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Progress towards clinical xenotransplantation

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1. ABSTRACT

Xenotransplantation has progressed from early heroic experiments on the path to meet the ever increasing demands of tissue and organ transplantation in patients with end-stage organ failure. The pig species is regarded as the most promising donor species. However, due to the evolutionary distance, innovative approaches are to be developed to permit life-supporting function in humans. Transplantation of organs from non-human primates has increased our knowledge on rejection mechanisms and provided opportunitities for testing modified immunosuppression of the host and genetic modification of the donor. The development of transgenic animals expressing human complement-regulatory factors, and of animals lacking the target for naturally occurring anti-pig antibodies, has essentially eradicated hyperacute rejection of solid organs. However, there is still a need for tolerable immunosuppression or immune-tolerance regimens to provide broadly available procedures in the clinical setting. Safety concerns especially cross-species transmission of infectious pathogens, in particular of porcine endogenous retrovirus. Many studies have indicated that this is highly unlikely. At present, cell and tissue transplantation of islets of Langerhans to diabetic patients is close to being tested in well-designed clinical trials. Further research is required before other porcine xenografts can offer a broadly available therapeutic option in clinical medicine.

2. INTRODUCTION

Xenotransplantation to humans is defined as any procedure that involves direct transplantation, implantation, or infusion of live cells, tissues, or organs from a non-human animal source. This term is also applied when human body fluids, cells, tissues, or organs are used that had come into contact with live non-human animal cells, tissues, or organs (e.g., vaccine preparations using cultures of xenogeneic cells) and might be contaminated by an infectious agent from another species. Here, we will discuss the xenotransplantation to humans without reviewing the second category.

The rationale for performing xenotransplantation is based on the limited availability of human cells, tissues or organs for the treatment of patients with end-stage organ failure. For instance, in the US, there are a total of 101,000 patients on the waiting list, among these 75,000 patients are on the kidney waiting list (status February 2007, data from the Organ Procurement and Transplantation Network (1)). In contrast, the number of kidney transplantation is about 16,000 each year, and the median waiting time is in the order of 4 years. Thus, the percentage of patients who actually receive a transplant is in the order of 25%, dependent on age, incidence of anti-donor antibodies, and ethnicity.

2.1. History

Organ transplantation is a relatively new procedure in clinical medicine. The first successful kidney transplants dated from the 1950s and the first heart transplant was conducted in 1963. These results showed that organ transplantation was a feasible approach from surgical-technical point of view. However, adequate immunosuppression to prevent and treat rejection was not yet available at that time. The development of therapeutic antibodies, the immunosuppressant cyclosporine (a calcineurin inhibitor), and effective anti-infection strategies in the late 1980s allowed clinical transplantation to become generally successful and develop to its present volumes. Then it became clear that donor supply could not meet the demands, and xenotransplantation was considered as a potential solution.

Early pioneering work in clinical xenotransplantation involved blood and bone marrow transfusions, but without clear evidence of efficacy (2, 3). Regarding solid organs, pioneering clinical research was performed in kidney, liver and heart transplants using nonhuman primates as donors. Well-known examples are kidney transplants conducted in the 1960s by Hitchcock *et al.* in Minneapolis MN (4), Reemtsma *et al.* at Tulane LA (5), and Starzl *et al.* in Denver CO (6). The first chimpanzee and baboon heart transplants were reported in 1964 by Hardy *et al.* in Jackson MI (7), in 1977 by Barnard *et al.* in Cape Town, South Africa (8), and in 1984 by Bailey *et al.* in Loma Linda CA (9). Starzl *et al.* performed three chimpanzee liver transplants between 1969 and 1974 in Denver CO (10), and subsequently performed two baboon liver transplants in Pittsburgh PA in 1992-93 (11). Survival times obtained in all these clinical experiments were rather poor, and typically measured in hours or days. The best results were reported by Reemtsma *et al.*, namely up to 9 months for a kidney transplant from a chimpanzee (12), and by Starzl *et al.*, namely 70-day survival for a baboon liver transplant (11). These isolated clinical "successes" compare very unfavorably with current results with clinical allografts. This relatively unsatisfactory data, combined with ethical issues (13) and subsequently with the prospect that endogenous nonhuman primate viruses pose a high potential risk of cross-species virus transmission (14), resulted in a moratorium in working with nonhuman primate donors. This opinion was accepted by the scientific community in the early 1990s.

Presently, the main focus in cell and tissue xenotransplantation is on the pig as donor. The choice of the pig is based on a number of arguments (15). Materials from pigs are widely used as therapeutics, for instance millions of diabetic patients have received pig insulin since the 1920s, over 1,000,000 patients have received pig heart valves since the 1950s, and pig skin has been used in treatment of over 20,000 patients with burns since the 1970s. Also, the pig has been domesticated for thousands of years, being a major source of meat in the food chain. Regarding their production, pigs are relatively easy to breed, with large litters and a quick growth to maturity (6-9 months). The physiology and anatomy of pigs makes them suitable as donor for humans, exemplified by species concordance for hormones like insulin, cardiovascular characteristics like cardiac output, and renal function parameters such as glomerular filtration rate. After the introduction of modern molecular biology technology, including transgenesis and cloning, it has become possible to generate genetically modified pigs, which was deemed necessary to reduce the immune attack by the recipient. Finally, there are no major religious objections or serious ethical obstacles (16, 17). Indeed, even those religions that proscribe consumption of pig-derived food products have approved the use of pigs as donor in treatment of end-stage disease.

First studies using pig donor organs (heart (18) and liver (19)) were reported in the early 1990s, after an exploratory pig heart transplantation by Ross *et al.* in London UK in 1968 (20). Since then, research on the use of pigs as donor in human transplantation has been intense, both in the academic setting and in industry. Much progress has been reported, but two major obstacles on this path remain before clinical trials: effective immunosuppression and demonstration of clinical safety and efficacy. These will be discussed as they pertain to each organ and tissue transplant type in the following sections of this review.

