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Induction of Tolerance to Parental Parathyroid Grafts Using Allogeneic Thymus Tissue in DiGeorge Anomaly

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Abstract

DiGeorge anomaly can affect both thymic and parathyroid function. Although athymia is corrected by allogeneic thymus transplantation, treatment options for hypoparathyroidism have been unsatisfactory. Parathyroid transplantation offers the potential for definitive cure but remains challenging due to graft rejection. Some allogeneic parathyroid grafts have functioned in adult recipients in the context of immunosuppression for renal transplants. Other efforts have attempted to reduce the allogenicity of the parathyroid grafts through manipulation of the parathyroid tissues before transplantation (using encapsulation or special culture techniques). Recently, we demonstrated the efficacy of parental parathyroid transplantation when combined with allogeneic thymus transplantation in an infant with complete DiGeorge anomaly. The recipient developed tolerance toward the parathyroid donor. The parathyroid graft has functioned for 5 years after transplantation without the need for continued immunosuppression or calcium supplementation. We observed that matching of the allogeneic thymus graft to the parathyroid donor HLA class II alleles that are unshared with the recipient appears to be associated with the induction of tolerance toward the parathyroid graft. Further work is needed to determine the optimal means for using combined allogeneic thymus and parental parathyroid transplantation to correct hypoparathyroidism in patients with both complete and partial DiGeorge anomaly.

Keywords

DiGeorge anomaly; parathyroid; thymus; tolerance induction; transplantation

Introduction

DiGeorge anomaly arises from abnormal embryologic development of the third and fourth pharyngeal pouches and the fourth pharyngeal arch, resulting in congenital defects involving (to varying degrees) the heart, parathyroid glands, and thymus[1, 2]. A hemizygous deletion at 22q11.2 can be found in approximately half of individuals with DiGeorge anomaly; in other cases the diagnosis can be made based on the clinical phenotype[3].

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DiGeorge anomaly infants who demonstrate fewer than 50 naïve ($CD45RA^+CD62L^+$) T cells/mm^3 or naïve T cell percentages less than 5% of total T cells in the peripheral blood have athymia and meet criteria for the definition of 'complete' DiGeorge anomaly[3]. This condition affects approximately 1% of infants with DiGeorge anomaly and leads to a primary immunodeficiency that is usually fatal due to infection by 2 years of age. Thymus transplantation using postnatal cultured allogeneic non-HLA matched thymus tissue provides immunoreconstitution that significantly improves survival[3]. In the recipients, host bone marrow-derived thymocyte precursors migrate to the donor thymus graft and develop into genetically host mature T cells. By 6 – 12 months after transplantation, naïve T cells appear in the circulation; the T cells proliferate normally to mitogens and have diverse T cell receptor repertoires. The recipients develop tolerance toward the thymus donors and do not require long-term immunosuppression[4].

Although athymia can be corrected with thymus transplantation, the parathyroid defect in DiGeorge anomaly remains a significant yet poorly addressed issue. Parathyroid deficiency has previously been identified in 17 – 60% of patients with DiGeorge anomaly[5]. In our own series of 60 infants with complete DiGeorge anomaly who received allogeneic thymus grafts, 48 (80%) have required calcium or vitamin D supplementation prior to thymus transplantation to prevent hypocalcemia. Hypocalcemia from primary hypoparathyroidism places children with DiGeorge anomaly at risk for seizures, which may lead to decreased mental capacity. Calcium supplementation itself can result in serious complications, including severe skin burns from intravenous infiltration, nephrocalcinosis, ectopic calcifications, and death. In addition, when children with DiGeorge anomaly and hypoparathyroidism undergo periods of physiologic stress, such as during infections, they often develop hypocalcemia despite ongoing calcium supplementation. Replacement of parathyroid hormone (PTH) could directly address the primary defect. However, exogenous replacement with recombinant human PTH presents a relatively poor option for treatment because of the associated potential risk for bone malignancies[6]. Allogeneic parathyroid transplantation would theoretically provide physiologic replacement of PTH without the risks associated with calcium and PTH supplementation.

