

Clinical results of parathyroid cells allotransplantation

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Abstract

Aim: To present the long-term allotransplant activity in seven patients who had undergone allotransplantation of macroencapsulated parathyroid cells for surgical hypoparathyroidism. Also, a modified technique to prepare parathyroid cellular allografts is described.

Methods: From December 2010 to November 2011, seven patients with severe postsurgical hypoparathyroidism underwent seven allotransplantations of cultured macroencapsulated parathyroid cells. Mean recipient age was 52 years (range: 39-59 years). Donors were selected from patients undergoing parathyroidectomy for primary (adenoma) and secondary (due to chronic renal failure) hyperparathyroidism.

Results: After cultivation and freezing, the parathyroid cells showed their high secretory activity of parathormone (2927.5 [1400.5-4847] pg/ml). The viability of cultured cells was 99%. For all patients, the median cellular allograft survival was three months (range: 1-9 months). In four patients (57.1%) the allografts retained their endocrine function for more than two months. None of the patients showed clinical symptoms of hyperparathyroidism in the post-transplantation follow-up period.

Conclusion: The present study has shown that in some patients parathyroid cell allotransplantation may be considered a method of treatment for permanent hypoparathyroidism after thyroid surgery. Allografted human cultured parathyroid cells showed evident but transient hormonal activity. Graft function and/or survival did not depend on the donor age and recipient characteristics.

Keywords: allotransplantation, parathyroid cells, surgical hypoparathyroidism.

Introduction

Hypoparathyroidism in most cases is a consequence of extensive thyroid or parathyroid surgery. Approximately 10% of patients who have had surgery for thyroid cancer develop hypoparathyroidism (1). Currently, the only treatment for hypoparathyroidism is the substitution therapy with calcium and vitamin D3, such treatment in the absence of feedback mechanisms can lead to overdose or insufficient dosing of calcium and vitamin D3 and to hypo- or hypercalcemic complications. To avoid such complications, various options of surgical treatment were attempted, including parathyroid fragments autotransplantation. In 1971, Wells used a parental allograft (2 parathyroid glands) which were obtained from father and transplanted to son with kidney transplant (2). Since then, there have been reports on the results of such transplantations (3-5). However, after allotransplantation of parathyroid fragments immunosuppression is needed, the use of which is not justified in cases of uncomplicated chronic hypoparathyroidism.

Rejection is one of the main problems of allotransplantation. Long-term survival and function of transplanted organs and tissues are possible with the elimination of antigen-presenting cells which express human leukocyte antigens (HLA) class II. Every endocrine organ consists of about 50% of immunogenic non endocrine tissues (vessels, fibroblasts and lymphocytes), while parathyroid cells only express HLA class I antigens, which are not involved in the induction of rejection reactions (6). Another immunoprotective approach is to encapsulate transplant cells in semipermeable membranes (7). The basic principles of cell immunoisolation include biocompatibility of the membranes, selective transport, permeability to elementary molecules and impermeability to large protein molecules. The most common geometric configuration of implanted immunoisolation devices are intravascular tubular or disc-shaped implants, hollow fiber and microspheres. The geometry of the first three implantable devices corresponds to

the concept "macrocapsules" and is characterized by a certain size (inner diameter ~ 0.5-1.5 mm, length ~ 1-10 cm) and capacity (thousands to millions of cells). Number of macrocapsules required for implantation depends on their capacity. Microencapsulation involves immobilization of cells in a thin spherical membrane, thereby forming microspheres in the form of small «beads», each of which contains one or more cells. The desired therapeutic effect is achieved by microencapsulating large number of cells (few thousand to millions). However, the technology of cell encapsulation, studied in detail in allo- and xenotransplantation of Langerhans islets (8), was not widely used in parathyroid transplantation.

In this article we present the clinical results of macroencapsulated parathyroid cells allotransplantation for patients with postsurgical hypoparathyroidism and also describe a modified method of preparing parathyroid explant by culturing in vitro.

