

Biomarker and surrogate development in vascularised composite allograft transplantation:

Current progress and future challenges

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The idea for this manuscript originated from a textbook chapter, written by the authors, entitled *“Biomarkers and surrogates of acute rejection in vascularized composite allograft transplantation”* (Reconstructive Transplantation and Regenerative Medicine) due for publication in 2020. However, this is an original article and shares only 3 % similarity when analysed using Turnitin™ software.

ABSTRACT

Vascularised composite allograft (VCA) transplantation is now a feasible reconstructive option for patients that have suffered significant soft tissue injuries. However, despite numerous technical advances in the field over two decades, a number of challenges remain, not least the management of transplant rejection. Part of the difficulty faced by clinicians is the early recognition and prevention of acute rejection episodes. Whilst this is potentially easier in VCAs than solid organ transplants, due to their visible skin component, at present the only validated method for the diagnosis of acute rejection is histological examination of a tissue biopsy. The aim of this review article is to provide an evidence-based overview of progress in the field of VCA biomarker discovery, including immune cell subsets, immune cell effector pathways, and circulating markers of allograft damage, and to discuss future challenges in the field.

Keywords: Biomarkers; surrogates; VCA; vascularised composite allograft transplantation

INTRODUCTION

In carefully selected patients that have suffered devastating tissue loss, vascularised composite allograft (VCA) transplantation is now a feasible reconstructive option in many centres around the world.¹ In addition to widely publicised progress in the fields of hand and face transplantation, abdominal wall, uterus and penis transplantation are now showing considerable promise.²⁻⁶ Despite encouraging clinical results the morbidity associated with long-term immunosuppression, an inability to prevent acute and chronic rejection episodes, and the limited life span of transplanted organs are starting to limit VCA progress and expansion in many units.⁷⁻⁹ Efforts are now focussed on trying to reduce side-effects and prolong VCA function and survival. Identifying biomarkers or surrogates that indicate imminent rejection or inadequate immunosuppression, allowing personalised treatment, is a priority both in solid organ transplantation (SOT) and VCA.

DEFINING BIOMARKERS AND SURROGATES IN TRANSPLANTATION

A unified definition of the terms biomarker and surrogate was proposed by the Biomarkers Definitions Working Group in 2001.¹⁰ They defined biological markers (biomarkers) as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. Biomarkers can be prognostic, diagnostic, or predictive of clinical outcomes. In the context of SOT, biomarkers currently exist that can predict or diagnose graft injury, including serum creatinine levels in renal transplantation, and serum aminotransferase levels (ALT and AST) in liver transplantation. These commonly used biomarkers aim to optimise graft function and survival by improving clinical decision making. They can also help individualise patient treatment, and ultimately decrease the morbidity associated with over immunosuppression. A number of advanced biomarkers are now available for the detection of subclinical rejection of renal transplants and the in depth characterisation of tissue immune responses and damage, although many are still undergoing clinical validation.^{11,12}

Biomarkers currently exist that are common to all transplants and include (1) markers of the morbidity of immunosuppression e.g. hypertension, hyperlipidaemia, renal biochemistry, glucose function tests, white cell count (2) markers of immunosuppressive compliance and dosing e.g. medication levels (tacrolimus) (3) immunological system markers e.g. donor specific antibodies and peripheral blood chimerism (4) diagnostic markers of rejection e.g. histological assessment by biopsy.

Surrogate end-points (surrogates) are a subset of biomarkers that are used as substitutes for a clinical endpoint and are of particular importance in transplant clinical trials.^{10,13} Surrogates are reliable indicators of longer-term transplant outcomes and are intended to replace these outcomes in studies.

Short-term surrogate endpoints must be predictive of long-term clinical outcomes such as graft dysfunction, graft survival or mortality. In SOT, surrogates include biochemical, histological, and immunological markers. For example, in renal transplantation creatinine levels or creatinine clearance is a surrogate for renal function; Banff histology score a surrogate for rejection or fibrosis; donor specific antibodies, perforin and granzyme B (GZMB) levels and microarrays or proteomic patterns are surrogates for alloresponse; hypertension, diabetes, hyperlipidaemia, infections and tumours can also be considered surrogates for quality of life and patient morbidity.¹⁴

THE CURRENT LANDSCAPE OF BIOMARKER DISCOVERY IN VCA

Acute rejection in VCA recipients is diagnosed using a combination of clinical assessment and tissue biopsy. Currently, histological assessment by tissue biopsy is the only clinical surrogate of rejection reliably used in the field of VCA. The utility of VCA skin as a monitoring tool to detect rejection, observing for rash, oedema or erythema, is unique in transplantation. Perhaps it is this increased visibility, and therefore recognition, that leads to the high acute rejection rates amongst VCA recipients, affecting 75-88% of patients in the first year after transplant.¹⁵ This is in contrast to acute rejection rates of 7.3% reported for renal transplantation in the first year after transplant.¹⁶

Reporting of VCA histopathology specimens is in accordance with the Banff 2007 working classification of skin-containing composite tissue allograft pathology.¹⁷ The Banff classification grades the histopathological features of acute rejection on a scale from 0 (no features of rejection) to 4 (frank necrosis of epidermis). There does not appear to be any meaningful pathological differences in skin rejection reported between VCA types.¹⁸⁻²⁰ However, unique to facial transplantation, mucosa appears to be one of the primary targets of acute rejection, and is always present in higher-grade skin rejection samples.²¹