3. CONTROL OF XENOGRAFT REJECTION MECHANISMS

As a point of reference, allograft rejection is primarily mediated by T-lymphocytes, i.e. the sensitization of T lymphocytes resulting in cytolytic T effector cells and T-cell help for *de novo* (induced) anti-donor B-lymphocyte responses. Before transplantation, a role of anti-donor antibodies in rejection is specifically excluded wherever possible, by matching donor and recipient for ABO antibodies and antibodies to major histocompatibility complex (MHC) antigens. Antibodies against other targets, including those towards "auto-antigens" that may be preferentially expressed on graft endothelium, or against other less well characterized antigens that may differ between donor and recipient, can play a role later after transplantation; some investigators hypothesize that they are especially important in the mechanism of chronic rejection. Substantial progress in transplantation across a positive crossmatch or against ABO blood group mismatch demonstrates that antibody-mediated rejection can be controlled under some circumstances. The lessons learned from this experience with respect to drug and treatment effects may be applicable to xenotransplantation.

The immune reaction towards a xenograft involves almost all branches of the immune system (21). The immune response to a xenograft is more vigorous than that to an allograft, at least in large measure due to existence of preformed "naturally-existing" antibodies. These anti-pig antibodies play a major role in xenograft rejection, and include both naturally existing antibodies and antibodies evoked after sensitization.

3.1. Hyperacute rejection

Each human individual has naturally existing antibodies towards porcine tissue (22, 23). The major

component comprises IgM class antibodies directed against carbohydrate antigens, similar to anti-ABO blood group antigens. Also, similar to anti-blood group antibodies, these antibodies have their main origin as a result of cross-immunization by bacterial flora upon colonization of the intestine after birth. Their effect on porcine tissue or cells is based on differences in glycosylation between humans and pigs, i.e. the expression of the major terminal sugar epitope galactose-alpha(1-3)galactose (Gal). Humans lack this terminal carbohydrate because they lack the enzyme galactosyl transferase (GalT) involved in its synthesis. Upon contact between these antibodies in the human circulation and a (vascularized) organ transplant, bound antibodies induce an immediate complement-mediated destruction, called hyperacute rejection (HAR), that is characterized by massive hemorrhage, deposition of terminal complement components, infiltration by polymorphonuclear granulocytes and thrombosis. HAR normally occurs within minutes and is completed within hours after transplantation (21).

The availability of animal models mimicking the pig-to-human situation is limited. Some small animal models, like guinea pig-to-rat organ transplantation, have been utilized, but such models might not correctly mimic the pig-to-human condition as disparity in complement regulation has a major role exceeding that of naturally existing antibodies (24). To most closely simulate a potential pig-to-human situation, old-world nonhuman primates are used as recipient, as they share with humans the absence of GalT, and their complement system is quite similar. In a number of studies it has been shown that these naturally existing anti-Gal antibodies occur in baboons and monkeys with high variability (25, 26), at similar or even higher levels when compared to humans (27).

A number of approaches have been initiated to prevent HAR. At first methods that deplete the antibodies from the circulation are worth mentioning. Extracorporeal perfusion through Gal-containing immunoadsorption columns has been used (28), as well as injection with Gal-containing polymers. One of these is NEX-1285, a synthetic Gal conjugated on a polyethylene glycol backbone developed by Nextran, Inc. (29), and another one is GAS914, a synthetic Gal on a poly-L-lysine backbone (Novartis Pharma AG) (30). Others have used Gal on a bovine serum albumin carrier (31). These conjugates proved to be effective in avoiding HAR. Their long-term use has been questioned, in particular in recipients with high levels of antibodies (32), amongst others because of the potential of incomplete removal and the high production costs. The clinical development of these compounds has been discontinued.

To make the donor less immunogenic or protect itself from immune injury, the technology of gene transfer ("transgenesis") has been utilized. One approach aimed to overexpress another sugar: H-transferase transgenic pigs have been generated and showed reduced expression of Gal (33, 34). As complement activation is a major component in rejection, complement inhibitors have been introduced. In the experimental setting cobra venom factor has been applied, a venom that essentially induces complement consumption and exhaustion of the complement synthesis (35): there have been some initiatives to discover and develop components in cobra venom factor towards clinically-acceptable drugs, but these initiatives are concurrently in early research phase. Various complement inhibitors have been investigated, including soluble complement receptor 1 (TP10, Avant Immunotherapeutics, Inc.) (36), complement activation blocker-2 (a chimeric protein from human decay-accelerating factor (hDAF, CD55) and membrane cofactor protein (MCP, CD46) (Millenium Pharmaceuticals, Inc.) (37), and an anti-C5 antibody (Alexion Pharmaceuticals, Inc.) (38). The anti-C5 antibody has recently been approved for the indication paroxysmal nocturnal hemoglobinuria. In various experimental conditions these compounds have shown to be effective in prevention of HAR. However chronic complement inhibition was insufficient to prevent graft injury at the doses used, since antibody-mediated rejection occurred. Another factor restraining clinical development is that the complement system is beneficial in combat bacterial infections, and long-term inhibition would presumably cripple this basic host defense.