Methods

From patients who underwent parathyroidectomy for primary or secondary hyperparathyroidism, six unrelated to recipients patients were chosen as donors (mean age: 54 years; range: 42-57 years; male-to-female ratio – 1:1). All donors gave informed consent to the use of parathyroid glands for culturing and subsequent transplantation. Prospective donors were examined for viral markers (HBs antigen, antibodies to HBs-, HBc-, HBe-antigen, hepatitis C virus, HIV) and anti-treponemal antibodies. Seropositive patients and those with a history of malignancy were excluded from the study. Four donors were on hemodialysis therapy due to renal failure and had secondary hyperparathyroidism; the other two donors had primary hyperparathyroidism (solitary adenoma). Parathyroid explants were carried out under sterile conditions during parathyroidectomy. Samples were delivered to the laboratory in the transport medium Dulbecco's Modified Eagle medium (DMEM). The storage time of biomaterial to cell seeding was not more than five hours at a temperature of +4°C.

To obtain a cell suspension, parathyroid gland was initially released from capsule, fat, connective tissue and blood vessels, and then mechanically disaggregated in a Petri dish by grinding scissors for three minutes to fragments of 0.1-2 mm³. Each of the parathyroid gland fragments was subjected to histopathological examination; parathyroid auto-transplantation into the forearm muscle was performed to patients with secondary hyperparathyroidism. Adenomatous parathyroid tissue served for working off of culture techniques and was not used as a graft due to the risk of functional autonomy and loss of physiological mechanisms of negative feedback between the level of serum calcium and parathyroid hormone (PTH) secretion, and also unknown risk of neoplastic transformation during cultivation process (9). For subsequent transplantation, three parathyroid glands of each of the four donors with diffuse hyperplasia underwent pretransplant treatment in order to obtain primary cell culture. Here is a brief description of our method for the isolation of cell biomass from grounded parathyroid tissue. Parathyroid tissue was treated with enzyme solution consisting of collagenase type II (1%), trypsin (0.25%) and DNase (0.01%). Under the action of the enzymes, intercellular connections were destroyed and parathyroid cells were released to the nutrient medium in the form of single cells or as aggregates consisting of different amounts (from 2-5 to 20-30) cells. The obtained cells were pelleted by centrifugation; the precipitate was resuspended in an appropriate nutrient medium. By exclusion of 0.4% trypan blue we evaluated the number of cells and their viability, the concentration was adjusted to 500-1000 thousands of cells in 1 mL culture medium. Cell suspension in 5 mL of medium was poured into culture flasks and cultured in a CO₂-incubator at 37°C. The growth medium in the flask with cultured cells was changed every 3-4 days. Assessment of the culture state was performed daily under phase contrast microscopy. The specific functional activity of parathyroid cells in the culture was confirmed by

immunoradiometric assay using PTH determination kit (Roche, PTH-DRG). Measurement was performed at initial culture state and during stimulation with Ca⁺⁺ (1 mM, and 3 mM). In order to determine cell phenotype, immunocytochemical study of culture smears was performed using monoclonal antibodies (Dako, Denmark) to human parathyroid hormone. Cell cultures monitoring was performed using microscope Olympus X51. Cell cultures (3-5 × 10⁶ cells/mL) with a high degree of production and cytochemical PTH expression were selected for subsequent cryopreservation. These cultures were placed in 2 mL cryovials and frozen under -70°C. Thawing of the culture was fast under water bath at a temperature of 56°C, cells were immediately placed in complete growth medium and their viability was determined. For transplantation, cells with high concentrations of PTH in the culture liquid (2927.5 [1400.5-4847] pg/mL) and with a viability of > 85% were selected. Based on the results of Wozniewicz et al. research (10), number of cells used for transplantation was equivalent to the number of cells in one normal parathyroid gland (~ 20-30 × 10⁶).

