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Review

Xenoreactive natural antibodies

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Abstract. Shortages of human organs for transplantation have made it necessary to examine the possibility of using nonhuman organs for xenotransplantation—the transplantation of tissues between different species. Pigs are now regarded as the most likely species to serve as donors for clinical xenotransplantation. However, rejection of pig tissues and organs, mediated by the host's immune system, remains a major barrier to successful xenotransplantation. The primary immunological hurdle to overcome is rejection mediated by antibodies in the host that recognize antigens present on xenogeneic tissues. Since these antibodies are produced naturally in the host without immunization, they are termed natural antibodies. Here, we review the nature of xenoreactive natural antibodies directed toward pig tissues, and summarize recent progress in the field of xenotransplantation directed at overcoming humoral rejection of porcine xenografts.

Key words. Natural antibodies; xenotransplantation; humoral rejection.

Introduction

The need for xenotransplantation

Presently, the United Network for Organ Sharing estimates that as of June 1999 in the United States alone there were 64,000 patients on waiting lists for life-saving organ transplants. However, in 1998, only ~20,000 transplants were performed using organs recovered from cadaveric or living donors. The acute shortage of transplantable human organs has stimulated a great deal of exploration into the possibility of using nonhuman donor organs for transplantation. While nonhuman primates may appear to be well suited for this purpose, several factors preclude their use as donors for clinical xenotransplants, including size, difficulty of breeding in captivity, status of some species as endangered and the potential to transmit infectious agents to humans [1, 2]. Pigs are now regarded as the most likely species to serve as donors for clinical xenotransplantation because they are similar to humans in size, physiology, breeding characteristics and because of ethical considerations [3, 4]. In this review we will focus specifically on xenotransplantation using pig tissues and organs.

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Humoral rejection is the major barrier to xenotransplantation across discordant species barriers

Early in the history of xenotransplantation it was observed that transplanting of organs between species that are phylogenetically very disparate resulted in immediate hyperacute rejection (HAR) of the transplanted organ. HAR, resulting in rapid vascular thrombosis and extensive interstitial hemorrhage, was histologically similar to the type of rejection observed when allografts were transplanted between ABO blood group-incompatible individuals. Since rejection of allotransplants between ABO-incompatible individuals was known to be due to the presence of preformed antibodies in the

host directed toward foreign blood group antigens, it was soon recognized that HAR was likely to be antibody-mediated and involve the complement system. Xenotransplantation across widely disparate species in which preformed xenoreactive natural antibodies exist in the host are now termed discordant, where those across more closely related species in which natural antibodies do not mediate HAR are termed concordant [5].

The carbohydrate epitope galactose α(1–3) galactose is the antigen recognized by xenoreactive natural antibodies (XNAs)

In the pig-to-primate discordant combination, XNAs recognize essentially a single carbohydrate antigen present on pig tissue, Galα1-3Galβ1-4GlcNAc-R, hereafter referred to as αGal [6–11]. This carbohydrate epitope is synthesized by the addition of a terminal galactosyl group to a preexisting galactose residue linked to an *N*-acetylglucosaminyl structure and is catalyzed by the glucosyltransferase uridine diphosphate (UDP) galactose:β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminide α(1-3)galactosyltransferase (EC 2.4.1.151), or αGT. As high as 10^6–10^7 αGal epitopes can be expressed on the surface of pig cells and tissues [12]. All placental mammals except humans, apes and Old World monkeys express a functional αGT enzyme and αGal epitopes on most tissues, including vascular endothelium [12]. Therefore, these animals are immunologically tolerant to αGal because their immune system develops in an environment in which this antigen is recognized as 'self'. Because animals that express a functional αGT enzyme are immunologically tolerant, they do not produce antibodies that bind αGal. In contrast, humans, apes and Old World primates carry a non-functional αGT gene whose function appears to have been lost approximately 30 million years ago [13]. Since these species therefore do not recognize αGal structures as self, they consequently produce αGal XNAs.