3.2. Genetic modification of donor animals

Modulating (inhibiting) deposition of terminal complement components only on the endothelial surface lining pig blood vessels would avoid the need for systemic complement inhibition. To accomplish this goal, pigs transgenic for human complement regulatory proteins were generated in the mid 1990s. Most studies have been performed with pigs transgenic for human decay-accelerating factor (hDAF, CD55) produced by Imutran-Novartis (39), pigs transgenic for human CD59 (protectin) by Alexion (40) and various strains of pigs transgenic for human CD46 (membrane cofactor protein, MCP) and human CD59 (preventing the prevents the complete assembly of the membrane attack complex of complement) by Nextran (41). hCD59-transgenic pigs have also been generated at the University of Hannover, Germany (42). Triple-transgenic pigs transgenic for H-transferase (see above), hDAF and CD59 have been generated at St. Vincent Hospital, Melbourne, Australia (33), and double-transgenic pigs transgenic for H-transferase and CD59 by Alexion (34). In most cases HAR was avoided working with organs from such complement-transgenic pigs, although in a review of a large series of transplants some baboons and cynomolgus monkeys hyperacutely rejected a hDAF-transgenic heart graft (43). Despite the persistent presence of the transgene, kidney and heart grafts showed histologic features of antibody-mediated injury later after transplantation, suggesting that the effect of the transgene in the long-term can be lost. Thus far, it is not known whether late graft injury represents an artifact (that is, formation of antibodies to the human transgene product by the nonhuman primate recipient because it is a xenogeneic antigen in this recipient species), or whether it represents a loss of function of the transgene with time after transplantation (44).

The most recent approach in genetic modification became possible after pig cloning techniques had been

developed. Nuclear/embryo transfer in cloning technologies has been performed to target GalT. Three groups have successfully created so-called GalT-knockout (GalT-KO) pigs: Immerge Biotherapeutics, Inc. (45), PPL Therapeutics (nowadays Revicor, Inc.) (46), and Nextran (47). Interestingly, besides the absence of Gal expression, GalT-KO minature swine showed naturally existing cytotoxic anti-Gal antibodies (48). Organs from two of these pig lines were not hyperacutely rejected after transplantation into baboons, and long-term survival was achieved under chronic immunosuppression (49-51). Relative to heterotopic hDAF heart transplants conducted using a similar immunosuppressive regimen (35), GalT-KO miniature swine hearts (49) showed better function after transplantation with less immunosuppression, but only a statistically insignificant trend toward increased survival time. Both heart and kidney GalT-KO grafts were finally lost despite chronic immunosuppression. The histology of rejected grafts showed thrombosis ("thrombotic microangiopathy") as a histologic feature. It is a key focus of current investigations whether coagulation pathway disparities or rather immune rejection mechanisms are primarily responsible for these lesions. An additional piece of evidence supporting the hypothesis that coagulation pathway dysregulation contributes to xenograft injury is the occurrence of disseminated intravascular coagulopathy observed in some studies (52). Thus, besides human complement modulators and GalT-KO modifications, proteins involved in modulating the coagulation system on the endothelial surface, maintaining an anticoagulant status and preventing a shift to a procoagulant status (53, 54), are subject for further investigations. If these studies identify protective pathways and specific molecules, additional genetic modifications might be warranted to protect the graft from injury by the coagulation cascade.

3.3. Rejection in solid organ xenotransplantation

In the case that HAR is prevented, the major cause of long-term dysfunction in the pig-to-nonhuman primate organ transplantation model is rejection. In contrast to allograft rejection, the majority of xenotransplant cases show histologic signs of antibody-mediated rejection with hemorrhage, deposition of immunoglobulins, fibrin, and complement components, infiltration by polymorphonuclear granulocytes, and thrombosis (21). Microvascular thrombosis with C4d deposition is often considered as a first sign of antibody-mediated rejection.

Various immunosuppressive regimens have been utilized by different groups. A major attempt has been undertaken by Imutran-Novartis and associated groups working with hDAF-transgenic organs in the late 1990s, using amongst others convential immunosuppressive regimens including induction with anti-thymocyte or anti-lymphocyte globulin, CD20 antibody or cyclophosphamide, and maintenance immunosuppression with different combinations comprising cyclosporine or FK-506 (calcineurin inhibitors), mycophenolate derivaties (inhibitors of inosine monophosphate dehydrogenase), rapamycin or rapamycin derivatives (inhibitors of growth factor-induced cell proliferation), FTY-720 (sphingosine 1-monophosphate receptor antagonist affecting lymphocyte recirculation), methotrexate, and steroids. In a number of studies animals were in addition subjected to splenectomy. On average, median survival times were in the order of 1-2 months, with antibody-mediated rejection as a major outcome. The group of McGregor *et al.* at Mayo Clinic, Rochester MN, studied heterotopic heart transplantation in baboons using CD46-transgenic pigs, and achieved a median survival of 3 months using induction with CD20 antibody and anti-thymocyte globulin, splenectomy, and chronic immunosuppression using rapamycin, FK-506, steroids and the Gal polymer NEX-1285 (41, 55). The group of Cooper *et al.* at Massachusetts General Hospital/Harvard Medical School, Boston MA, achieved prolonged survival of heart grafts from hDAF-transgenic pigs or GalT-KO miniature swine in baboons using a regimen of anti-thymocyte induction, thymic irradiation, and maintenance immunosuppression with an anti-CD154 antibody in combination with mycophenolate mofetil and steroids (35, 49, 56). Also heparin and aspirin were given as anticoagulants. Survival of informative cases in these studies ranged between 2 and 6 months.

Interestingly, differences in beneficial effects of anticoagulant use in the medication have been reported by different groups. In contrast to Cooper *et al.,* who ascribe their best outcomes to the use of anticoagulation (57), the group of McGregor *et al.* concluded that anti-coagulation had no beneficial effect (58, 59). It remains to be established whether this difference is related to the source of the animal or to the components in the very different immunosuppressive regimens and pig lines. Of note, the commercial development of CD154 antibodies was discontinued because of thrombo-embolic complications in first clinical explorations and in some nonhuman primates. The Harvard group observed high incidence of arterial thrombosis in a kidney allograft series that was prevented simply by use of ketorolac (60). If CD154 blockade proves necessary to accomplish organ xenografting and alternative strategies are ineffective (CD40 blockade, for example), this relatively trivial intervention may prove pivotal to clinical success.