During the period from December 2010 to November 2011, seven patients underwent parathyroid cells allotransplantation, indication for which in all cases was iatrogenic hypoparathyroidism after thyroid surgery for cancer (5), Graves' disease (1) and Hashimoto's thyroiditis (1). ABO and HLA matching was not performed. All chosen cases for parathyroid cells allotransplantation met the criteria of postoperative hypoparathyroidism. The development of characteristic symptoms was observed immediately after thyroid surgery, the symptoms relieved after administration of calcium-containing drugs, return of symptoms after discontinuation of drug therapy. Low levels of plasma PTH were recorded, patients had hypocalcemia and hyperphosphatemia. Prior to transplantation, all patients received oral substitution therapy, including 20 (10-20) µg of vitamin D3 and 2000 (1200-4000) mg of calcium. In two cases, symptomatic hypocalcemia required intravenous

administration with 40 mg of calcium chloride at least once a week.

The study was approved by the Ethics Committee for Biomedical Studies at the Belarusian State Medical University. All patients gave their informed consent to participate in the study.

The macrocapsule designed as a cylindrical tube 15-20 mm in length and 3-4 mm in diameter from microporous, 157 microns thick, polyvinylidene difluoride artificial membrane with a pore diameter of 0,55-1,37 μm , and the porosity of 28.2%. Then $\sim 20\text{-}30 \times 10^6$ parathyroid cells were injected to the lumen of the capsule.

Under spinal anesthesia, encapsulated graft was implanted into the lumen of the deep femoral artery followed by plastic repair of the arteriotomy with autovenous patch. Artery patency was confirmed by palpation at the end of the operation, as well in postoperative period by Doppler sonography. Moderate postoperative pain was controlled with NSAIDs. Thrombosis prophylaxis was performed with subcutaneous Dalteparin for 5 days.

The location for allograft was chosen due to three factors: the bloodstream is an immunological privilege place (11), no risk of circulatory disorders of the lower limb in the case of thrombotic complications, and the possibility of adequate oxygenation and nutritional provide to cell allograft. In pre- and post-transplant period, blood samples from the cubital vein were taken for the measuring of serum calcium and PTH. Criteria of graft functioning was considered an increase in the concentration of serum calcium ≥ 2 mmol/L, knockdown in the need for calcium-containing drugs, increase in serum PTH compared with pretransplant indicators, relief or improvement of the main symptoms (paresthesia, tetany). Criteria