Characteristics of αGal XNA in humans

αGal XNAs make up at least 80–90% of anti-pig antibodies in humans and primates lacking a functional αGT gene [9]. The specificities of the other antibodies remain uncertain [14]. αGal XNAs comprise ~1–8% of circulating immunoglobulin in humans, and it has been shown that approximately 1% of Epstein-Barr virus-transformed peripheral blood B cells make antibodies that bind αGal [15, 16]. αGal XNAs appear to be encoded for in humans by a restricted set of immunoglobulin Vh genes from the V_H3 family [17]. αGal XNAs are thought to be, at least in part, T-independent antibodies because of the structural nature of the epitope on glycoproteins and glycolipids. However, somatic hypermutation has been observed in Vh genes encoding these antibodies [17]. Transplantation of humans or primates with tissues expressing αGal epitopes has been shown to lead to an increase in the titer of αGal XNAs [18–20], and these antibodies have been implicated in chronic rejection of tissue grafts [19].

Characteristics of XNA-mediated rejection

Binding of XNAs to αGal epitopes on pig tissues or the vascular endothelium of solid organs results in the activation of processes that ultimately lead to rejection. Binding initiates activation of the complement system, which in turn induces rapid type I endothelial cell activation and destruction and loss of vascular integrity, culminating in HAR [21]. In discordant species combinations, host complement-mediated destruction is extremely potent because the regulators of complement expressed on pig tissues, such as CD55, CD59 and CD46, that normally protect tissues from complement assault, appear to be either ineffective in their ability to inhibit human complement or are expressed on pig tissues at levels inadequate to confer protection [22–24]. In the absence of complement, XNAs are the major cause of a delayed form of graft rejection referred to as delayed xenograft rejection (DXR) or acute vascular rejection (AVR) [25]. Binding of XNAs to αGal epitopes expressed on vascular endothelium leads to type II endothelial cell activation. In DXR, endothelial cell activation requires transcription and translation of gene products, which does not appear to occur during HAR. Histologically, DXR is associated with less interstitial hemorrhage than observed with HAR, but vascular thrombosis appears to be similar in both DXR and HAR. In nonhuman primates, αGal XNAs have been shown to be involved in chronic rejection of nonvascularized porcine and bovine cartilage grafts [19, 26]. Thus, while it has been known for some time that αGal XNAs play a major role in rejection of vascularized xenografts, it now appears that these antibodies are also important in rejection of nonvascularized tissues.

The role of natural antibodies in primate immunity

Production of αGal XNAs is believed to be elicited in response to normal bacterial flora that colonize the human gastrointestinal tract [27, 28]. The presence of αGal XNAs in serum and secretory fluids, such as colostrum and saliva, suggests that these antibodies have evolved to play a protective role in primate immunity. Consistent with this hypothesis, it has recently

been shown that αGal XNAs may be involved in viral immunity. Enveloped viruses produced in αGal-expressing animal cells, such as lymphocytic choriomeningitis virus, Newcastle disease virus and vesicular stomatitis virus, as well as C-type retroviruses, have all been shown to be susceptible to inactivation by serum αGal XNAs [29, 30]. Based on these studies, it appears that incorporation of glycoproteins modified with αGal structures into virus envelopes renders the virus susceptible to attack by αGal XNAs and complement. Thus, αGal XNAs may play an important role in preventing cross-species infection by pathogens, termed zoonosis. However, it has not yet been determined to what degree αGal XNAs are required to protect against various pathogens.

Attempts to prevent antibody-mediated xenograft rejection

Modification of donor organs and tissue

Eliminating the αGal epitope from porcine tissue through genetic engineering of pigs would be the most direct way to prevent HAR or DXR. However, it is not yet possible to conduct gene-targeting experiments in pigs to knock out the αGT gene because porcine embryonic stem (ES) cell lines capable of achieving germline transmission of mutations induced by homologous recombination are not available. Even if the appropriate ES cell lines were available, it is not clear that αGT-deficient pigs would be viable. Attempts have therefore been made to remove αGal epitopes on pig endothelial cells enzymatically in vitro [31]. However, this approach is inefficient and does not prevent reexpression of αGal epitopes.

Attempts have been made to eliminate or reduce αGal expression by replacing αGal epitopes with other carbohydrate moieties to which humans are immunologically tolerant. This approach has been termed competitive glycosylation [32], and involves expressing as a transgene the α1,2-fucosyltransferase gene (αFT) that is capable of competing with αGT for its common substrate oligosaccharide, lactosamine. Fucosylation of lactosamine generates the O blood group antigen (αFuc) to which the vast majority of humans are tolerant. This approach has been explored in cell culture [32], and in transgenic mice and pigs [33–35]. It has been shown that expression of αFT in vascular endothelium can eliminate approximately 90% of cell surface αGal epitopes. Although this reduction is impressive, between 10^5 and 10^6 αGal epitopes may still be expressed on the cell surface, a number that is sufficient to permit DXR mediated by XNAs. Indeed, it has been shown that even very low levels of αGal epitopes on transplanted tissue may be sufficient to cause DXR or chronic rejection [19, 26]. There is also some concern because expression of αFT may be associated with the early development of cancer in transgenic pigs [C. Koike, personal communication].