Allogeneic Parathyroid Transplantation

Observations in Animal Models

Animal models have yielded important insights regarding factors that contribute to rejection of – as opposed to tolerance toward – allogeneic parathyroid grafts. Not long after the MHC antigens were discovered, it was demonstrated that the degree of histocompatibility between the recipients and donors plays a significant role in determining long-term parathyroid graft survival[7]. This understanding has resulted in efforts to reduce recipient allorecognition toward mismatched parathyroid graft MHC antigens. Various strategies have been tested in both animal models and human recipients (discussed in the following section). Of interest, in one approach, Goss and colleagues were able to demonstrate 50% indefinite survival of allogeneic parathyroid grafts in calcium-deficient rats after engrafting the parathyroid tissues within the endogenous recipient thymuses and administering a single dose of antilymphocyte serum[8]. This finding revealed the potential for using the thymus to "protect" parathyroid grafts from rejection due to mismatched MHC antigens.

Allogeneic Parathyroid Transplantation in Humans

Early attempts to perform allogeneic parathyroid transplantation met limited success. Allogeneic parathyroid transplantation was reported as early as the beginning of the 20^{th} century[9]. A review of parathyroid allotransplantation published in 1963 was not encouraging since recipients failed to demonstrate long-term graft function[9]. However, in 1967 Amos

and Bach reported the discovery of human MHC antigens (HLA). As the understanding evolved concerning how these antigens contribute to graft rejection, investigators began to develop strategies to overcome HLA mismatches between recipients and parathyroid graft donors. The most commonly used approaches include parathyroid transplantation within the context of immunosuppression for renal allografts, encapsulation of the parathyroid tissues, and culture of the parathyroid tissues before transplantation.

Persistent parathyroid graft function has been observed when allogeneic parathyroid tissues are transplanted into patients who have received renal allografts. The first case was reported in 1973; the recipient was given an allogeneic parathyroid graft within 2 months of allogeneic renal transplantation (summarized by Torregrosa et al.[10]). The patient was receiving immunosuppression at the time of parathyroid transplantation to prevent rejection of the renal graft. The immunosuppression was maintained indefinitely, allowing the parathyroid graft to function for over 21 months after transplantation. In 1979, Wells et al. similarly performed parathyroid allotransplantation after allogeneic renal transplantation (reviewed[10]). The parathyroid graft function was lost when the recipient developed acute rejection of the renal graft. Since that time, multiple other cases have been reported in which renal allograft recipients were given allogeneic parathyroid transplants[10]. In almost all cases that have demonstrated parathyroid graft function beyond 2 years post-transplantation, the patients were maintained on long-term immunosuppression. As a possible exception, Zeng discontinued immunosuppression in 9 patients after 2 years. The clinical data suggested persistent parathyroid graft function in the patients up to 6 years post-transplantation, although PTH data were not provided[10]. Thus, transplant surgeons have been able to take advantage of the fact that renal allograft recipients require indefinite immunosuppression after transplantation to perform subsequent transplantation of allogeneic parathyroid tissues. Although the parathyroid grafts can function for as long as 13 years[10] after transplantation using this approach, the recipients fail to develop tolerance to the parathyroid grafts, and persistent parathyroid graft function has not been demonstrated in the absence of the immunosuppression.

In efforts to overcome the need for immunosuppression, other investigators have attempted to encapsulate the parathyroid graft tissues to limit exposure of any mismatched parathyroid HLA antigens to the recipient immune system. Initial experiments used alginate, a potential carcinogen. Later work transitioned to the use of a different form of sodium-alginate. In 1997, Hasse et al. reported the first use of alginate-encapsulated allogeneic parathyroid tissues in human recipients (see brief review[10]). The allografts were well-tolerated and demonstrated normal parathyroid function without the need for immunosuppression, although long-term follow-up data are not available. Long-term function of encapsulated allogeneic parathyroid grafts may be complicated by the development of fibrosis for unclear reasons[11]. Meanwhile, the need to extensively manipulate the parathyroid graft tissues prior to transplantation increases the risk for environmental contamination or loss of viability of the tissues. Thus, while encapsulation shows promise, further work is required to optimize its use in human recipients.