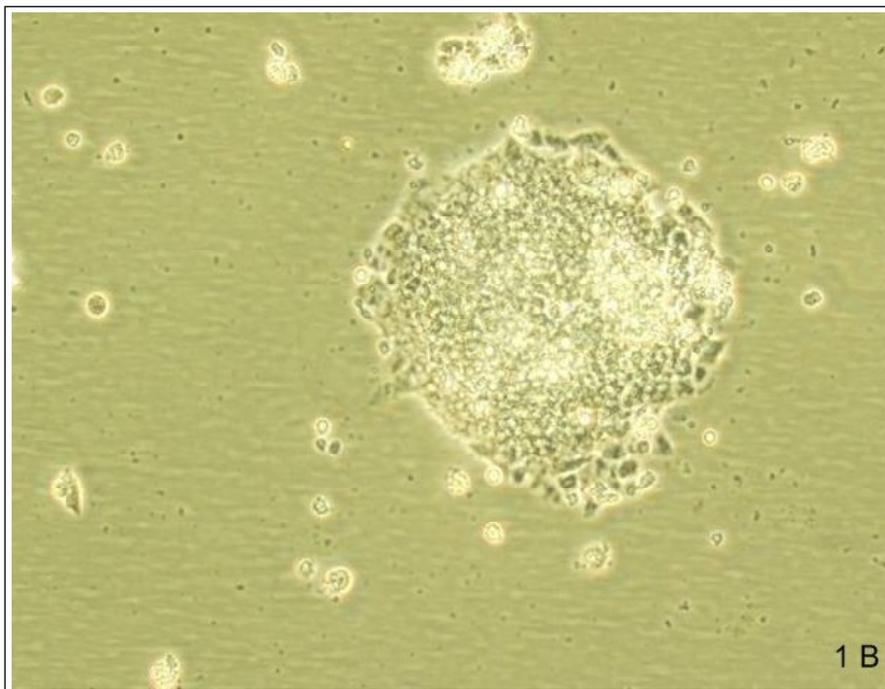
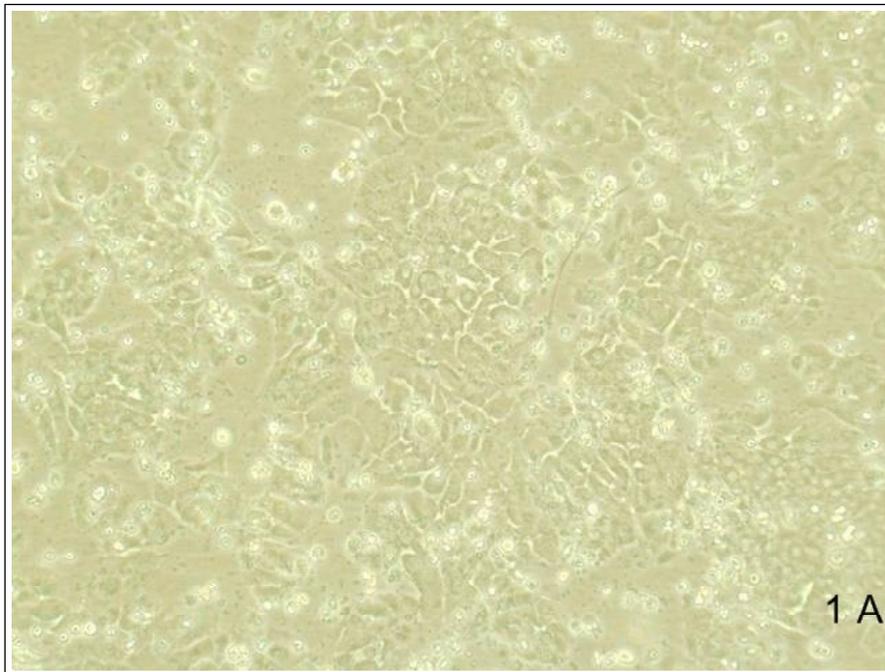
of graft dysfunction was considered a very low or undetectable levels of serum PTH, maintenance or renewal of clinical symptoms of hypocalcemia and increased demand for calcium therapy.

Data are presented as median and percentiles (Me [25-75]).

Results

The cell culture obtained from human parathyroid glands within the 1st day of cultivation was predominantly presented by the floating cells and single fibroblast-like cells. From the second day the adherent fraction of irregular cells with contoured nuclei formed quickly, and represented multiple foci of cell growth, tightly adjacent to each other. By the 4th-10th days of cultivation cells had formed a solid monolayer presented by polygonal epithelial cells intimately adjacent to each other. Data analysis showed that most cells of the parathyroid tissue fragments were obtained during 18-hour enzyme incubation at 4°C followed by 10-minute incubation at 37°C. In this case, we separated cells in concentration of $3\text{-}5 \times 10^6$ in 1 mL and with viability of 99%. On the third day the formation of cell aggregates consisting of 20 or more cells in the culture obtained from human parathyroid samples was observed under the microscope (Figure 1A). Relative homogeneity of cell culture was noted, manifested in the same size and stacking density of cells, so cells almost reached the monolayer (Figure 1B). Vesicular structures – microfollicles began to form. The cells filling the cavity of the follicles were very tightly adjacent to each other and had cubic form. After testing of specific functional activity of cell culture, the average concentration of PTH was 2927.5 (1400.5-4847) pg/mL.