In conclusion, various immunosuppressive protocols have shown to be efficacious in pig-to-primate solid organ transplantation, but survival is at present insufficiently long or consistent to justify clinical trials. The major achievements have been reached in the effectiveness of immunosuppression to prevent appreciable cellular and antibody graft responses, with improving adverse side effects and better tolerability by the host. Antibody-mediated rejection, or thrombo-embolic complications that might be associated with rejections, are still a major obstacle in reaching long-term survival. Cellular rejection, featured by infiltration of (CD8-positive) T cells in combination with macrophages, is generally unusual (21). In a number of studies it has been shown that antibody-mediated rejection is associated with newly-generated antibodies to other donor antigens than anti-Gal antibodies, as documented in assays using endothelial cells as target cells (51, 61-63).

Interestingy, with survival rates increasing, phenomena of chronic rejection have become evident (21, Hisashi *et al.*, manuscript submitted). Histologically, this is featured by blood vessel abnormalities including thickening of the vascular intima and (partial) vessel obstruction. In the clinical allograft setting chronic rejection has a multifactorial origin including immune factors and non-immune factors such as the condition of the graft (e.g., age of donor, cold ischemia time). In the xenotransplantation models described above most of non-immune factors can be minimized, so that we presume that the main contributing factor is a smouldering ongoing rejection reaction.

3.4. Xeno-tolerance

As an alternative to chronic immunosuppression, tolerance induction has been proposed. Tolerance is more difficult to achieve in the xenotransplantation setting than in allotransplantation. As an example, costimulatory blockade with an CD154 antibody can be mentioned. In the nonhuman primate allogeneic kidney transplant model, a 3-month course of CD154 treatment can result in long-term allograft function without rejection (64), although chronic rejection is not prevented (Alan Kirk, personal communication). This level of graft protection has thus far been approached but not equaled in pig-to-nonhuman primate studies despite the combination of CD154 treatment with other immunosuppressives (35, 49).

Deletional tolerance is the major focus of the group at Massachusetts General Hospital/Harvard Medical School (Sachs, Sykes, *et al.*) working in mouse, miniature swine and nonhuman primate models (65-67). In such models, nonmyeloablative induction combining whole-body and thymic irradiation, followed by bone marrow transplantation, anti-thymocyte globulin treatment and a short course of cyclosporine resulted in long-term multilineage hematopoietic chimerism and long-term kidney allograft survival after discontinuation of immunosuppression. This progress has advanced to the clinical situation in patients with hematologic malignancies (68) and end-stage renal disease associated with multiple myeloma (69): in the latter condition, long-term kidney allograft function after weaning of immunosuppression was achieved using pretransplant conditioning with thymic irradiation, cyclophosphamide, anti-thymocyte globulin, and cyclosporine, followed by MHC-matched donor bone marrow and kidney transplantation, and a short course of anti-thymocyte globulin and a 2-months period of cyclosporine.

The translation of these successes to a pig-to-nonhuman primate kidney transplantation model has proven difficult, in first instance related to the complications in establishing hematopoietic chimerism. In the so-called thymo-kidney approach, a thymo-kidney was prepared 2-3 months before transplantation by implanting the thymus under the renal capsule of the pig donor. Upon subsequent transplantation in baboons after conditioning with thymectomy or thymic irradiation, splenectomy, and continued treatment with cobra venom factor, cyclophosphamide, mycophenolate, steroids, and T-lymphocyte depletion and anti-Gal antibody depletion, survival up to one month after transplantation was obtained (66, 70). *In vitro* evaluation revealed signs of cellular unresponsiveness towards the donor, and the grafts showed antibody-mediated rejection. In the first series of 14 transplants using GalT-KO kidney donors, three long-term survivors (till 68, 81 and 83 days post-transplantation) were observed, with an apparently normally functioning kidney graft without clear histologic signs of rejection (50). These animals received either a pre-prepared thymo-kidney or a combination of a kidney and vascularized thymic lobe transplant, after a conditioning regimen including thymectomy and splenectomy, whole body irradiation, cobra venom factor and T-cell depletion by anti-thymocyte globulin and anti-T cell antibody, and subsequent chronic treatment with mycophenolate mofetil, a CD154 antibody and steroids. Sacrifice of these animals was mandated not by graft dysfunction but rather by technical complications (catheter malfunctions), acute myocardial infarction, or pneumonia, which would be more readily diagnosed and treated in a human organ recipient. These data are promising and warrant more studies on xenotolerance induction.

3.5. Islet cell transplantation

Islet replacement in patients with type 1 diabetes (insulin-dependent diabetes), and to a less extent type 2 diabetes (insulin-independent diabetes) is considered the best treatment option, because insulin-secreting beta-cells are assumed to be under optimal control of glucose homeostasis. Treatment of defective islet cell function with insulin is nowadays common practice for decades, but does not prevent acute complications like hypoglycemia unawareness in poorly controlled patients, and vision, cardiovascular, and renal complications in the long-term of diabetes management. Pancreas transplantation with/without a kidney transplant has been pioneered in the 1970s, and first pancreatic islet transplants date from the 1980s. Despite the large patient population (in the US there are presently about 30 million patients with diabetes, out of which about 2 million patients with type 1 diabetes), pancreas transplantation has not evolved to a widely applied procedure. The outcome is affected by the quality of donor organ, complications of immunosuppression, and the risk-benefit balance between disease control relative to insulin and complications of immunosuppression suggesting a narrow therapeutic index (relative benefit of therapy compared to conventional treatment). The same applies for islet transplantation (71-73). However, after the development of the so-called 'Edmonton protocol' in 2000 (74), which is a steroid-free regimen comprising rapamycin, FK-506 and an anti-IL2 receptor antibody, and which protocol turned out to be quite well tolerated, activities in islet cell transplantation have substantially increased. Today, phase III multicenter registration trials are in progress in the US under guidance of the National Institutes of Health, and the approval as a clinical procedure is on the horizon. The Edmonton protocol has also triggered islet cell xenotransplantation research, with the main rationale to compensate for the limited human donor supply.

