoated plates to measure the spontaneous degradation of the radiolabeled protein. After incubation at 37°C in 5% CO2, the culture media were precipitated with 15% TCA in the presence of 1% BSA, and the radioactivity levels of the degradation products in the supernatants were counted. Specific cell-mediated degradation of 125I–β2-m was determined by subtracting degradation in the absence of cells. The cells were washed with ice-cold PBS, and cell-associated radioactivity was measured by counting 125I–β2-m after solubilization with 1 N NaOH. The experiments were performed in triplicate.

Bioengineered Subcutaneous Implantation of L2 Cells in Nude Mice

The gelatin microspheres were sterilized with ethylene oxide gas and impregnated with bFGF as described previously (21). The bFGF-containing gelatin microspheres were suspended in 0.15 ml of PBS and incorporated by injection into a 1 × 1 × 0.5-cm type I collagen sponge sheet sterilized in advance with ethylene oxide gas. The sponge sheet was subcutaneously implanted into the backs of female 5-wk-old BALB/c A Jcl-nu mice (Clea Japan, Inc., Tokyo, Japan). One week later, L2 cells grown in DMEM with 10% FCS were removed from culture plates with trypsin-EDTA (Life Technologies) and suspended in PBS (1 × 10⁷/ml). The cell suspension (0.15 ml) was injected into the collagen sponge sheet in the mice. Only PBS was injected into the sponge sheet in the control mice. All animal experiments in this study were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals or the equivalent.
The mice were killed by systemic perfusion with saline or tissue fixation solution. The implanted cell tumor or other tissues were resected for the following specific experiments.

**Histoautoradiography**

Three and 6 h after nephrectomy and injection with $^{125}$I–$\beta_2$-m, the cell-implanted mice were anesthetized and killed by systemic perfusion with 0.25% glutaraldehyde/4% paraformaldehyde in PBS. The implanted cell tumors were resected, and the tissues were fixed with 0.25% glutaraldehyde and 4% paraformaldehyde in PBS. The paraffin sections processed from the fixed tissues were covered with emulsion NTB-2, which was exposed to radiation for 60 d in a dark room. The sections were developed with developer D-19, counterstained with hematoxylin, and microscopically observed.

**SDS-PAGE Analysis**

Three and 6 h after $^{125}$I–$\beta_2$-m injection into the cell-implanted and nephrectomized mice, blood samples were obtained and the mice were killed by systemic perfusion of saline. The implanted cell tumors were resected and homogenized using Ultra Turrax (Ika Labortechnik, Staufen, Germany) in Laemmli sample buffer supplemented with 4% β-mercaptoethanol. The $^{125}$I–$\beta_2$-m used for the original peritoneal injection and the blood samples were also mixed with the same Laemmli sample buffer. Each sample was prepared at the same radioactivity ($1 \times 10^3$ cpm) for loading on SDS-PAGE using 15% Ready Gel J. After electrophoresis, the gel was fixed with 5% glycerol/40% methanol/10% acetic acid and subjected to autoradiography with Hyperfilm MP.

**Measurement of $^{125}$I–$\beta_2$-m Uptake by the Implanted L2 Cells and Other Tissues**

Three, 6, and 14 h after $^{125}$I–$\beta_2$-m injection into cell-implanted or control nephrectomized mice, blood samples were obtained from the heart and the mice were killed by systemic saline perfusion ($n = 5$ or 6 for each time specified in both cell-implanted and control groups). The sections of the implanted cell tumor, heart, lung, liver, and skeletal muscle were weighed and subjected to radioactivity counting. Comparisons among the tissue radioactivity levels in the cell-implanted group at each time were made using the Scheffe method, a conservative statistical method for multiple comparisons of the ANOVA. Comparisons of the radioactivity levels of heart, lung, liver, and skeletal muscle between the cell-implanted and control groups were made using unpaired $t$ test. $P < 0.05$ was considered significant.