Figure 1. Morphology of parathyroid cell culture, 3-day growth in vitro; Phase contrast microscopy [magnification, $\times 4$ (A); magnification, $\times 20$ (B)]



These results of the investigations are compatible with data from the literature. Depending on the methodic conditions of cell separation from human parathyroid it is possible to obtain cells in

concentration from 1×10^5 to 5×10^6 per mL with viability from 90% to 100% (10). The results of our investigation have shown that application of the method with 18-hour preincubation with enzymes

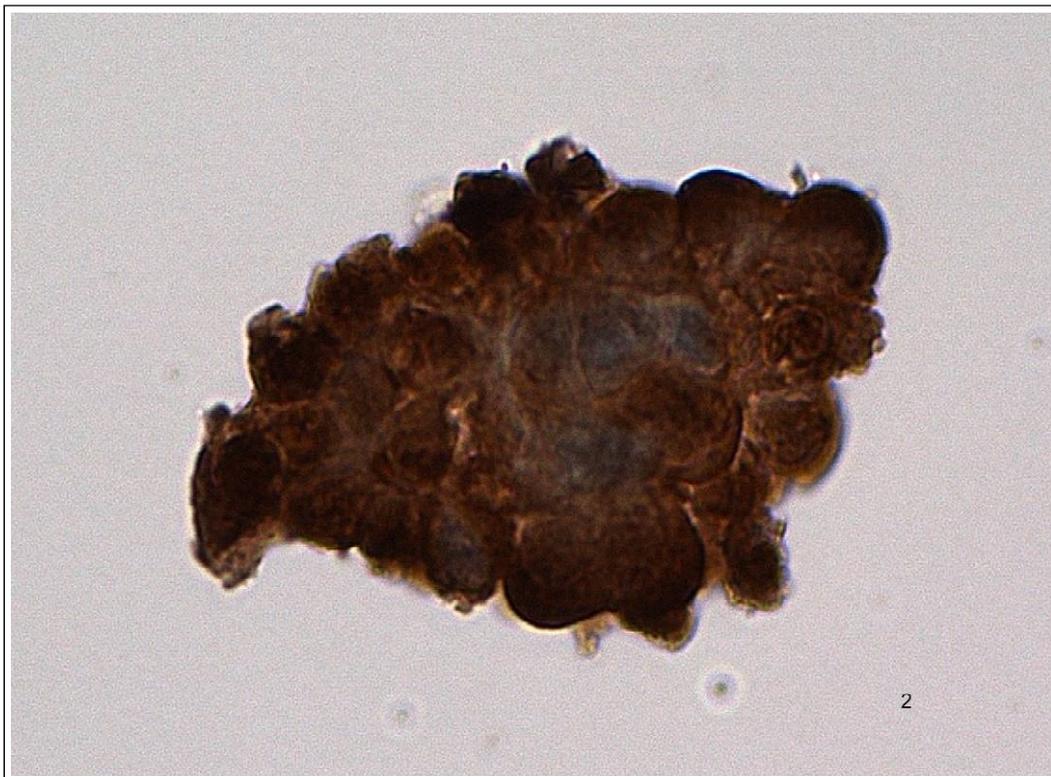
made it possible to obtain cells not only of high concentration and of a high viability degree (99%), but also of high functional activity in comparison with other methods of selection. Nawrot et al. (3) noted the absence of calcification foci and low stromal components, CD3, CD4, CD8 positive lymphocyte passagers and CD68 positive macrophages in the parathyroid donor tissue with diffuse hyperplasia. Moreover, cultivation and cryopreservation reduced the HLA class I parathyroid cells antigen expression and eliminated HLA class II positive cells.

Cell cultivation of more than six weeks leads to growth impairment and progressive decrease of PTH secretion (3). Such a loss of functional activity is normal for the primary long-existing cell cultures, that is explained by the difference in the features

of microenvironment in vitro and in vivo (12). Attempts to add soluble components to the nutrient medium were made and co-cultivation with endothelial cells or into extracellular matrix (sandwich culture) was performed in order to bring conditions of cell culture in vitro further towards the natural one (13).

Golden chromogen staining of cytoplasm of the cells studied in the impression smears and determined under the microscope was considered to be positive reaction in the immunocytochemical investigation with antibodies to human parathyroid hormone (Figure 2). It indicated that cultivated cells phenotypically corresponded to parathyroid cells. Cell membrane tortuosity of parathyroid cells was the important confirmation of the secretory phase.