A second approach to modification of pig donors has been to construct transgenic pigs that express human regulators of complement activation (RCA) on vascular endothelium. Expression of human RCA on vascular endothelium prevents activation of human complement, thereby preventing HAR. Human CD55 (human decay-accelerating factor, hDAF) and CD59 (membrane inhibitor of reactive lysis) transgenic pigs have been constructed [36–40], and reports suggest that organs from RCA transgenic pigs when analyzed ex vivo or when transplanted into primates do not undergo HAR. However, all organs eventually undergo DXR despite intensive pharmacologic immunosuppression [38, 41–46]. Thus, while hDAF, CD59 and αFT transgenic pigs will most likely play an important role in clinical xenotransplantation, it would appear that methods to prevent production of αGal XNAs in the host must be developed to completely prevent antibody-mediated xenograft rejection.

Modification of the host

In terms of modifying the host, attempts have been made to deplete XNA by perfusing the host's blood through pig organs [47, 48] or affinity columns comprised of either αGal oligosaccharides or anti-human immunoglobulin (Ig) covalently coupled to a solid matrix [48–51]. Plasma exchange has also been used to reduce levels of XNAs [52]. Attempts have also been made to neutralize XNAs by administering soluble α-galactosyl carbohydrates or anti-XNA idiotypic antibodies to the host [53–56]. While these approaches have made it possible to remove or reduce the levels of existing serum XNA or inhibit XNA, and will most likely be an important part of host preparative regimens for xenotransplantation, they do not prevent XNAs from returning, beginning within 24 h following treatment [49, 50, 57]. Furthermore, it has not been possible to prevent the return of XNAs following host conditioning by using nonspecific immunosuppressive agents [57, 58].

A murine model to test methods of preventing production of αGal-reactive natural antibodies by modification of the host

The αGT knockout mouse

In order to examine methods to prevent production of αGal-reactive antibodies, mutant mice lines lacking a functional αGT gene (GT^0 mice) were generated by

gene targeting in embryonic stem cells [59, 60]. It has been shown that in GT^0 mice, disruption of the αGT gene completely eliminates expression of αGal epitopes on tissues in homozygous mutant mice, permitting development of IgM and IgG antibodies that bind αGal without the need for immunization [59, 61]. These mice therefore represent a unique small animal model to study methods of preventing development of αGal-reactive antibodies

Induction of hematopoietic chimerism prevents the development of αGal-reactive antibodies

It has been known for many years that a state of mixed (donor and host) hematopoietic chimerism, induced by bone marrow transplantation, can lead to permanent tolerance of T and B cells across allogeneic and concordant xenogeneic species barriers [62–64]. The establishment of mixed chimerism through bone marrow transplantation in adult animals leads to specific tolerance in an otherwise fully immunocompetent host [65, 66], and even low levels of chimerism are sufficient to induce transplantation tolerance [67–71]. Recently, it was shown that following reconstitution of lethally irradiated GT^0 mice with a mixture of GT^0 and $GT^{+/0}$ (normal littermate) bone marrow, production of αGal-reactive antibodies is completely inhibited in the GT^0 host [72]. Mixed hematopoietic chimeras in these studies were shown to be tolerant to the αGal epitope because immunization with pig cells failed to elicit production of αGal-reactive antibodies, although antibody responses to other non-αGal pig antigens were normal. These results demonstrate that expression of αGal epitopes on bone marrow-derived hematopoietic cells is sufficient to induce tolerance to the αGal epitope.