**Evaluation of the Undegraded Forms of $^{125}$I–$\beta_2$-m in the Blood Samples**

Ten microliters of each blood sample obtained as described above was immediately mixed with 200 µl of heparinized saline and precipitated with 15% TCA in the presence of 1% BSA. The undegraded forms of $^{125}$I–$\beta_2$-m in the TCA precipitations were counted and compared between the cell-implanted and control groups at each time specified. The results were analyzed by unpaired $t$ test. $P < 0.05$ was considered significant.

**Results**

**Internalization and Degradation of $\beta_2$-m via Megalin in Cultured L2 Cells**

$\beta_2$-m is known to be a low-affinity ligand for megalin (18,19), although no direct evidence has been presented demonstrating that megalin mediates its cellular internalization and degradation. We investigated this using a cultured rat yolk sac epithelial-derived cell line (L2) (27), which has been shown to express megalin on the surface (26,28) and is well characterized for its endocytic function (29). By cell association and

![Figure 1](image-url)
degradation assays, we found that L2 cells effectively took up 125I-labeled β2-m, and the radioactive degradation products appeared in the culture medium increasingly with time (Figure 1A). After 3 h of incubation, cell association and degradation of 125I-β2-m were blocked at 70 and 85%, respectively, by anti-megalin IgG (200 μg/ml) but not by nonimmune IgG (200 μg/ml; Figure 1B). Also, β2-m cell association and degradation were blocked at 61 and 84%, respectively, by 2.0 μM receptor-associated protein, a competitor to megalin ligand binding, as a fusion protein with GST but not by 2.0 μM GST (Figure 1B). These data indicate that the cellular uptake and degradation of β2-m is dependent on megalin activity.

**Bioengineered Implantation of L2 Cells within the Subcutaneous Tissues of Nude Mice**

To colonize L2 cells in vivo, we used recently developed methods (21–23) for cell implantation in nude mice. Gelatin microspheres were impregnated with bFGF, an angiogenic factor, and incorporated into a type I collagen sponge sheet. The sponge sheet was subcutaneously implanted in the backs of the nude mice. One week later, when angiogenesis was induced in and around the sponge sheet (data not shown), L2 cells were injected into the collagen sheet. As a control, only PBS was injected into the sponge sheet. After a 2-wk incubation, hemispherical tumors were found with an average diameter of 3 cm. From this value, we estimate that the masses had the average volume of 7 cm³, which amounts to approximately 7 g. The average weight of the mice with the implanted cells was 22.9 g. Thus, the tumor burden was approximately 30% of the mouse body weight. The density of the implanted cells was calculated as 1 × 10⁹ cells/cm³. Implantation of L2 cells into the sponge sheet without the local bFGF delivery resulted in the development of undetectable or significantly smaller tumors (data not shown). There was no evidence of distant metastasis of the implanted L2 cells.

**Expression of Megalin in the Implanted L2 Cells**

Two weeks after L2 cell implantation, the mice were killed by systemic perfusion with tissue fixation solution, and the implanted cell tumors were resected for histologic analysis. The implanted L2 cells were distinguished from host-derived infiltrating cells by the following characteristics: (1) large cell size; (2) large, weakly hematoxylin-stained nucleus; and (3) vacuole-rich cytoplasm (Figure 2). Immunohistochemical analysis revealed that the implanted L2 cells expressed megalin on the outer membranes (Figure 2A). The type I collagen sponge sheets, implanted with bFGF-containing gelatin microspheres in the control mice, were infiltrated by host-derived lymphocytic cells at the margin (Figure 2B), but these cells were not stained by the anti-megalin antibody.

