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Early Fetal Gene Delivery Utilizes both Central and Peripheral Mechanisms of Tolerance Induction

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Abstract

(1) Objective—We previously reported the induction of stable immune tolerance following the direct injection of retroviral vectors into preimmune fetal sheep. In the present studies, we conduct detailed analysis of the thymus of recipients of in utero gene transfer (IUGT) to delineate the mechanism of the observed immune tolerance and assess the impact of recipient age on this process.

(2) Methods—Fetal sheep at varying gestational ages received the MSCV-NeoR-RFP retroviral vector. The thymus was then collected from these animals at 27–30 days post-injection and analyzed for evidence of transduction of key immunoregulatory thymic cells.

(3) Results—Our results reveal that both thymic epithelial cells (TEC), crucial for presentation of self-antigen during T cell thymic selection, and the cells comprising the Hassall's corpuscles, which can present antigen directly and also instruct dendritic cells to induce the formation of CD4+CD25⁺ T-regulatory cells in the thymus, were only efficiently transduced if IUGT was performed early in gestation.

(4) Conclusions—Our findings thus demonstrate, for the first time, that early IUGT can potentially take advantage of multiple tolerogenic avenues in the fetus, transducing both TEC, which promote central tolerance, and Hassall's corpuscles, which induce formation of Tregs that could act to maintain peripheral tolerance to the transgene products.

Keywords

In Utero; Gene Transfer; Immune Tolerance; Thymus; Hassall's Corpuscles

INTRODUCTION

A bane of gene therapy trials is the generation of a recipient immune response, causing elimination of transduced cells and cessation of transgene expression. While immune responses to foreign marker genes are not surprising, therapeutic transgenes have proven equally problematic. For example, in the hemophilias, both gene therapy and protein replacement

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frequently trigger formation of inhibitory antibodies, rendering subsequent treatments ineffective. While targeting transduction to desired cell types and thus avoiding transduction of antigen-presenting cells (APC) promises to one day circumvent this problem, we and others are exploring in utero gene therapy (IUGT) as an alternate approach to circumvent these immunological barriers [1-5]. IUGT offers the advantage of delivering the therapeutic vector at a time in development that allows both correction prior to disease onset and transgene expression during the period of so-called “preimmunity”, thereby precluding an immune response. Indeed, we and others have demonstrated that IUGT during this period leads to durable immune tolerance to marker and therapeutic transgenes, with resultant long-term transgene expression [2,5-10]. However, no studies have yet delineated the mechanism responsible for the immune tolerance observed following IUGT, nor defined the temporal window during which IUGT transduces cells responsible for durable immune tolerance.

MATERIALS AND METHODS

In Utero Gene Transfer

Fetal sheep at 54-59, 60-65, 70-74, and 100-114 days of gestation (term 150 days, 5-6 fetuses per gestational age range) received the MSCV-NeoR-RFP retroviral vector as described [3, 11,12]. In short, once a surgical plane had been achieved through anesthesia, the fetal peritoneal cavity was localized by palpation, and an i.p. injection of retroviral supernatant at a titer of 1×10^6 pfu/ml (1ml for 54-59 day-old fetuses, 2ml for 60-65 day-old fetuses, and 3ml for 70-74 day-old fetuses) was given to each fetus, and the incisions were closed. As in our prior studies, these vector doses were based on literature suggesting that, based on fetal growth kinetics, we would obtain a relative constant ratio of vector particle number to gram weight, at least from 54-74 days of gestation [13-16]. Due to limitations in the volume of vector that could safely be injected, animals at 100-114 days of gestation also received 3ml of vector supernatant. Also, due to risks and difficulties with attempting to perform a hysterotomy at greater than 70 days of gestation, these pregnant ewes were prepared and anesthetized as above, but the vector was injected percutaneously under ultrasound guidance, similarly to other previously published studies in sheep, rabbits, and monkeys [17-20]. In ongoing in utero gene transfer studies and our studies on in utero hematopoietic stem cell (HSC) transplantation, we have alternatively employed injection by palpation and injection under ultrasound guidance. In our hands, these two approaches produce essentially identical results as far as accuracy of injection into the peritoneal cavity, and also with respect to the success of the procedure, be it gene transfer or HSC engraftment. In the present studies we employed primarily the surgical/palpation method, since we are more efficient with this approach. Animals were euthanized at 27-30 days post-injection and the thymus collected for analysis of transduction. This study was approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Thymus Preparation and immunohistochemistry