Other investigators have sought to extend the survival and function of allogeneic parathyroid grafts without long-term immunosuppression by culturing the parathyroid tissues before transplantation. In normal, healthy parathyroid tissue, HLA class I antigens are expressed in 23% of parenchymal cells and 62% of stromal cells; HLA class II antigens are not expressed in parenchymal cells but are ubiquitously expressed in the stromal cells[12]. Culturing the parathyroid tissues *in vitro* or *in vivo* prior to transplantation significantly reduces the expression of the HLA antigens by unclear mechanisms, although loss of passenger donor leukocytes may play a role[12, 13]. Feind et al. reported the first series of human *in vitro* cultured allogeneic parathyroid graft recipients (see review[10]). The investigators were not

able to demonstrate graft function through 6 months post-transplantation. Several subsequent reports of cultured allogeneic parathyroid transplantation without immunosuppression have emerged. Sollinger et al. cultured irradiated parathyroid allografts *in vivo* (in nude mice) before placing the tissues into human recipients (summarized by Torregrosa et al.[10]). The recipients demonstrated PTH production without immunosuppression but continued to require calcium supplementation after transplantation. The largest series of cultured parathyroid allotransplantation was reported in 2007 by Nawrot et al.[13] Dissected parathyroid tissues were cultured *in vitro* and transplanted into 85 recipients. The optimal culture period was 6 weeks, and reduction of parathyroid tissue HLA expression was demonstrated. Four of 85 recipients had parathyroid graft function that persisted beyond 24 months after transplantation. Thus, cultured allogeneic parathyroid transplantation remains an unsatisfactory treatment option for hypoparathyroidism at this time.

Combined Allogeneic Thymus and Parental Parathyroid Transplantation

We recently used parental parathyroid transplantation in combination with allogeneic thymus transplantation to treat 4 infants with congenital athymia and primary hypoparathyroidism from complete DiGeorge anomaly[14]. Although one subject received immunosuppression due to the diagnosis of atypical complete DiGeorge anomaly[3], all immunosuppression was discontinued in the recipient by 6 months after transplantation. The other 3 subjects did not receive post-transplantation immunosuppression. The surgical procedures were tolerated well by the recipients and donors. One subject died at 9.6 months post-transplantation from respiratory failure related to congenital rib defects. The remaining 3 recipients have done well clinically aside from the development of autoimmune hypothyroidism in 2 of the 3 survivors.

Results

All recipients demonstrated function of the thymus and parathyroid grafts after transplantation[14]. Two of the 3 survivors developed alloreactivity toward their parathyroid donors in mixed lymphocyte cultures (MLCs) and lost function of the parathyroid grafts[14]. The third survivor, however, has demonstrated continued parathyroid graft function at 5 years after transplantation together with persistent recipient tolerance toward the parathyroid donor in MLCs[14].

Matching of the allogeneic thymus tissue to the parental parathyroid graft for HLA class II alleles not shared between the parathyroid donor and the recipient appeared to be required for the development of recipient tolerance toward the parathyroid donor and long-term parathyroid graft function. The 2 subjects who received parathyroid grafts that had HLA class II alleles not shared with either the recipient or the thymus donor rejected their parathyroid grafts. On the other hand, in the subject who developed tolerance toward the parathyroid donor and long-term parathyroid graft function, all of the HLA class II alleles for the parathyroid graft matched with either the recipient or the thymus graft.

A Model for Tolerance

The abrogation of alloreactivity toward allogeneic parathyroid grafts may be mediated through central or peripheral tolerance mechanisms[15]. In central tolerance, developing T cells are negatively selected within the thymus and deleted if they demonstrate strong reactivity toward intrathymically presented antigens. In peripheral tolerance, a variety of mechanisms exist to suppress self-reactive T cells that are not eliminated within the thymus. These mechanisms include suppression by regulatory T cells, anergy, and activation-induced cell death. Regulatory T cells maintain tolerance by inhibiting the activity of self-reactive T

cells through contact-mediated or cytokine-mediated processes. Regulatory T cells can be stimulated or induced by so-called "tolerogenic" dendritic cells[16]. In anergy, self-reactive T cells are functionally inactivated but not deleted. Lack of costimulatory signaling is believed to play a role in inducing anergy. The inactivated T cells can escape anergy *in vitro* through the combination of IL-2 supplementation, engagement of their T cells receptors, and costimulation. Finally, in activation-induced cell death, T cells that demonstrate strong affinity for self-antigens not only become functionally inactivated but also undergo apoptosis.