Cell and tissue transplantation differs from solid organ transplantation as there is no blood vessel connection made between graft and recipient during surgery, and hence no involvement of donor endothelium lining the blood vessels. This condition appears to be quite beneficial for pig islet insulin-producing beta-cells, even more because such cells do not express Gal (75, 76). This observation reflects the much lower relative content of glycolipids in porcine pancreas when compared to other tissues like intestine (Diswall *et al.*, manuscript submitted). As a consequence, islets are not hyperacutely rejected upon conventional administration, which is injection in the portal vein followed by lodging in the liver (77). There is an inflammatory reaction called 'Instant Blood Mediated Inflammatory Reaction', which could result in loss of islets before they lodge in the liver, and which is apparently independent of xenoreactive antibodies (78). The subsequent rejection of pig islets in non-immunosuppressed monkeys is cell-mediated with influx of T cells and macrophages, without histologic signs of antibody-mediated rejection (77). Interestingly, material contaminating transplanted pig islets can be Gal-positive but is apparently unable to trigger an antibody-mediated rejection by naturally existing anti-Gal antibody, or by a boost in anti-Gal antibody synthesis. The microvasculature in transplanted islets expresses Gal at transplantation, but appears to be replaced by Gal-negative donor-derived endothelium later after transplantation.

Thus immunosuppression in islet cell transplantation has thus to focus mainly on cell-mediated rejection. Prevention of xenograft rejection needs a higher extent of rejection than that required in allotransplantation. This has been elegantly demonstrated in the comparison of monkey and pig donors in transplantation in diabetic cynomolgus monkeys: a regimen comprising a CD25 antibody, a rapamycin derivative and FTY-720 yielded long-term diabetes reversal and function of an isl*et al*lograft (79) but not that mediated by an islet xenograft (80). Recently, long-term diabetes reversal in diabetic monkeys has been achieved by the group of Larsen *et al.*, Atlanta GA, transplanting porcine neonatal islets and using a regimen including a CD25 antibody and CD154 antibody in induction, and maintenance immunosuppression with rapamycin and CTLA4-Ig (81). In a similar approach the group of Hering *et al.* (Minneapolis MN) achieved similar results by transplanting adult porcine islets and a regimen including a CD25 antibody in induction, and maintenance immunosuppression with a CD154 antibody, a rapamycin derivative, FTY-720 and leflunomide (80). In both studies there were no evident adverse effects of drugs documented. Based on these results, further refinement of the immunosuppressive regimen aiming on a clinically acceptable protocol is presently under investigation.

Islet cells, like other cell and tissue transplants, have the potential advantage that encapsulation allows cells to be shielded from rejection by the host's immune system. Encapsulation should be performed in such a way that nutrients and oxygen can reach the transplant, but also that pore sizes prohibit the access by components of the rejection reaction. Alginate is a component that is pursued at this moment. Promising data have been recorded in the pig-to-monkey transplant model, but long-term survival data have not yet been published (82). A case report claiming long-term (~10 years) survival of alginate-encapsulated neonatal porcine islets in a diabetic patient has recently been published (83). A number of companies have announced their intention to proceed with encapsulated porcine islets into clinical trials. The wisdom of proceeding to the clinic at this time, given the current best published results in preclinical studies, has recently been disputed (84).

The testes has been described as a so-called immuno-privileged site, where grafted cells can survive without being rejected (85). Related to this, Sertoli cells have been proposed as inducing immunomodulation with little or no requirement for immunosuppression in the transplant setting. This potential effect of Sertoli cells has been claimed in small laboratory animal models but not yet in large animal pig-to-nonhuman primate transplantation models. Clinical trials in children with diabetes have been performed using porcine Sertoli cells in combination with islet cells, inserted in an autologous collagen-covered device (86). This device consists of a stainless steel mesh tube, with a polytetrafluoroethylene rod in its interior, implanted in the upper anterior wall of the abdomen two months before injection of islet cells and Sertoli cells. Although success has been claimed (86, 87), various ethical and scientific aspects of this study have been challenged by the scientific community, with the recommendation that 'further preclinical research and development is needed before transplants of this nature are performed in humans' (88).

3.6. Liver (cell) transplantation

Although a pig-to-human liver transplant failed within 2 days (19, 89), pig livers have been used to support patients with acute hepatic failure as a bridge to transplant or recovery of their own liver function. As an alternative to liver transplantation, extracorporeal perfusion through an intact liver or through a device filled with hepatocytes has been used. Complete or partial reversal of coma was observed in 7 of 10 patients whose blood was perfused *ex vivo* through a normal pig liver (90). Overall experience in extracorporeal liver perfusion suggests physiologic efficacy similar to that achieved using human or baboon livers (91-92). Interestingly, there appears to be no equivalent of HAR in porcine liver subjected to perfusion with human blood (93), and humoral-mediated damage starts later after start of perfusion. Donor liver injury in these procedures is usually delayed and altered in character when livers from hDAF-transgenic or CD55/CD59-transgenic pigs are used (93-95).

As an alternative to whole liver, extracorporeal perfusion though devices filled with hepatocytes has been proposed (96-98). A number of instruments have been developed and are in clinical development (Arbios Systems, Inc.; and Excorp Medical, Inc.). Safety and efficacy has been shown in phase I-II clinical trials in patients with acute liver failure (99-102).

Besides extracorporeal perfusion, liver xenotransplantation has been explored in preclinical transplantation models. In a life-supporting pig-to-primate liver transplantation model using a liver from a hDAF-transgenic donor, survival up to 8 days has been reported (103). Although this survival is much less than that reported for other solid organs like heart and lung, this survival is remarkable in view of the many metabolic functions of the liver and species disparities in metabolic processes under guidance of liver function. Also, long-term survival, up to 8 months, has been noted for porcine hepatocytes after transplantation in cynomolgus monkeys under chronic immunosuppression (104). These data form a positive indication that liver (cell) xenotransplantation could be used as a bridge in patients with acute hepatic failure awaiting a human donor transplant.

