ADVANCES IN HEPATOLOGY

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Insights Into Pig Liver Xenotransplantation

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G&H Currently, how large is the shortage of human livers for transplantation in the United States?

RM There are currently more than 11,000 patients on the waiting list for a liver transplant in the United States. Last year, more than 2000 patients were removed from the list prior to transplantation because of clinical deterioration or death.

Most importantly, these numbers only reflect the patients who were listed for transplantation. Many patients are too sick to be added to the list. There are also patients with hepatocellular carcinoma beyond the Milan criteria and patients who have not met the sobriety requirements for listing. In total, chronic liver disease is responsible for approximately 35,000 deaths per year in the United States. If unlimited organs were available, most of these deaths could be prevented.

G&H Why are pigs being considered for xenotransplantation?

RM The main reason for xenotransplantation, or transplantation between 2 species, is the shortage of organs for transplant. Even though nonhuman primates are genetically more similar to humans, it would be impossible to fulfill demand with those animals.

Pigs have a similar size, anatomy, and physiology to humans. Also, the availability of pigs is much greater than nonhuman primates, as pigs reach maturity and human size faster (at 6 months vs 15 years for a maximum size of 4 ft 11 in) and have a larger number of offspring per litter (10 vs 1). In addition, pigs have a much lower risk of zoonosis transmission and are most likely associated with fewer cultural objections for use in transplantation. All of these factors, in combination with the advancement of genetic engineering, make pigs the species of choice for xenotransplantation.

G&H What are the genetic barriers of pig-to-human liver xenotransplantation?

RM There are 3 main groups of genetic barriers to xenotransplantation. The first is the presence of pre-formed antipig antibodies in humans, leading to hyperacute rejection and graft demise. The genes mainly responsible—*GGTA1*, *CMAH*, and *B4GalNT2*—can be erased with the aid of clustered regularly interspaced short palindromic repeats (CRISPRs). Additional protection can be granted by adding human complement regulatory proteins such as CD55, CD46, and CD59 to the pig graft.

The second barrier is the incompatibility of pig proteins and enzymes with the human system. Currently, the main result of this incompatibility is thrombotic microangiopathy, which is primarily caused by the inactivity of pig thrombomodulin and hyperfunction of pig von Willebrand factor, both of which lead to thrombosis of the graft. By adding human versions of those genes, the issue may be corrected.

The last barrier is delayed xenograft rejection, which is similar to cellular rejection in allotransplantation, but is much stronger and requires additional immunosuppressive agents, some of which are not approved by the US Food and Drug Administration (FDA) for human transplantation. Manipulation of the swine leukocyte antigen (SLA) class I and SLA class II may be beneficial; however,

most researchers believe that this will come in a later phase after initial success of pig-to-human xenotransplantation.

Other gene manipulations to prevent innate immune system responses, such as adding human CD47 and HLA-E, can also help improve outcomes. Specifically in livers, massive thrombocytopenia can be caused by internalization of platelets on liver sinusoidal endothelial cells. This can be minimized by knocking out, or erasing, *ASGR1*.

G&H How can CRISPRs be used for genetic modifications?

RM CRISPRs can be used to knock out genes or generate targeted gene insertions to create transgenic pigs. Essentially, CRISPRs are enzymes that can find and cut predetermined sequences of DNA based on a guide RNA. Scientists identify the genes they want to erase and construct a guide RNA that matches the targeted sequence. Multiple guide RNAs can be constructed per experiment, meaning that multiple genes can be disrupted at the same time. The CRISPR enzymes and guide RNAs are introduced into cells (or embryos) to cut the DNA at the chosen locations.

When the DNA is cut, 2 things can happen. Either the cell will correct itself perfectly using the other allele as a mold and no genetic modification will occur, or the cell will correct itself with random additions or subtractions of nucleotides. In the second case, the gene reading may be completely impaired, and the gene will be disrupted, creating the knockout.

To create a targeted transgene insertion, a DNA fragment that contains both the transgene of interest and a region identical to the targeted location can be used in addition to the CRISPR and guide RNA. When the cut is made, the complementary sequence of the extra DNA fragment may be used as the mold to correct the DNA cut. If that happens, the transgene will be integrated at the desired location as part of the cell correction.

G&H How should pigs be selected for genetic manipulation?

RM Pig size, blood group, and the presence of endogenous infections need to be considered when selecting pigs for posterior genetic manipulation. Miniature pigs are considered ideal to better match the size of human organs. Pigs with the universal blood group O should be selected. Also, all pigs have endogenous retroviruses incorporated in their DNA. There are 3 types: PERV A, PERV B, and PERV C. All pigs have PERV A and PERV B in different amounts, but not all pigs have PERV C. Ideally, pigs that have lower amounts of PERV A and PERV B and lack PERV C should be selected.

