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Detecting chimerism in transplantation: looking for needles in a haystack

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ABSTRACT

Endothelial chimerism in transplanted organs is a fascinating phenomenon, indicative of a mechanism by which progenitor recipient cells replace the donor endothelium. It has been hypothesized that this replacement could lead to a decrease in alloreactivity and thus would positively influence graft outcome. However, recent studies have shown that the amount of recipient-derived endothelial cells found in donor organs is relatively small. What effect on graft survival can we expect from this low number of chimeric cells? There are several hypotheses that address this question, but distinguishing the true effect of donor endothelial replacement on outcome from other factors affecting graft survival is difficult. Furthermore, "contamination" of chimeric cells from sources other than the recipient would have to be excluded before the effect of donor endothelial replacement by recipient cells can be accurately assessed. Pregnancies and blood transfusions are the other sources that may induce chimerism. Most of the techniques currently used to detect chimeric cells in donor organs are not specific enough to distinguish chimeric cells that may have been present in the graft before transplantation and recipient-derived chimeric cells that replace the endothelium after transplantation. Also the sensitivity of these techniques may be questioned: Do we really detect all chimeric cells that are present? This review will elaborate on these questions and discuss future perspectives of research into chimerism.

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INTRODUCTION

In the early 1960s, Medawar hypothesized that cells in a transplanted organ could be replaced by cells of the recipient and that this replacement could lead to the induction of graft tolerance.[1] Chimerism —i.e., the presence of cells from one individual in another individual— has since then grown into a frequently discussed phenomenon in relation to transplantation. Important issues to address are the mechanisms of chimerism induction, the possible effect of pre-transplant chimerism in the organ graft, and the best way to detect the chimeric cells and determine their possible sources. It is likely that damage —caused by vascular rejection, for instance— could induce chimerism in the graft because damaged endothelial cells may be replaced by recipient progenitor cells.[2,3] Once chimerism is present, it may reduce alloreactivity and make the organ less prone to further rejection episodes, thereby improving graft survival.[4] To investigate whether chimerism (defined as the presence of recipient-derived cells in a donor organ) is indeed beneficial for graft survival, it is important to be sure that the chimeric cells are uniquely derived from the recipient. Recently it has been demonstrated, however, that chimerism may already be present in the graft before transplantation.[5] Most of the methods used today to detect chimeric cells in transplanted organs focus on mismatches between donor and recipient; they are not specific enough to exclude the possibility that a portion of the chimeric cells originated from a source other than the recipient. In this review, we discuss findings and methodological pitfalls that influence investigations on chimerism in transplantation.

CHIMERISM IN TRANSPLANTATION

Various studies have been performed to unravel the role of chimerism in solid organ transplantation, especially in the kidney. Lagaaij et al.[2] demonstrated the presence of recipient-derived endothelial cells in 38 kidney allografts. Seventy-six percent of the specimens showed chimeric endothelial cells. These cells were found significantly more often in grafts of patients who had experienced rejection episodes. In six out of seven grafts (86%) with vascular rejection, extensive chimerism was present, defined as

more than 33% of the endothelial cells being of recipient origin. In contrast, extensive chimerism was only present in one out of six grafts (17%) with interstitial rejection. These results suggest a mechanism by which damaged endothelial cells are replaced by circulating recipient progenitor cells.

Evidence for such a mechanism is found in the work of Woywordt et al.[6] and Popa et al.[3]. The first group showed that damaged endothelial cells are, in fact, shed during vascular rejection. They studied 129 blood samples and biopsies of patients who had received a renal transplant and showed that patients with acute vascular rejection in the biopsy had the highest number of circulating endothelial cells. However, they did not further identify whether the circulating endothelial cells were of donor or recipient origin. Thus, how many of the circulating endothelial cells were damaged endothelial cells of the donor organ and how many were progenitor endothelial cells of recipient origin remained in question. Popa et al.[3] did distinguish circulating endothelial cells of donor origin from those of recipient origin by typing their HLA-DRB alleles using single-cell nested polymerase chain reaction (PCR); they found that in nine patients with a kidney allograft, the majority (71%) of the total number of circulating endothelial cells were of donor origin. In this experiment, sera of patients with a history of acute rejection were examined. The amount of circulating endothelial cells was associated with periods of acute rejection; however, the time points at which the samples were tested varied from 0-141 days after the rejection episode. The fact that in some patients, numbers of circulating endothelial cell are still relatively high even 100-plus days after rejection may indicate a role for endothelial maintenance in addition to repair in the induction of endothelial chimerism.