Related to its metabolic function, the liver is the primary source of many proteins crucial to normal coagulation and biochemical functions, and most pig and human protein sequences differ substantially. This obstacle might obligate extensively re-engineering pigs to produce a broad array of human rather than pig proteins, which is a formidable task if necessary and if possible. Also, the fact that human erythrocytes are rapidly taken up by pig hepatic macrophages (105, 106) poses a potential complication to liver xenotransplantation besides those shared with other types of xenografts. A solution to this problem would greatly facilitate extracorporeal pig liver support or pig liver transplantation. Nonetheless, extracorporeal perfusion of a transgenic pig liver or a device containing porcine hepatocytes may be more efficacious than currently available treatments for patients with acute liver failure. Many patients on the liver transplant waiting list who nowadays die due to rapid deterioration of liver function could benefit if the liver functions well, and also might occasionally prevent the need for a liver transplant if the injured liver recovers (for instance, in the case of acetaminophen overdosing). For these reasons clinical trials of liver perfusion for acute hepatic failure seem justifiable.

3.7. Lung transplantation

Lung xenotransplantation is a rather unexplored field in experimental xenotransplantation, and has shown differences from other organs in rejection mechanisms. Animal models of lung transplantation are complicated in technical aspects, and only a few transplant studies have been conducted in nonhuman primate recipients. In the orthotopic model, swine lung can provide respiratory support for a short time period exceeding the normally measured time limit for hyperacute rejection (107). Transgenic expression of hDAF and CD59 positively effects lung function in this model (108), and also a beneficial effect for GalT-KO donors has been recorded (108). In contrast to the transplantation of other solid organs, depletion of naturally existing anti-porcine antibodies is not generally sufficient to prolong survival of lung grafts beyond 24 hours, although it is also possible that the procedures required for antibody removal in animal models could be detrimental to lung xenograft function (109).

More detailed studies on rejection mechanisms have been conducted in the model of *ex vivo* perfusion of swine lungs with human blood (110). Although antibody binding and complement activation are important, preventing both of these processes is not sufficient to protect pig lung from acute injury within minutes of exposure to human or baboon blood. This conclusion was reached using antibody depletion from blood, using lungs from hDAF-transgenic or CD59-transgenic pigs or GalT-KO miniature swine, and using complement inhibitors (40, 111-115). Considerable work has been conducted to reveal the mechanisms by which thrombin and platelets interact with resident macrophages in the lung (macrophages within blood vessels, so-called pulmonary intravascular macrophages, and/or in the pulmonary interstitial areas). Inhibitors of platelet coagulation protein receptors (GPIB, GPIIBIIIA) (116), direct thrombin inhibition (117) and depletion of pulmonary intravascular macrophages (118-119) each had a protective effect on lung function. Thus, innate immune mechanisms involving platelets and macrophages and coagulation play a major role in hyperacute lung rejection. The current working hypothesis is that pulmonary intravascular macrophages trigger local coagulation pathway activation when activated by either antibody, complement, or by local coagulation and thrombin formation. The resulting expression of tissue factor and subsequent activation of coagulation pathway products, along with binding of human platelets to even quiescent pig endothelium, amplifies platelet activation, aggregation and clot propagation. Because clot dissolving systems function poorly across the species disparity between human and pigs (53, 120) the entire coagulation cascade is inappropriately amplified, causing local thrombosis and inflammation. Efficient strategies to block these intersecting injurious feedback loops must be developed before clinical lung xenografting can be envisioned.

4. SAFETY

The transplantion of viable porcine tissue to a human patient has the intrinsic risk of transmission of infectious pathogens. Thus, there are strict guidances by regulatory authorities to be followed in release of xenogeneic life material for administration to humans, and monitoring of patients after transplantation. At the regulatory level in the US, there is the *Guide for the care and use of laboratory animals* (121) describing items like animal environment and medical care of animals, the *PHS Guidelines on infectious disease issues in xenotransplantation* (122) addressing amongst others items related to animals sources and clinical protocols, and the *Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (123) presenting a comprehensive approach to the safety of viable materials from animals for clinical use in humans. In addition there are publications in literature with recommendations by experts in the field on microbial safety specification of potential xenotransplantation products (124, 125). Regarding preclinical studies it is anticipated that the assessment of transmission of infectious pathogens in

preclinical transplantation not only is an important aspect in the approval process of clinical studies by regulatory authorities, but also contributes to the design of the monitoring protocol in patients.

4.1. Biosecure barrier pig production

Generally, source pigs for clinical xenotransplantation are reared and maintained in barrier units, under strict microbial control to avoid entry of pathogens, via amongst others air, water, food, and contact with animal care takers. Infectious agents in conventionally reared pigs that have zoonotic potential are among the first that have to be excluded, i.e. *Erysipelotrix rhusiopathiae*, *Leptospira sp*, *Brucella sp*, *Salmonella sp*, *Streptococcus suis* and Influenza.

Besides bacteria, fungi and parasites, a large list of viruses has been recommended to be excluded from the source pig herds, based on their ability of known zoonotic potential, ability to replicate in human cells, oncogenic potential, or the possible detrimental effects on the herd in case of a breakdown in biosecurity (124-126). The removal of viral pathogens is feasible by advanced reproduction technologies including embryo transfer technology, cesarian derivation, and/or early weaning. This has been shown for cytomegalovirus (127), circoviruses and lymphotropic gamma-herpesviruses (128). Early weaning, albeit being the most simple approach, is not always successful (129).

A number of these pathogens have been excluded from pig donors in preclinical transplantation studies in nonhuman primates. Some research groups utilized source pigs from barrier units and have suggested that the pathogen-free status of donors positively affects the efficacy of transplantation (41). This particularly concerns viruses like porcine cytomegalovirus, that upon reactivation can induce pathology in grafted tissue, but appears not to show pig-to-nonhuman primate transmission (130). Cytomalovirus exclusion is particularly relevant in the setting of nonhuman primates, as antiviral agents like ganciclovir appear less efficacious in nonhuman primates than in man (131).