In combined allogeneic thymus and parental parathyroid transplantation, several of these mechanisms may play important roles in promoting the development of recipient tolerance toward the parathyroid grafts. The work by Goss et al.[8] showed that placement of the parathyroid tissues – including the parathyroid MHC antigens – within the thymus can induce recipient tolerance toward the parathyroid grafts. In allogeneic thymus transplantation, microchimerism develops within the thymus graft consisting of thymus donor medullary thymic epithelial cells and recipient antigen presenting cells (*e.g.*, dendritic cells). Thus, if all of the parathyroid donor HLA class II alleles are shared with either the thymus graft or the recipient, the HLA class II antigens of the parathyroid graft will be expressed within the thymus graft, producing a model similar to the one created by Goss et al. The mechanisms by which expression of the parathyroid donor HLA class II alleles within the thymus graft leads to tolerance toward the parathyroid graft remain incompletely understood. Negative selection within the thymus graft likely deletes recipient alloreactive T cells that recognize the parathyroid donor HLA class II antigens shared with and expressed by the medullary thymic epithelial cells and recipient antigen presenting cells. This hypothesis remains difficult to test directly. In our studies, appropriate matching appeared to induce anergy in some T cells of the recipient who developed tolerance to the parathyroid donor that were capable of rejecting the parathyroid graft.[14] Regulatory T cells did not appear to be required to suppress alloreactivity by the subject toward the parathyroid donor when tested at 17 months after transplantation[14], but our more recent assessments suggest that the role of regulatory T cells cannot be completely excluded (unpublished data). We have not excluded a possible role for activation-induced cell death.

Other Considerations

It remains unclear why the presence of HLA class I mismatches between the parathyroid donor, recipient, and thymus donor did not appear to create a barrier to tolerance induction. It is known that the alloreactivity observed in MLCs comes from HLA class II differences[17]. Thus, HLA class I mismatches alone may be insufficient for the generation of significant allorecognition responses in certain conditions[18], such as in parental parathyroid transplantation. In this manner, our results are similar to findings in a registry of cardiac transplant recipients that showed a significant effect of HLA class II – but not class I – mismatches on long-term outcomes after transplantation[19].

We observed the development of autoimmune hypothyroidism in 2 recipients: in 1 of the 2 who rejected the parathyroid graft and in the recipient who developed tolerance to the parental parathyroid. The etiology for this finding remains unclear. In reviewing all subjects who have received allogeneic thymus grafts without parathyroid transplantation at our institution, we identified 38 who have survived beyond 1 year. Thirteen of the 38 recipients have developed autoimmune thyroid disease ([3] and unpublished data). The appearance of thyroid disease in 2 subjects who received combined allogeneic thymus and parental parathyroid transplantation is not statistically greater in prevalence and thus is not likely to have been due to parathyroid transplantation.

Other potential explanations for our observations remain under consideration. The recipients each received a single parental parathyroid gland. As a result, no significant dose differences should account for the disparate outcomes. On the other hand, graft failure due to failure to vascularize or other factors cannot be excluded in the subjects who lost parathyroid function. In addition, loss of parathyroid function due to rejection of parathyroid tissue specific proteins, such as PTH or the calcium-sensing receptor, remains possible. However, our MLC data argue that allorecognition is a key mechanism of graft rejection and that tolerance toward the parathyroid donor HLA antigens is required for persistent parathyroid graft function.