For this approach to work in a pig-to-primate setting, pig bone marrow cells or stem cells would have to be used to make a pig-human bone marrow chimera. However, bone marrow transplantation across discordant xenogeneic barriers may not be currently acceptable for clinical use in humans as a means of inducing tolerance to allo- or xenografts for a variety of reasons, including (i) the severity of the preparative regimen required, (ii) the potential for inducing severe graft-versus-host disease (GVHD) [73], (iii) high rates of engraftment failure following T cell depletion from the donor bone marrow to prevent GVHD [74, 75], and (iv) difficulty in establishing pig bone marrow engraftment and long-term hematopoietic chimerism in primates [76, 77]. Insofar as tolerance induced by mixed chimerism depends on engraftment of donor bone marrow [64, 78, 79], the difficulties in establishing pig bone marrow engraftment represent a major barrier to exploiting cellular chimerism as a means to tolerize B cells producing XNA in primates.

Gene therapy as part of a tolerance-inducing regimen to prevent production of αGal-reactive antibodies

Since it has not yet been possible to establish pig-to-primate mixed hematopoietic chimerism, an alternative approach using retroviral gene therapy has recently been developed [80]. This approach involves introducing a functional αGT gene by retroviral gene transfer into autologous bone marrow-derived cells to establish molecular rather than cellular chimerism. The retrovirally transduced αGT gene synthesizes αGal epitopes that can be expressed on the surface of bone marrow-derived cells or on secreted proteins that in turn can tolerize B cells that produce αGal-reactive antibodies. This approach has at least one significant advantage over cellular chimerism, because establishing molecular chimerism involves modification of autologous bone marrow stem cells, thereby overcoming difficulties associated with engraftment of pig bone marrow in primates.

To examine whether genetic engineering of bone marrow could be used to inhibit production of αGal-reactive antibodies, bone marrow cells from GT^0 mice treated in vivo with 5-fluorouracil prior to harvest were transduced with retroviruses carrying the gene encoding pig αGT or control retrovirus carrying the neomycin resistance gene (NEO) only [80]. Transduced bone marrow cells were then recovered and used to reconstitute lethally irradiated syngeneic GT^0 mice. At 7 weeks post bone marrow transplantation, αGal-reactive antibodies were readily detectable in the sera of control mice reconstituted with NEO-transduced bone marrow. In contrast, αGal-reactive antibodies were not detected in the sera of mice reconstituted with pig αGT-transduced bone marrow. While the titer of αGal-reactive antibodies in mice reconstituted with NEO-transduced bone marrow increased steadily over time up to 12 weeks after reconstitution, over the same time period mice reconstituted with pig αGT-transduced bone marrow failed to produce αGal-reactive antibodies. Mice reconstituted with pig αGT-transduced bone marrow maintained undetectable levels of αGal-reactive antibodies measured by several different methods for over a year following reconstitution. These data demonstrate that production of αGal-reactive antibodies can be inhibited using a gene therapy approach without additional immunosuppression.

While the mechanism by which natural antibody production is inhibited by a gene therapy approach is not yet defined, it is likely that expression of αGal epitopes on bone-marrow derived cells encoded by the transduced pig αGT gene inhibits the ability of B cells to make αGal-reactive antibodies. As mentioned above, antibodies reactive with αGal are believed to be produced in response to normal bacterial flora present in

the host. Thus, even with constant antigenic stimulation, the effect of gene therapy on αGal-reactive antibodies is maintained. While more experimentation is required to elucidate the mechanism by which antibody production is inhibited in this system, we suggest that B cells producing αGal-reactive antibodies may undergo deletion, anergy or receptor editing as has been observed in other systems [81]. Nevertheless, this is the first demonstration that production of preexisting natural antibodies can be inhibited by a gene therapy approach. Similar approaches may be applicable to the induction of tolerance in other disorders, such as autoimmune disease.

Conclusion

It is now clear that processes leading to humoral rejection of xenografts, such as HAR and DXR, have become better defined in recent years. Methods to overcome the XNA hurdle to successful xenotransplantation are now being intensively examined. Advances in both the modification of donor tissues and organs through genetic engineering techniques and of the host immune response are being achieved, and it is likely that a combination of approaches will be necessary if xenotransplantation is to become a clinical reality. If production of αGal XNAs can be prevented in primates, it will be important to examine how this alteration affects susceptibility to pathogens. After the humoral barrier is overcome, host cellular immunity will represent the next major barrier to xenotransplantation. To date, it has not been possible to assess whether conventional immunosuppression will be sufficient to prevent cell-mediated xenograft rejection. While it is obvious that much work needs to be done to solve the problems inherent in xenotransplantation, the advances that are taking place provide hope that this approach to the donor shortage will fulfill its immense promise.

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