**Uptake of Circulating 125I-Labeled β2-m by the Implanted L2 Cells in Renal Failure**

To show that the implanted L2 cells take up circulating β2-m in renal failure, we carried out the following experiments: Two weeks after the cell implantation, the mice were nephrectomized and received an peritoneal injection of 125I-β2-m. Three and 6 h after the tracer injection, the mice were killed by systemic perfusion with tissue fixation solution. Histomorphology of the implanted cell tumors revealed that the cells effectively took up the radiolabeled tracers (Figure 3). No differences in these results were noted between tissues obtained 3 and 6 h after the tracer injection (data not shown). The implanted cell tumors were also resected after systemic saline perfusion, homogenized in a Laemmli sample buffer supplemented with 4% β-mercaptoethanol, and analyzed using SDS-PAGE (Figure 4). When compared with the 125I-β2-m sample used for the original peritoneal injection (lane 1), the implanted cells were found to contain the monomeric form of 125I-β2-m and low-molecular-weight degradation products (lane 2). However, the blood samples from the cell-implanted mice (lane 3)
showed the oligomeric forms of $\beta_2$-m with a significantly lower amount of degradation products. The apparent difference in the radiolabeled protein pattern between the implanted cells and the blood sample indicates that the radioactivity in the implanted cells was not due to blood contamination. The results were basically the same between the samples obtained 3 and 6 h after the tracer injection (data not shown).

To demonstrate the efficacy of L2 cell implantation for the uptake and clearance of $\beta_2$-m in renal failure, we carried out the following experiment: Two weeks after cell implantation or PBS injection for the control, mice were nephrectomized and received a peritoneal injection of $^{125}$I-$\beta_2$-m. Three, 6, and 14 h after the tracer injection, the blood samples were obtained and the mice were killed by systemic saline perfusion ($n = 5$ or 6 for each time specified in both cell-implanted and control groups). The implanted cell tumor, heart, lung, liver, and skeletal muscle samples were resected for analysis. At the time specified, the radioactivity per weight of the implanted cell tumor was significantly higher than that of other tissues (Figure 5). The radioactivity levels of the heart, lung, liver, and skeletal muscle were not statistically different between the cell-implanted and control groups. The blood samples were precipitated with TCA to evaluate the amounts of the undegraded forms of $^{125}$I-$\beta_2$-m. The radioactivity in the precipitation was significantly decreased in the cell-implanted group 6 and 14 h after injection of $^{125}$I-$\beta_2$-m, compared with the control group (Figure 6). The ratio of the $^{125}$I-$\beta_2$-m clearance rate from the 6- to 14-h points between the cell-implanted and control groups was calculated as $\ln (I_{6}/I_{14})/\ln (C_{6}/C_{14}) = \ln (3580/1780)/\ln (5511/3635) = 1.68$; $I_{6}$ and $I_{14}$ are the mean radioactivity (cpm/μl blood) in the TCA-precipitated blood of the control group at 6 and 14 h, respectively. Thus, the $^{125}$I-labeled $\beta_2$-m clearance rate from the 6- to 14-h points in the cell-implanted group was calculated as 68% greater than that in the control group. These results indicate that elimination of $\beta_2$-m from the blood was facilitated by the implantation of L2 cells in renal failure.

**Discussion**

Accumulation of uremic toxin proteins, such as $\beta_2$-m, which causes dialysis-related amyloidosis, is one of the most crucial problems in current dialysis therapy. In current therapy modalities, $\beta_2$-m removal is insufficient even with high-flux membrane hemodialysis devices and direct absorbent columns, because they are used for a limited dialysis time and the effects are transient. Therefore, the development of more continuous types of therapeutic models is required for future clinical application. In the present study, we demonstrated that megalin, a multiligand endocytic receptor, specifically mediates cellular uptake and degradation of $\beta_2$-m in a cultured rat yolk sac epithelial-derived cell line. Through the use of a three-dimensional extracellular matrix scaffold of collagen and a drug delivery system for localized bFGF application, we successfully implanted the megalin-expressing cells in the subcutaneous tissue of nude mice and found that the implanted cells maintained their
Figure 5. Tissue uptake of circulating $^{125}$I-$\beta_2$-m. Three, 6, and 14 h after $^{125}$I-$\beta_2$-m injection into the cell-implanted and control nephrectomized mice, implanted cell tumor and other organs were obtained (n = 5 or 6 for each time specified in both cell-implanted and control groups). The $^{125}$I counts per weight of the implanted cell tumor (T), liver (L), lung (Lu), heart (H), and skeletal muscle (S) are indicated. Uptake by the implanted cell tumor was significantly higher than in other tissues at all time points (Cell-impl.: *P < 0.05). The $^{125}$I counts in the heart, lung, liver, and skeletal muscle were not significantly different between the cell-implanted and control (Cont.) groups.