From the older literature the argument emerges that chimerism would be beneficial.[1] It is difficult to find evidence in favor of or against this hypothesis because many factors influence graft outcome (e.g., age, sex, number of HLA mismatches, cold ischemia time, and many others).[7] Therefore, the effect of chimerism on graft outcome can only be studied within the large spectrum of factors that together determine how long the graft will survive. To our knowledge, only two studies have investigated the possible relationship between the occurrence of chimerism and graft outcome in humansand

both concluded that the frequency and the amount of chimerism bore no relationship to outcome.[8,9] These results, as mentioned above, may have been influenced by the variability of factors known to influence graft outcome in the studied patient groups. Assuming that chimerism is the result of damage to the endothelium, the factor that induced this damage first of all has its own effect on outcome. For instance, if endothelial chimerism follows vascular rejection, which is known to be negatively associated with graft outcome, the effect of chimerism alone would first have to overrule the negative effect of vascular rejection to be considered a positive predictor for graft outcome. Timing may play an important role in these effects. For instance, there is evidence that vascular rejection occurring soon after transplantation could be beneficial for graft survival,[4] possibly indicating that replacement of the donor endothelium soon after transplantation is favorable.

Not only can the endothelium of a renal graft be replaced by cells of recipient origin, but tubular epithelial cells in a renal graft also may become chimeric.[8] This finding parallels results of other studies on chimerism in transplanted organs: chimeric endothelium, duct epithelium, and hepatocytes were found in transplanted livers;[10-12] chimeric bronchial epithelium and type II pneumocytes were found in transplanted lungs;[13] and chimeric cardiomyocytes and smooth muscle cells were found in transplanted hearts.[14-18] Thus far, the importance of these various forms of chimerism for graft survival remains unknown.

The differences in the reported amounts of chimeric cells in solid organs after transplantation in various studies are remarkable; e.g., they range in heart allografts from no chimeric myocytes[14] to a low level of 0.04% chimeric myocytes[15] to a high level of 9% chimeric myocytes.[17] These different results may partly be caused by the various techniques used to detect the chimeric cells. We will further discuss these disparities below, but first, we will concentrate on the phenomenon of pre-transplant chimerism.

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Pre-transplant chimerism from various sources

To investigate whether transplantation-induced chimerism is beneficial for graft survival, the chimeric cells present in the organs used for these studies should be derived uniquely from the recipient. Recent studies demonstrated that chimerism may already be present in the graft before transplantation, and these chimeric cells may be derived from various sources, such as blood transfusions, or in the case of a female donor organ, from previous pregnancies.[5] In transplantation studies, the method most often used to detect chimeric cells in organ allografts is to perform in situ hybridization targeting the Y chromosome on organs transplanted from women into male recipients.

Blood transfusions could be a source for chimeric cells in donor organs,[19] although they contain an extremely low proportion of nucleated cells that would contain the Y chromosome. However, if only one or two of these nucleated cells were stem cells, proliferation and differentiation of these cells would be possible, resulting in pre-transplant chimerism of donor organs. We previously found no relationship between the number of chimeric cells in organs of healthy women and the number of blood transfusions they had received.[5] However, in a study by Lee et al.[19], multi-trauma patients were found to have chimeric cells in their circulation shortly after blood transfusions.

In pregnant women, fetal cell DNA and fetal cells are present in the circulation.[20,21] Fetal cells may remain in the maternal circulation up to 27 years after delivery.[22] Male fetal stem cells also have been detected in the bone marrow and rib sections of women who had carried a male fetus up to 45 years earlier.[23] One point of criticism of studies on chimerism in transplants has been that the detected male cells in transplanted organs might be derived from the sons of the female donors, and not from the male recipients. Indeed, we recently provided evidence of the presence of male chimeric cells in normal female organs (kidneys, hearts, and livers) that theoretically could have been used for transplantation.[5]