Figure 2. Immunocytochemistry investigation of cell culture; positive cytoplasmic staining of parathyroid cells is observed (magnification $\times 400$)



Before transplantation serum level of PTH and calcium in the patients was at subnormal range of 10.7 (6.5-23) pg/mL and 1.71 (1.59-1.79) mmol/L. Average parathyroid graft functioning was 3

(1-9) months. In 4 (57.1%) patients cell allografts have maintained their endocrine function for more than 2 months (Table 1). Post-transplant clinical symptoms of hyperparathyroidism were not

identified in none of the cases. Allograft dysfunction in different periods of observation demanded resumption of oral substitution therapy with vitamin

D3 and calcium, but at the same time, no parenteral administration of calcium salt solutions were needed.

Table 1. Duration of allograft functioning

	Allograft functioning (months)		
	0-2	3-6	>6
Quantity of transplantations	3 (42.8%)	2 (28.6%)	2 (28.6%)

Ultrasound Doppler performed in 12 months after the operation, confirmed the patency of the deep femoral

artery at the site of capsule implantation without significant hemodynamic disturbances (Figure 3).

**Figure 3. Femoral artery ultrasonography
(the arrows indicate deep femoral artery with the graft)**



The muscles in the forearm are considered to be the place of parathyroid auto- and allotransplantation according to the opinions of many authors, that's why another objective criteria of graft functioning – Casanova test – has been offered. It is a comparative analysis between the levels of serum PTH in the forearm with a graft and without it (14). In this case, the concentration gradient of half or more has

reflected satisfactory function of the grafted parathyroid tissue. Casanova test examined in two patients with long-term functioning allografts (6 and 12 months) in a month after the transplantation was 1.1 and 1.2. Probable cause of such low levels of PTH concentration gradient can be regarded as the inability to achieve correct recall of the described test in the case of parathyroid cells transplantation to the

deep femoral artery because blood sampling for the investigation was performed at considerable distance from the cell graft (superficial veins of the foot) in the absence venous outflow reduction by application of sphygmomanometer cuff. At the same time, increasing of PTH concentration (14.4 [12.4-21.1] pg/mL) in the systemic blood circulation and normocalcemia (2.03 [1.96-2.09] mmol/L), along with the absence (in one case) or by reducing the requirement of vitamin D3 and calcium-containing medicines (15 [8.75-22.5] µg and 1500 [875-2250] mg) indicated the functioning of the allograft.

Discussion

Until now, the reproducible method of successful parathyroid allotransplantation without recipient's immunosuppression has not been developed yet, and clinical results of such transplantations are still unacceptable and presented in the literature in the form of few reports of cases from practice (case reports) (4,15). Several experimental studies were concerned with the investigation of immune privilege of the anterior chamber of the eye, kidney capsule, cerebral ventricle at allotransplantation of endocrine tissue (16). However loci developed for transplantation can not be used for clinical purposes due to high trauma surgery and the risk of serious postoperative complications. Our method of parathyroid allotransplantation made it possible to avoid the aforementioned problems and, at the same time, confirmed the hypothesis of arterial lumen affiliation to the immunologically privileged sites.

Short-term immunosuppression with cyclosporin A, pre-transplant cultivation with 5% CO₂ and X-ray irradiation of the graft were done to increase the allograft functioning terms (17,18). Subsequently Nawrot et al. (3) rejected immunosuppression because the development of graft dysfunction had not been associated with any particular reason including immune response in the majority of the patients, at the same time cyclosporin caused serious side effects. Now the authors are conducting the prospective research of the efficacy of local

immunosuppression with tacrolimus, which, in their opinion, will decrease or reduce the rate of side effects presenting within oral or intravenous administration of immunosuppressants.