Future Work

Further efforts will be needed to optimize the approach for combined allogeneic thymus and parathyroid transplantation. First, our proposed matching strategy needs to be confirmed with additional transplants or in animal models. Next, if matching of the allogeneic thymus graft to the parathyroid donor HLA class II alleles that are unshared with the recipient is required for the induction of tolerance toward the parathyroid graft, allogeneic thymus tissues may need to be cryopreserved for transplantation. Protocols would need to be developed to create and use these tissue banks. Finally, in order for combined allogeneic thymus and parental parathyroid transplantation to work as a therapy in patients with partial DiGeorge anomaly, an immune conditioning or ablation protocol will need to be developed that will allow the allogeneic thymus tissue to be accepted by the recipient so that it can promote, in turn, tolerance toward the parathyroid graft. The fact that complete DiGeorge anomaly recipients of allogeneic thymus transplantation do not reject their grafts when host T cells develop demonstrates that tolerance toward the thymus grafts is an achievable goal.[4]

Our model for combined allogeneic thymus and parathyroid transplantation suggests other potential avenues for future research that could be focused on creating intrathymic donor and recipient HLA chimerism. According to our paradigm, placement of parathyroid donor HLA class II antigens directly within the thymus should result in tolerance to the parathyroid allograft without the need to match the parathyroid to the thymus or the recipient. Thus, in complete DiGeorge anomaly recipients, a completely mismatched parathyroid allograft could be wrapped within the thymus tissue and transplanted within the same quadriceps incision. For other patients who have endogenous thymus tissue, parathyroid donor bone marrow cells or HLA class II peptides could be injected intrathymically together in combination with heterotopic parathyroid transplantation. The induction of tolerance after allopeptide injection into endogenous thymic tissue has been reported in animal models.[20] This approach may be difficult in patients who have thymic hypoplasia or who have significant replacement of the thymic tissue with adipose tissue. As an alternative technique to intrathymic injection, patients could receive bone marrow transplantation from the donor of the allogeneic parathyroid graft. In this approach, the parathyroid donor bone marrow cells would presumably generate dendritic cells to populate the endogenous thymus tissue, inducing tolerance to the parathyroid graft. Such a method would be similar to efforts in combined bone marrow and tissue transplantation for other solid organs, such as heart, lungs, or kidneys, that have been studied by multiple other investigators over the past several years.[20] Combined bone marrow and renal co-transplantation has been reported in human subjects with mixed success.[15, 20] Post-transplantation analyses have focused on bone marrow chimerism, and it remains unclear how well these evaluations gauge intrathymic chimerism. As a final option, recipients could be infused with dendritic cells from the parathyroid donor or from the recipient that have been primed using parathyroid donor HLA class II peptides. Circulation of the dendritic cells into the thymus would then lead to the induction of tolerance. The successful use of allopeptide-pulsed immature dendritic cells to induce tolerance to cardiac, pancreatic islet,

and renal allografts has been described in animal models but will need to be further studied in human subjects[20].

Finally, adjunctive therapies should be considered to support and enhance the induction of tolerance to allogeneic parathyroid grafts by co-transplanted thymus tissue in DiGeorge anomaly patients. Recipient regulatory T cells may be collected, expanded *ex vivo*, and then re-infused. This process is being developed for bone marrow transplantation recipients but has not yet been reported in human solid organ transplantation.[21] Alternately, recipient regulatory T cells may be induced *in vivo* using rapamycin.[15, 20] As a third method for promoting regulatory T cell-mediated suppression of graft rejection, "tolerogenic" dendritic cells may be generated *in vitro* and infused into the recipients. This technique has only recently been examined for use in transplantation[16]. Another adjunctive therapy may include costimulatory blockade to induce anergy. This approach is being tested in human renal[20] and hepatic[15] transplantation recipients and has been complicated by reports of thromboembolic events. Finally, methods may be explored to silence HLA class II expression in the allogeneic parathyroid tissues using small molecules. Continued technological advances could make this approach more promising in future years.

Conclusions

Efforts have increased over the last 20 years to offer the possibility of successful parathyroid allotransplantation. Combined allogeneic thymus and parental parathyroid transplantation holds promise for the treatment of primary hypoparathyroidism due to DiGeorge anomaly – especially complete DiGeorge anomaly. Further work is needed to identify optimal means for manipulating the thymus to enhance its ability to induce tolerance to parathyroid grafts.

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Non-Standard Abbreviations

MLC	mixed lymphocyte culture
PTH	parathyroid hormone

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