Y chromosome-positive cells can be detected in variable amounts in women carrying male fetuses[20,21] and in women who carried male fetuses in the past.[22,24] There are also

reports of women who have Y chromosome-positive cells but who state they have never carried a male fetus.[25,26] One hypothesis to explain this phenomenon is that these women may have carried a male embryo without knowing it, having perhaps lost the embryo early in pregnancy. How likely is this explanation? In the case of pregnancy with a male embryo, Y chromosome-positive cells are present in the maternal circulation as soon as 6 weeks after conception.[20] It is possible, therefore, that unrecognized pregnancies give rise to chimeric cells, and there is evidence that unrecognized pregnancies occur relatively frequently. By testing continuously for hCG levels in 221 women, Wilcox et al.[27] found that 22% of 198 pregnancies identified by an increase in hCG level ended before the pregnancy was detected clinically. The possibility of a very early abortion giving rise to circulating chimeric cells in the mother's circulation is further emphasized by the fact that pregnancy termination is in particular the event at which fetal cells enter the circulation. This fact was elegantly demonstrated by Bianchi et al.,[20] who linked pregnancy duration to the number of Y chromosome-positive cells in the maternal circulation in cases of induced abortions. In an earlier study, this group did not find an association between gestational age and the mean number of transfused fetal nucleated cell equivalents detected when the mother's blood was sampled during an ongoing pregnancy.[28] They suggested that the fetal-maternal hemorrhage that occurs as a result of pregnancy termination results in a sudden increase of chimeric cells in the maternal circulation.[20]

A critical note should be made regarding the "earliest" and "latest" time points at which fetus-derived cells may be present in the maternal circulation in relation to pregnancy, as identified in the Bianchi et al.[22] and O'Donoghue et al.[23] papers. Because these time points were determined by detection of Y chromosome-positive cells and not by detection of DNA specific to the fetus in question, it is possible in either case that these cells originated in yet another pregnancy. More specifically, it cannot be ruled out that the Y chromosome-positive cells detected at 6 weeks of pregnancy originated from a previous, possibly unrecognized, pregnancy; neither can it be ruled out that the Y chromosome-positive cells detected 45 years after delivery might have been derived from an unrecognized pregnancy that occurred later or earlier than the reported final pregnancy.

A possible consequence of chimeric cells being transferred from the fetus to the mother and vice versa is that during each subsequent pregnancy, chimeric cells from the previous pregnancy can be transferred to the new fetus. Thus, even newborns may already have chimeric cells in their circulation from their older siblings. In the case of a twin pregnancy, chimeric cell transfer from one sibling to the other is even more likely to occur. Van Dijk et al.[29] detected blood group chimerism in 32 of 415 (8%) twin pairs, and 12 of 57 (21%) triplet pairs.

As long as we lack evidence at the DNA-level that chimeric cells actually correspond to the genetic properties of the mother's offspring, the other possible sources of chimeric cells present in women cannot be ruled out. It may well be that what we have regarded until now as a homogeneous group of fetus-derived chimeric cells is actually a very heterogeneous group of cells derived from multiple pregnancies and possibly from other sources as well.

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METHODS

Measurable mismatch between donor and recipient is used to distinguish between cells of the recipient and cells of the donor, and this can be a mismatch of gender, HLA type, or blood group. Assessing individual differences at the DNA level is also possible, but so far, this approach has not been used extensively. As mentioned previously, the method most often used to detect chimeric cells in transplantation studies is in situ hybridization using Y chromosome-specific probes on grafts of sex-mismatched patients. Most authors perform fluorescence in situ hybridization (FISH) using α-satellite probes for the centromeric regions of the Y chromosome (DYZ3, Yp11.1-q11.1),[30,31] satellite III probes for the heterochromatic regions of the Y chromosomes (DYZ1, Yq12),[17,32] or an LSI probe for the Y chromosome SRY locus (SRY, Yp11.3).

A closer look at studies using FISH reveals remarkable differences in the sensitivity of the Y chromosome-specific probes. Not all nucleated male cells will give a Y chromosome-positive FISH signal because the histological sections are 4-6 μm thick, and the Y

chromosome will not be present in all nuclear fragments. Indeed, looking at the results for FISH targeting the Y chromosome and performed on male control tissue, a striking range in percentages of nuclei positive for the Y chromosome occurs (see Table 1), varying from 28.5% to >95%.[14,16-18,31,33-36] This range makes it difficult to compare the results of the different studies. Some authors used a correction factor to estimate the likely proportion of Y chromosome-positive cells in the female grafts, based on the results of the male control tissue.[8,15,17,31,34] Others only include slides if a specific percentage of nuclei (e.g., 75%) contained fluorescent signals, an approach that could lead to bias.[25,37]