Normal parathyroid gland contains 60% endocrine cells and 30% – vascular endothelial cells surrounded by 10% fat and fibrous tissue. Lymphocytes and macrophages are found out in the vascular lumen only. Parathyroid cells phenotype is characterized by the weak degree of HLA class I antigen expression, while the components of the stroma have HLA class II antigen superexpression (30% – normal and >50% – hyperplastic parathyroid tissue) (3,6). The results confirmed the data of other investigations about the highest parathyroid cell growth activity cultivated in vitro and obtained from parathyroid tissue with diffuse hyperplasia. The idea of using parathyroid cells from hyperplastic parathyroid tissue has arisen on the basis of the reports indicated the presence of cell reserve in any functioning organ which can recover lost cells by means of apoptosis.

A logical assumption about the practicability of using the parathyroid glands obtained from young donors, was confirmed in the current study. Recipient's age did not influence the terms of graft functioning, while in patients with a long history of hypoparathyroidism the results of parathyroid allo-transplantation were better. Prolonged (12 months) secretory activity of the parathyroid allograft was observed in two pairs of recipient/donor with B (III)/O(I) blood group phenotype. At the same time, Nawrot et al. (3) did not find out the influence of donor age on the allograft survival, but minimum and maximum terms of its functioning were being observed in cases of donor age is not the only factor that determine the viability of the graft. Prolongation of the functional activity of the graft can improve the viability of the cultivated parathyroid cells by preventing their aging. At the same time, our results are quite invalid, due to the heterogeneity of the study group and a small number of recipients.

Another factor that has determined graft survival is cell adhesion. Each layer of cells in the normal parathyroid glands is surrounded by the capillary

net. The tendency toward the high degree of adhesion was noted in the population of cells cultivated in vitro, however, in vivo their viability depended on the speed of graft neovascularization (19). Parathyroid cells which did not get enough nutritional support from the bloodstream formed aggregates of 1000 microns, which led to their death in the result of metabolic starvation. The results of our experimental studies show the important role of the pericapsular avascular fibrosis, which has influenced on the functional activity and viability of the encapsulated graft are to be considered the confirmation of this case. Death of certain cells has occurred in the so-called period of "nutrient starvation" – when graft nutrition by means of diffusion is impossible due to the established capsular fibrosis and its neovascularization has not yet occurred (11).

Donor parathyroid tissue for transplantation, has been subjected to freezing and storing in liquid nitrogen for six months or more, after which it was defrosted and cultivated (20). We used the experience of the Polish colleagues (3). From another point of view, pre-cultivation of "fresh" parathyroid glands with subsequent cryopreservation of the obtained parathyroid cells can improve the results of clinical allotransplantation. Unfortunately, careful analysis of the data has not revealed significant prognostic factors affecting the survival of allogeneic parathyroid cells. Perhaps it requires further investigations aimed at more complex immunological phenomena which determine the viability of the transplanted cells. For example, it is necessary to clear up the role of HLA class I antigen re-expression in the development of implanted parathyroid cells rejection in the long

Conflicts of interest: The authors declare no conflicts of interest.

term. At the same time, the results of experimental xenotransplantation of human parathyroid cells into the abdominal cavity of mice showed the possibility of long-term (15 months) storage of their endocrine function within the absence of rejection (21). According to the authors' point of view, pre-cultivation of parathyroid tissue and injection of anti-CD4 monoclonal antibody cause the elimination of antigens on the surface of parathyroid cells. As Nawrot et al. (3) showed, re-allotransplantation of the parathyroid cells obtained from the same donor was very effective. It was the indirect evidence of the absence of recipient's immunization to the donor antigens after the first transplantation.

This investigation shows that parathyroid allotransplantation is an effective therapeutic alternative to standard therapies for some patients with hypoparathyroidism developed after thyroid surgery. It should be noted that despite of the allogeneic nature of the graft, the patients did not require immunosuppression in the postoperative period. The study of PTH secretion allowed to finding out obvious, but transient functional activity of the transplanted allogeneic parathyroid cells in more than half of the patients. The age of donors and recipients, the duration of hypoparathyroidism had no significant effect on the duration of graft functioning. Some questions which are concerned with porosity, biocompatibility and long-term stability of microporous membranes as well as the need of HLA- and ABO-typing of donor-recipient pairs and the need of immunosuppression require further research.

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