Figure 6. Significant elimination of $^{125}$I-$\beta_2$-m from the blood by the cell implantation. The blood samples were obtained 3, 6, and 14 h after $^{125}$I-$\beta_2$-m injection into the cell-implanted and control nephrectomized mice. The radioactivity levels of TCA-precipitated forms of $^{125}$I-$\beta_2$-m in the blood are indicated. The levels deceased significantly in the cell-implanted group (○) at 6 and 14 h after injection with $^{125}$I-$\beta_2$-m, compared with the control group (●, *P < 0.05). The $^{125}$I-$\beta_2$-m clearance rate from the 6- to 14-h points in the cell-implanted group, represented as the slope of the graph, was calculated as 68% greater than that in the control group.

levels of megalin expression after in vivo growth. Moreover, these cells effectively endocytosed $^{125}$I-$\beta_2$-m from the circulation in an experimentally induced state of renal failure. Our data clearly demonstrate that megalin can clear circulating $\beta_2$-m in vivo and establish the feasibility of a cell implantation model to remove uremic toxin proteins for dialysis therapy.

Implantation of megalin-expressing cells is essential for $\beta_2$-m clearance because megalin is expressed only in highly specialized epithelial cells and typically has no access to the vasculature in vivo. In the lung, for instance, megalin is apically expressed in type II alveolar pneumocytes (13) and exposed to surfactants. The absence of vascular exposure in lung is consistent with our data indicating that pulmonary uptake of circulating $^{125}$I-$\beta_2$-m in the nephrectomized mice was low. The liver, which does not express megalin, was also found not to take up circulating $\beta_2$-m efficiently, indicating that it is not internalized by hepatic scavenger receptors, including LDL receptor–related protein that shares multiple common ligands with megalin (30). Therefore, for efficient $\beta_2$-m clearance, it is necessary to implant megalin-expressing cells in the matrix scaffold and supply these cells with blood vessels to provide exposure of circulating $\beta_2$-m to megalin.

Exposing plasma proteins to megalin, an efficient endocytic receptor, could also result in the degradation of high-molecular-weight proteins. Using ligand blot analysis, however, we have found that rat plasma proteins smaller than approximately 30 kD were preferentially bound by $^{125}$I-megalin, with very little binding of proteins >50 kD (data not shown). Leheste et al. (19) also reported that megalin did not bind albumin. However, suppressing functions of cubilin, an albumin receptor cooperating with megalin (31), may be needed for the cells to be clinically used. Also, the regulation of the vascular permselectivity for large plasma proteins supplied to implanted cells should be investigated.

Our relative evaluation of the $^{125}$I-labeled $\beta_2$-m clearance rate from the 6- to 14-h points after tracer injection showed a 68% increase by cell implantation. This would correspond to the effect of a 40% reduction (1/1.68) in the steady-state $\beta_2$-m level, which seems almost compatible to the reduction rate of the time average concentration of $\beta_2$-m by high-flux dialysis: 35 to 55% (32). Because high-flux dialysis is effective in prolonging the onset of dialysis-related amyloidosis (33), the cell implantation therapy with such relevant efficiency would also be promising for combination use with high-flux dialysis. Development of a continuously efficient system of cellular $\beta_2$-m uptake and the evaluation of its absolute degradation rate are requisite for clinical application.

This cell implantation system is applicable not only to dialysis patients but also to those with renal insufficiency at predialysis stages. This would serve to prevent the actions of uremic toxin proteins even in early stages of renal damage. It could be beneficial for those patients to protract deterioration of renal functions by compensating for protein metabolism overloaded with residual nephrons, which likely progresses the dysfunction (34).
In summary, we confirmed the molecular function of megalin for the cellular uptake and metabolism of β_2-m, a uremic toxin protein. By bioengineered subcutaneous implantation of megalin-expressing cells, we demonstrated that the cells are able to clear effectively β_2-m from the blood in renal failure. We consider this system potentially applicable for assisting current dialysis therapy, which is at present suboptimal for uremic toxin protein removal. We are currently engineering cells to express much greater levels of megalin and less endogenous β_2-m, which should dramatically improve this procedure for clinical applications.

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