Table 1. Results for fluorescent in situ hybridization (FISH) targeting the Y chromosome on male control tissue in different studies

Study	Percentage of male control tissue positive for the Y chromosome	Type of cells examined	DNA probe used
Thiele et al.,[18] 2004	28.5	cardiomyocytes	DYZ (not further specified)
Poulsom et al.,[34] 2001	34	cortical tubular cells	not specified
Glaser et al.,[14] 2002	34.7	heart nuclei	DYZ1
Quaini et al.,[17] 2002	44	cardiomyocytes	DYZ1
Ng et al.,[31] 2003	52.2	liver nuclei	DYZ3
Müller et al.,[16] 2002	66	cardiomyocytes	Y3.4
Stevens et al.,[36] 2004	86	liver nuclei	DYS1
Khosrotehrani et al.,[33] 2005	>90	male control tissue (probably skin)	not specified
Rubbia-Brandt et al.,[35] 1999	>95	parenchymal and non-parenchymal liver cells	not specified

Slide thickness was not specified in the Quaini, Khosrotehrani, or Rubbia-Brandt papers. All other slides were reported to be 4–6 μm thick. There was no association between the thickness of the paraffin embedded slides and the percentage of Y chromosome-positive cells in male control tissue. Different DNA probes targeting the Y chromosome were used.

For several of our own studies, we have used digoxigenin (DIG) in situ hybridization targeting the Y chromosome. The DIG-labeled probes were visualized with PO-labeled anti-DIG and developed with NovaRed staining.[5,38] The advantage of this technique is that the strength of the positive signal (as denoted by dots) can be fairly easily

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established on the basis of color, size, and the location in the nucleus, as well as by a histological evaluation of the type of cells. The histomorphology of the tissue also can be evaluated. Moreover, the NovaRed read-out technique has the advantage of long-term preservation.

Chimerism in transplanted organs can also be studied without a sex mismatch, but with a mismatch in HLA or blood group antigens, as has been performed using immunohistochemistry for HLA class I antigens[2,11,39] and for ABO blood group antigens.[2] It is important to study the presence of chimerism in various mismatches in order to exclude selection bias. It is generally assumed that the results obtained in the study group with the selected mismatch are representative of all other mismatches. For instance, the results obtained in studies with male recipients of female grafts are considered representative of male recipients of male grafts, female recipients of female grafts, and female recipients of male grafts. However, a recent study on chimerism after renal transplantation in blood group-mismatched donor and recipients found that female recipients induce chimerism in their grafts significantly more extensively and earlier than male recipients.[9] This finding shows that certain selected mismatches may exhibit particular patterns of chimerism not shared by other mismatches, and these differences can only be investigated by using various methods to detect the chimeric cells.

PCR is another method for detecting chimeric cells. Nested PCR may be preceded by microdissection to select a specific cell type. As with other approaches, most authors search for Y chromosome-positive cells with this method,[5,39] although other markers have been used, such as SE33[8,13] or HLA-DR alleles.[39,40] Extreme caution is required to avoid and detect possible PCR contamination resulting from the known risk of PCR product carryover and false-positive amplification. Possible precautionary measures are to have the work performed by female technicians only (in case of Y chromosome-specific PCR), to carry out preamplification steps in a separate room under a laminar flow hood, and to include several negative control samples (amplification mixture without DNA) in each experiment.[41]

Conclusion

The research field of chimerism in transplantation is developing rapidly. Many studies, both clinical and experimental, are being conducted to investigate the effect of chimerism in allografts and its significance for graft outcome. It seems that chimeric cells in transplanted organs are not as extensively present as was assumed many years ago when Medawar suggested chimerism might explain graft tolerance.[1] However, thorough investigation is required to determine whether chimerism in transplantation reflects an experienced rejection episode, is a tool for the recipient to make the graft more "self" and increase graft tolerance, or is a combination of both. Various difficulties arise, however, in studies dealing with this subject. The effect of chimeric cells derived from pregnancies or blood transfusions may influence the results, especially because of the small amount of chimeric cells found. We must develop new techniques with higher specificity for determining the source of the chimeric cells detected and higher sensitivity to ensure that all chimeric cells present are detected. Only then can we determine the therapeutic measures for inducing chimerism that may be the key to improving graft survival.[42] At this point, looking for the few chimeric cells of recipient origin that can influence graft outcome under certain conditions is similar to looking for needles in a haystack, although the result may ultimately be rewarding.

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