Thymic tissues were dissected, rinsed, and fixed in paraformaldehyde. After sucrose cryoprotection, tissues were embedded and frozen in OCT (TissueTec). Cryosections were prepared (7 to 10 μ m), washed with PBS, and blocked with normal goat serum. Sections were incubated with primary antibodies [pan-Cytokeratin (BioGenex, San Ramon, CA); Thymic stromal lymphopoietin (TSLP) (R+D Systems, Minneapolis, MN); and neomycin phosphotransferase II (NPTII) (Upstate, Charlottesville, VA)] overnight, washed, and detected with Alexa-conjugated (Invitrogen, Molecular Probes, Eugene, Oregon) secondary antibodies. Images were acquired with an Olympus Fluoview confocal microscopy system (Olympus America, Melville, NY, USA).

RESULTS AND DISCUSSION

We previously reported that retroviral-mediated IUGT in preimmune fetal sheep induces stable transgene-specific immune tolerance, allowing long-term persistence of transduced cells and transgene expression, even following post-natal boosting with purified transgene product [6]. We hypothesized that the tolerance observed following IUGT during the period of so-called “pre-immunity” was due to transduction of key thymic APC which could present the vector-encoded antigens to developing T cells. To test this hypothesis, IUGT was performed on sheep at various points in gestation. Thymus collected from 3 recipients in each group was analyzed by immunohistochemistry to determine the levels of gene transfer and the transduction pattern within the thymus. We employed an antibody to the vector-encoded NPTII, a pan-cytokeratin antibody to identify TEC, and a CD45 antibody to identify thymic hematopoietic cells. At least 2000 cells in each of 3 representative sections from each animal were counted under fluorescence microscopy to quantitate the percentage of TEC and hematopoietic cells transduced as a result of IUGT at each gestational age.

Although significant numbers of thymic cells were transduced regardless of the age at which IUGT was performed, and the overall level of thymic transduction remained relatively constant throughout gestation, the recipient age dramatically affected which cell types were transduced. Animals that received early IUGT (54-65 days) exhibited mean levels of TEC transduction of 5.98 ± 0.88 , while those animals receiving late IUGT (72-114 days), averaged only $0.65 \pm 0.21\%$. Early IUGT thus yielded nearly ten-fold higher levels of TEC transduction. Figure 1 shows representative photomicrographs of thymus from sheep injected at gestational day 54 (Figure 1A) or 74 (Figure 1B), stained with anti-NPTII (red) and anti-pan-Cytokeratin (green). Significant numbers of transduced cytokeratin-positive TEC can be seen in the fetus that received early IUGT, while very few transduced TEC are present in the fetus that received late IUGT. A graphical summary of these findings appears in Figure 1C.

Examining these thymic sections with anti-NPTII and anti-CD45 again revealed two temporal gestational windows during which marked differences existed in the levels of thymic hematopoietic transduction. However, in contrast to TEC transduction, animals that received early IUGT (Figure 1D; representative section from an animal receiving IUGT at 54 gestational days) possessed very few, if any, transduced hematopoietic cells, while in animals that received IUGT later in gestation, the vast majority of the transduced thymic cells were of hematopoietic origin (Figure 1E; representative section from an animal receiving IUGT at 74 gestational days). To ascertain the identity of the transduced hematopoietic cell within the thymus, we performed dual labeling with an antibody to the NPTII transgene (red) and a panel of antibodies against various hematopoietic markers (green). As shown in Figure 1F, the vast majority of the transduced hematopoietic thymic cells expressed CD4 (green), suggesting they were T helper or T regulatory cells (Tregs). To determine whether these were transgene-expressing Tregs, we labeled serial sections with anti-NPTII (red) and anti-CD25 (green). As can be seen in Figure 1G, which is a representative section of thymus dual-labeled with anti-NPTII (red) and anti-CD25 (green), very few if any of the transduced hematopoietic cells expressed CD25, indicating either that they have not yet matured into full-fledged Tregs, or that they are not in fact Tregs.