Besides the microbial status of the source pigs, there is a potential safety risk of the processing of procured tissue to cellular products, in particular in case of using advanced isolation and cell culture technologies. This applies to e.g. pancreatic islet cells and hepatocytes, and preparation of extracorporeal perfusion devices.

4.2. Porcine endogenous retrovirus

Besides exogenous pathogens, there is an intrinsic risk of endogenous pathogens, the most relevant one being porcine endogenous retrovirus (PERV) (132, 133). The observation that PERV can infect human cells upon coculture *in vitro* with porcine cells has raised concern about the safety of any xenotransplantation procedure. This *in vitro* transmission to human cells applies to two of three subgroups identified thus far, PERV-A and PERV-B; the third established subgroup, PERV-C, only shows transmission to porcine cells. Reproducible transmission at detectable levels is only demonstrated using sensitive molecular biologic technology, and only in a few human cell lines, in particular the human renal epithelial 293 cell line.

Nowadays, about one decade after the first concerns were expressed, extensive research has resulted in the situation that PERV transmission is no longer considered a major obstacle for clinical xenotransplantation, and that the possible risk of PERV transmission and subsequent induction of disease upon xenotransplantation appears manageable. This conclusion is based on the fact that there is no disease condition in pigs associated with PERV (even not in heavily immunosuppressed animals), and that in various models using small and large animal species no transmission of PERV could be demonstrated (134, 135). This has been demonstrated not only in pig-to-nonhuman primate transplantation models (136, 137; Long *et al.*, unpublished observations), but also in specifically designed infectivity studies in immunosuppressed nonhuman primates (138). Also in animal models in which a chimeric state of porcine and human cells/tissue was generated *in vivo*, no pig-to-human cross-species PERV transmission has been reported (139). However, PERV transmission to human cells in a human-*scid* mouse model upon transplantation of porcine islets has been claimed (140-142), but this could subsequently be related to pseudotyping due to xenotropic murine leukemia virus (143, 144). Also in a number of clinical trials using viable porcine material, no evidence of pig-to-human PERV transmission has been demonstrated (94, 145-148), despite the fact that genomic porcine DNA could be detected as an indicator of chimerism, up to 8 years after subjecting patients to extracorporeal porcine spleen perfusion (148).

Most research on *in vitro* PERV transmission to human cells has been performed using material from the Massachusetts General Hospital miniature swine herd. Blood cells from these animals transmit PERV *in vitro*, both to the porcine ST-IOWA cell line and to the human 293 cells. Recently, the molecular analysis of PERV in human 293 cells has shown that the virus is a recombinant of the PERV-A and PERV-C subgroups (149, 150). Interestingly, this recombinant does not occur in the germline DNA of miniature swine. This observation indicates that infectious PERV in these animals might be an exogenous virus and not an endogenous virus. This is a very important observation, as it essentially opens the possibility that PERV could be eliminated from the herd by advanced reproduction technologies mentioned above. This observation also points to the fact that PERV-C, albeit itself unable to enter human cells, is important in risk assessment, as it might represent the infectious driver in pig-to-human PERV transmission. This has been emphasized in a recent study showing that small changes in the PERC-C envelope can result in *in vitro* binding to human cells (151).

Another risk estimate is the possibility that human endogenous retroviral genomic segments could recombine with PERV genomic segments resulting in new

viruses with increased infectivity potential to human cells. This issue can only be studied *in vitro*, in human cells that are susceptible to PERV transmission, i.e. the above-mentioned 293 cells. These cells bear human endogenous retroviral genomic segments. But, after PERV transmission and chronic high level virus production there was no evidence of such recombinations detected (152): this observation is considered an additional safety benefit.

In conclusion, knowledge gathered after the first observation that PERV can transmit to human cells in an *in vitro* cell culture experiment has led to the conclusion that the potential risk of cross-species PERV transmission to human recipients of a xenograft appears unlikely and manageable. It has therefore been suggested that xenotransplantation can progress toward a clinical procedure in patients when clinical trials are performed in carefully designed protocols with appropriate monitoring of cross-species infectious pathogen transmission.

5. CONCLUSIONS AND PERSPECTIVES

Research in all aspects of xenotransplantation has shown tremendous progress during the last decade. At first, hyperacute rejection of vascularized solid organs xenografts appears manageable thanks to the development of genetically modified pigs, either pigs transgenic for complement regulatory proteins or GalT-KO pigs. Essentially, the use of Gal-containing polymers has become obsolete after the introduction of GalT-KO pigs, and naturally-existing anti-Gal antibodies do not play a role any more. It is anticipated that GalT-KO pigs will be the platform of further derivations in genetic modification of donor animals focusing at first on complement regulation or modulation of the coagulation cascade.

Second, in models that are most close to the human situation, namely transplantation in nonhuman primates, chronic immunosuppression using low-molecular weight drugs (such as cyclosporin, FK-506, rapamycin and rapamycin derivatives, mycophenolate derivatives, FTY-720) has shown some success in achieving functional survival, but biologicals affecting co-stimulatory pathways have shown to be quite effective and required in combination treatment. This has been shown in models of heart and kidney transplantation, but even more in models of porcine islet cell transplantation. First data on diabetes reversal and functional survival up to 6 months by porcine islets transplantation in diabetic monkeys form a solid basis for continued investigations on immunosuppression protocols that are both effective and well tolerated. Co-stimulatory blockade of the CD40-CD154 pathway proved to be quite effective using anti-CD154 antibodies, but the development of such antibodies has been discontinued because of thrombo-embolic complications. Thus, there is a need to implement new biologicals, for instance CTA4Ig and others interfering in the CD28/CD86 costimulatory pathway. Not only should tailored immunosuppression protocol focus on an increased window between efficacy and tolerability of the regimen, but also the risk-benefit balance between diabetes control by insulin and safety of immunosuppression in islet cell replacement strategies. Interestingly, costimulatory blockade can have immunomodulatory aspects resulting in a reduced need for immunosuppressives. Although xeno-tolerance appears to be more difficult to achieve than allo-tolerance, this aspect of immune modulation should get a special place in the agenda of future studies. Deletional tolerance is evidently part of such studies building on the promising first data on graft survival and xenogeneic unresponsiveness *in vitro,* for instance in the kidney transplantation model using GalT-KO miniature swine.