G&H What guidelines or regulations are available regarding xenotransplantation or the modification of pig genes?

RM The FDA has nonbinding recommendations on its website regarding the care of pigs for xenotransplantation. These recommendations involve the origins of the animals, housing in germ-free facilities, programs for the prevention of infections and screening for infectious agents, the importance of keeping animal feeding records for at least 2 generations prior to utilization in clinical grade studies, and quarantining animals for at least 3 weeks prior to harvesting cells, tissues, or organs for xenotransplantation. In addition, animal tissues and samples should be stored for at least 50 years to trace back any sources of infections.

Regarding genetic modifications, the FDA recommends that potency be tested to reflect the desired biologic activity. This is very important because there is currently wide variability on transgene expression profiles in different cells of the same animal, which could potentially be an issue for FDA approval.

G&H Thus far, what appears to be the biggest barrier to transplanting pig livers into humans?

RM With the resolution of the first major barrier (hyperacute rejection), the current roadblock is severe thrombocytopenia caused by capture of most of the human's platelets by pig Kupffer cells and liver sinusoidal endothelial cells. After the resolution of this barrier, other barriers may become apparent.

G&H What research has been conducted on xenotransplantation of pig livers to other animals (eg, nonhuman primates)?

RM There have been several preclinical trials of pig-to-nonhuman primate orthotopic liver xenotransplantation. Survival ranged from 3 days with liver xenotransplantation from unmodified pigs in the 1960s to 8 days with the first CD55 transgenic pig in 2000 to 4 to 7 days with the first *GGTA1*-knockout pig in 2010.

Survival started improving when genetic modifications were supplemented with measures to prevent thrombotic microangiopathy and thrombocytopenia with continuous infusion of human prothrombin concentrate complex and co-stimulation blockade, initially with belatacept (survival of 25 days) and later with anti-CD40 (survival of 29 days).

G&H What is the current status of pig-to-human liver xenotransplantation?

RM Because the liver has more than 500 vital functions, xenotransplantation of this organ is very complex, even more than xenotransplantation of the kidneys or heart. With this complexity and limited survival in nonhuman primate trials, pig-to-human research is behind.

Even though there is much to be done before orthotopic pig-to-human xenotransplantation can even be considered, bridging patients to allotransplantation with extracorporeal pig liver perfusion is close to reality. In 1999, 2 patients who underwent extracorporeal perfusion with transgenic pig livers (with human complement regulatory proteins CD55 and CD59) were successfully bridged to allotransplantation. That was a very limited trial, but with the current knowledge in immunosuppression in xenotransplantation, pharmacologic therapies to prevent thrombotic microangiopathy, and modern genetically modified pigs, additional similar trials can soon become reality.

G&H What are the most important next steps in research in this area?

RM The next steps are to resolve severe thrombocytopenia as well as thrombotic microangiopathy. These issues have been minimized with additional genetic modifications; however, no pig-to-nonhuman primate liver xenotransplantation trials have been performed to evaluate the effectiveness of these modifications in vivo. Our group at Miami Transplant Institute/University of Miami, in collaboration with the Schiff Center for Liver Diseases, is researching more reliable methods of transgene expression to overcome these important issues.

G&H Are there any misconceptions in the gastroenterology/hepatology community about this topic?

RM Yes, there is a misconception that all transgenic pigs with modifications of the same gene are equivalent. Different pigs from the same source have a wide range of gene expression patterns, some with minimal patchy expression of the desired transgene. There have been reports of transgenic pigs having gene expression ranging from 8% to 96% positive cells, and all were considered to have positive transgene expression. There was even a case of a kidney transplanted into a nonhuman primate in which the gene of interest was later found not to be expressed at all on that particular organ. Documenting transgene expression with immunohistochemistry for each organ and each transgene should become standard practice for xenotransplantation research; however, it is rarely performed.

Concern for unreliable transgene expression is important enough that some experts believe in generating multiple knockout animals without transgene expression for clinical trials. The FDA's recommendations for good manufacturing practices require determining the potency of the xenotransplant product, which has been shown to be very difficult at the present time. This is because there are variations of expression from animal to animal (even with identical genotypes), and even a decrease of expression during the animal's life span.

Because of the importance of this issue, our group is currently investigating new methods of achieving consistent and reliable transgene expression in all organs.

Disclosures

Dr Miyashiro Nunes dos Santos has no relevant conflicts of interest to disclose.

Suggested Reading

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