One caveat that applies to this data on hematopoietic cell transduction within the thymus is the complex issue of trafficking of prelymphocytes, a process that is presumably in motion throughout gestation, with hematopoietic cells within the thymus being transduced and exiting the thymus by the 30-day time point we chose for analysis. Thus, the cells we see at the time of analysis may represent only a small percentage of those that were originally transduced. Since the rate of exit should remain fairly constant throughout the period of gestation we studied, the percentage of transduced hematopoietic cells exiting the thymus by the 30-day

time point should remain fairly constant, indicating that we transduced a much higher percentage of thymic hematopoietic cells when we injected the vector later in gestation. Furthermore, we know from prior unpublished studies that performing IUGT early in gestation (54-60 days) results in higher levels of transduced hematopoietic cells within the bone marrow and peripheral blood after birth than does IUGT later in gestation (70-72 days). Because all of the hematopoietic cells exiting the thymus are continually being replaced by new hematopoietic cells from the circulation, it is thus possible that at least a percentage of the entering hematopoietic cells may in fact have been transduced prior to entering the thymus. If this is the case, some of the transduced hematopoietic cells we see following IUGT later in gestation could theoretically reflect migration of hematopoietic cells that were transduced earlier in gestation that then entered the thymus and appeared as transduced thymic hematopoietic cells at our 30-day time point. However, we do not think it likely that this migratory process can explain our observations, since high levels of circulating hematopoietic cells were only seen if IUGT was performed prior to about 65 days of gestation, the same time period during which we see almost exclusively epithelial cell transduction within the thymus. Thus, if the majority of the transduced hematopoietic cells we see in the thymus at the 30-day time point are due to exit of resident hematopoietic cells and entry of new hematopoietic cells from the circulation, we should have seen higher levels of transduced hematopoietic cells within the thymus of the animals that received IUGT at the earliest time points, not the ones that received IUGT later in gestation.

We next hypothesized, based on our findings with TEC transduction, that early IUGT could also be inducing immune tolerance through transduction of Hassall's corpuscles, epithelial-derived thymic structures that play a critical role in instructing dendritic cells to induce formation of CD4+CD25+ regulatory T cells (Tregs) in the human thymus [21-24]. Transduction of these thymic structures could then presumably lead to the generation of Tregs that could prevent a transgene immune response. Furthermore, recent studies by Chentoufi and colleagues[25] have now shown that the TEC comprising the Hassall's corpuscles are also the thymic cells responsible for the presentation of proinsulin, and this presentation is in fact required to avoid autoimmune-induced diabetes. Further support for the ability of Hassall's corpuscle TEC to function as tolerance-inducing APC comes from recent studies by DeVoss et al.[26], Kekalainen et al.[27], and Zhang et al.[28], which have collectively shown that Aire-expressing TEC are required for induction of tolerance to self-antigens, that these Aire-expressing TEC are found at the outer borders of the Hassall's corpuscles, and that these TEC co-label with TSLP. Furthermore, it is known that expression of soluble antigens by these cells can lead to not only direct antigen presentation, but also cross-presentation of antigen to neighboring dendritic cells with subsequent Treg formation[28], suggesting that expression of foreign antigen by these specific TEC could potentially promote tolerance through multiple mechanisms. To test the hypothesis that early IUGT could result in transduction of this immune regulatory population, we performed immunohistochemistry on thymus sections from 6 animals in each age group (54-65, and 70-114 days of gestation, term: 150 days) using anti-NPT II to identify transduced cells and an antibody to the Hassall's corpuscle-specific protein thymic stromal lymphopoietin (TSLP). At least 500 cells were counted under fluorescence microscopy in each of 3 representative sections from each animal to score the percentage of TSLP+NPTII+ cells following IUGT at each age. Following early IUGT (54-65 days of gestation), $23 \pm 3.14\%$ of the TSLP+ cells were transduced compared to a mean percentage of transduction of only $2.87 \pm 1.4\%$ when IUGT was performed late (72-114 days). These results demonstrate an 8-fold difference in the levels of transduction of cells within the Hassall's corpuscles depending on the age at which IUGT is performed. Figure 2A presents a graphical summary of these findings. A representative section from an animal that received IUGT at 61 days of gestation is shown in Figure 2B. In this figure, numerous TSLP+ (green) Hassall's corpuscles are visible, many of which were successfully transduced as a result of early IUGT

and express of the vector-encoded NPT II transgene (red). Nuclei are counterstained with DAPI (blue).