Third, lessons from solid organ transplantation are that disparities in factors affecting coagulation should be addressed besides modulation of immune reactivity. This conclusion comes from the data in porcine kidney and heart transplantation into nonhuman primates, and from the lung perfusion model using human blood. It remains to be established whether this aspect can be adequately addressed by pharmacologically active drugs or need the development of donors transgenic for human anti-coagulant molecules on the endothelial cell surface. Considering the differences in pathways between kidney, heart and lung, different approaches and solutions to this complication might be required.

Fourth, cell/tissue transplantation has progressed further than solid organ transplantation of vascularized grafts. This might be related to the incomplete understanding of reactions at the endothelial cell surface upon contact with blood from the xenogeneic host, e.g., initiation of coagulation pathways. On the other hand the absent or low expression of Gal on respective cells has been a facilitating factor, because this enabled to work with wild-type pigs and not genetically modified animals. Further refinement of immunosuppressive regimens to a clinically acceptable protocol is warranted for porcine islets transplantation: on the other hand more preclinical data on e.g. long-term survival appear to be needed for encapsulated islets. Regarding the application of porcine livers, extracorporeal perfusion through devices containing hepatocytes have already been in first clinical trials, and continuing this approach for patients with acute liver failure is justifiable.

Finally, substantial progress has been made in safety aspects, in particular pig-to-human pathogen transmission. Using source animals from biosecure barrier facilities, most pathogens including porcine viruses are already eliminated, so that the main issue of concern regards porcine endogenous retrovirus. It appears that this concern is manageable. This conclusion is based on studies on *in vitro* virus transmission to human cells, in particular the characteristics of the virus recovered from human cells after *in vitro* transmission, combined with the inability to show transmission *in vivo* either in patients exposed to living porcine tissue or in experimental animal models.

Challenges in moving forward in clinical studies is not only based on scientific developments, but by its very nature also on ethical and regulatory aspects. It is logical that safety and ethics of clinical xenotransplantation have received special attention, both by (global) scientific

societies, regulatory authorities and international organizations. In many countries regulatory advisory committees have been established, which is reviewed elsewhere (153, 154). Recent activities worth mentioning are the activities of a Working Party of the Council of Europe (155) and in the US the activities of the Secretary's Advisory Committee on Xenotransplantation (discontinued in June 2005) (156, 157). The Ethics Committee of the International Xenotransplantation Association (IXA) has developed a position paper (158). In 2004, the 57th World Health Assembly of the World Health Organization (WHO) has published the Resolution WHA57.18, urging Member States 'to allow xenogeneic transplantation only when effective national regulatory control and surveillance mechanisms overseen by national health authorities are in place' (159), which was supported by the IXA (160) being in accord with their recommendation for international cooperation on xenotransplantation (161). The establishment of an Inventory of Human Xenotransplantation Practices, a collaborative effort of the University Hospital Geneva, IXA and WHO, fits with this desire for international cooperation and exchange of information. The information in this inventory 'is intended only to gather information and not for legal or judgemental purposes' (162), 'ultimately be used to inform national health authorities, health care staff, and the public, with the objective of encouraging good practices, with internationally harmonized guidelines and regulation of xenotransplantation' (163). With this safety, ethical and international regulatory framework in place, clinical trials in xenotransplantation can proceed using carefully designed protocols that include appropriate monitoring of patients for cross-species transmission of infectious microorganisms.

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Abbreviations: DAF: decay-accelerating factor, Gal: galactose-alpha(1-3)galactose, GalT: galactosyl transferase, GalT-KO: GalT knock-out, HAR: hyperacute rejection, IXA: International Xenotransplantation Association, MCP: membrane cofactor protein, MHC: major histocompatibility complex, PERV: porcine endogenous retrovirus, WHO: World Health Organization

Key Words: Antibody-Mediated Rejection, Anticoagulants, Anti-Gal Antibody, Anti-Pig Antibody, Baboon, Cell/Tissue Transplantation, Cellular Rejection, Chimerism, Chronic Rejection, Cloning, Coagulation, Complement, Complement inhibitors, Complement

Regulatory Protein, Costimulatory Blockade, Cross-Species Pathogen Transmission, Cynomolgus Monkey, Cytomegalovirus, Disseminated Intravascular Coagulopathy, Encapsulation, Endothelial Cells, Endothelial Cell Activation, Ethics, Extracorporeal Perfusion, Extracorporeal Liver Perfusion, Ex Vivo Perfusion, Ex Vivo Lung Perfusion, Galactose-alpha(1-3)Galactose, Galactosyl Transferase, GalT Knock-out, Gene Targeting, Genetic Modification, Heart Transplantation, Hepatocyte Transplantation, Hyperacute Rejection, Innate Immunity, Immunosuppression, Immune Tolerance, Islet Transplantation, Islet Cell Encapsulation, Kidney Transplantation, Liver Assist Device, Liver Transplantation, Lung Transplantation, Lymphotropic Gamma-Herpesvirus, Macrophage, Miniature Swine, Naturally Existing Antibody, Nonhuman Primates, Pig, Porcine, Porcine Endogenous Retrovirus, Pulmonary Intravascular Macrophages, Regulatory Guidance, Rhesus Monkey, Sertoli Cells, Solid Organ Transplantation, Species Disparity, Swine, Thrombosis, Transgenesis, Xenosis, Xeno-transplantation, Xeno-Tolerance, Zoonosis

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