Our results demonstrate, in a large animal model, that several key thymic immune cell types are transduced as a result of IUGT at various points in gestation, suggesting multiple possible mechanisms to explain our achievement of immune tolerance in our previous studies. Our results show that the optimal time frame for performing IUGT to achieve immune tolerance is likely early in gestation, between 54 and 65 days in this model, since this is the period during which maximal transduction of cells within the thymus responsible for presentation of self-antigen occurred. This window for tolerance induction would correspond to roughly 13-16 weeks in humans, once adjustments have been made due to the difference in gestational length between the two species. These data also provide the first evidence to date that IUGT early in gestation may offer the opportunity to take advantage of multiple tolerogenic pathways present in the fetus, since it results in transduction of not only TEC, which could present the vector-encoded transgene products during the immunologically critical period of thymic selection, thus promoting deletion of strongly reactive clones, but also Hassall's corpuscles. The transduction of Hassall's corpuscles has not, to our knowledge, yet been reported following gene therapy and may have significant clinical implications, since the TEC within these structures can both directly present antigen in a tolerogenic fashion, and induce formation of Tregs, at least in part, through the secretion and cross-presentation of soluble antigen by closely associated dendritic cells within the Hassall's corpuscles, that could then act to maintain peripheral tolerance to the transgene products. This dual presentation by the TEC and cross presentation of soluble antigen could conceivably promote both central and peripheral Treg-mediated tolerance and suggests that this in utero approach could be useful for inducing prenatal tolerance to clinically relevant soluble proteins such as Factor VIII or Factor IX. Based on the role Hassall's corpuscles play in instructing dendritic cells to form Tregs, studies are currently underway delineating what impact transgene expression in the Hassall's corpuscles has upon the levels and specificity of circulating Tregs within these sheep. These preliminary analyses have thus far revealed that animals that received IUGT early in gestation (prior to 70 days) have higher percentages of CD4+CD25+ cells within their periphery than do control animals or animals transduced later in gestation. This finding is particularly interesting, given recent studies demonstrating that CD4+CD25+ cells in sheep express the Foxp3 transcription factor and possess the functionality and cytokine profile of functional Tregs [29].

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Figure 1. Gestational age of recipient dictates which thymic cell types are transduced

Thymic sections obtained from sheep receiving IUGT at varying gestational ages were subjected to immunohistochemistry with antibodies to the vector-encoded NPTII and to markers for thymic epithelium (TEC) or thymic hematopoietic cells to assess the impact of recipient age on the transduction of each of these cell types.

1A: Representative thymic section from an animal receiving early IUGT at 54 days of gestation, showing large numbers of cytokeratin-positive (green) TEC are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT early in gestation. Nuclei are counterstained blue with DAPI.

1B: Representative thymic section from an animal receiving IUGT at 74 days of gestation, showing that very few cytokeratin-positive (green) TEC are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT late in gestation.

1C: Graphical summary of quantitative results examining impact of recipient age at time of IUGT on the levels of TEC transduction. Please see text for details.

1D: Representative thymic section from an animal receiving early IUGT at 54 days of gestation, showing very few, if any, CD45-positive (green) thymic hematopoietic cells are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT early in gestation.

1E: Representative thymic section from an animal receiving IUGT at 74 days of gestation, showing that the majority of thymic cells that are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT late in gestation are CD45-positive hematopoietic cells. Nuclei are counterstained blue with DAPI.

1F: Representative thymic section from an animal receiving IUGT at 74 days of gestation, showing that the majority of thymic hematopoietic cells that are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT late in gestation are CD4-positive. Nuclei are counterstained blue with DAPI.

1G: Representative thymic section from an animal receiving IUGT at 74 days of gestation, showing that the thymic hematopoietic cells that are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT late in gestation do not express CD25. Nuclei are counterstained blue with DAPI.

Figure 2. IUGT early in gestation results in transduction of Hassall's corpuscles

2A: Graphical summary of quantitative results examining the impact of recipient age at time of IUGT on the efficiency of transduction of Hassall's corpuscles. Please see text for details. 2B: Representative thymic section from an animal receiving IUGT at 61 days of gestation, showing numerous TSLP-positive (green) cells of the Hassall's corpuscles are transduced and expressing the vector-encoded NPTII (red) as a result of IUGT early in gestation. Nuclei are counterstained blue with DAPI.