Additionally, when VCA skin pathology specimens are reported, there is inherent intra- and inter-observer error, as inflammation seen is not specific to rejection.²² This issue is not unique to VCA, with borderline changes being a challenge to differentiate from true acute rejection, necessitating clinical correlation.^{23,24} Further problems arise as rejection may be sub-clinical and therefore may not manifest

as classical skin changes. This is more common for grade 1 biopsies, which are often only detected on protocol surveillance biopsies. The extent of visible VCA skin changes do not always correlate with histopathological VCA skin rejection severity, particularly given that common skin-intrinsic pathologies may manifest with features similar to rejection. However, recent advances in molecular phenotyping may help differentiate between rejection and intrinsic skin inflammation.^{25–27}

There appears to be limited correlation between the VCA rejection grade on biopsy and recommended treatment or outcomes, particularly as VCA rejection can be managed using topical immunosuppression alone, or in combination with traditional therapies.²⁸ Indeed, some authors base their assessment of rejection severity not on biopsy but on response to therapy.²⁹

The true clinical relevance of rejection in VCA is still unknown. However, it is presumed that untreated rejection will lead to graft injury and loss of function, due to a combination of chronic rejection and vasculopathy, but also as a direct result of inflammatory immune injury. As such, efforts are being made to identify and treat rejection as early as possible, or even better to predict and treat rejection prior to visible skin changes occurring.

Given the accessibility and ease of skin biopsy in VCA the need for non-invasive biomarkers or surrogates is not as great as in SOT, but there remains a clear need for a more sensitive and specific diagnostic marker of rejection that could replace, or augment information obtained from biopsy, helping guide early clinical decision making.

A general approach used to identify potential VCA biomarkers has been to look at immune cell phenotypes within tissue and blood and to attempt to identify cells indicative of rejection.

Immune cell subsets in tissue biopsies

In hand and face transplantation, acute rejection is associated with CD3⁺ Tcell infiltration into the skin. There is some evidence to suggest that the ratio of CD8⁺ to CD4⁺ cells is increased in mild rejection, while this is reversed in severe rejection.^{30–32} However, other authors have reported the opposite pattern to be true.¹⁹ Other cells detected in tissue biopsies in smaller numbers include RNA binding protein (RBP) T-cell intracellular antigen 1 (TIA-1) expressing T cells, FOXP3⁺ regulatory T cells (Tregs), CD68⁺ macrophages, CD20⁺ Bcells and CD14⁺ myeloid dendritic cells.³³ However, transplanted skin even when not rejecting has high numbers of T lymphocytes with a predominance of CD8⁺ over CD4⁺ cells, CD68⁺ macrophages, and CD20⁺ B cells, and myeloid dendritic cells.³⁴ As a result, these cell

sub-types are less useful as potential predictors of acute rejection. The role of human leukocyte antigen-DR isotype (HLA-DR) expression during rejection episodes is unclear. Some groups have published no increase in the expression of this marker of activation during rejection, whilst others report a correlation with severe rejection episodes.^{19,32} Analysis of face transplant rejection found abundant skin-resident T cells (Trm) of donor origin at vascular, pilosebaceous and epidermal sites of injury associated with rejection.³⁵

Although Forkhead box P3 (FOXP3) is traditionally associated with immune regulation, there is some evidence that its upregulation can correlate with episodes of acute rejection. Muthukumar *et al* highlighted the utility of urinary FOXP3 mRNA measurement as a useful biomarker of outcomes following episodes of acute rejection in human renal transplant recipients.³⁶ Lower levels of urinary FOXP3 mRNA was associated with irreversible acute rejection and even graft failure.

Analysis of clinical samples found FOXP3 mainly in samples undergoing severe rejection and also that expression of FOXP3 increased with time after transplantation.^{30,37,38} There was low or no correlation with grade 1 rejection, which makes FOXP3 or its effector expression less useful as a diagnostic marker. In a rat hind-limb model treated to stimulate Treg proliferation, the ratio of FOXP3 to cytotoxic/effector genes (GZMB, IFN- γ and Prf1 [perforin]) was higher in rejecting skin transplants that spontaneously resolved compared to those that developed progressive rejection.³⁹

The search for a sensitive and specific marker for rejection on histological analysis is likely to require more detail than can be provided by cell type alone. This type of analysis may be assisted by the molecular examination of the functional response of the cells.

Immune effector signalling pathways in tissue biopsies

As expected from SOT studies, interferon-stimulated genes/enzymes/products and immune effector functions are upregulated in rejection but remain non-specific and can be upregulated in transplanted tissue that is not rejecting.⁴⁰ A longitudinal examination of a single VCA recipient who developed both acute cellular rejection and (possibly) antibody mediated rejection identified activation of the interferon gamma (IFN- γ) signalling pathway, overexpression of the genes involved in cytotoxic cell recruitment, and genes associated with cytotoxicity.^{33,40}

But IFN- γ , the chemokine receptor CXCR3/C-C chemokine receptor type 5 (CCR5) pathway, CD8⁺ cytotoxic cells and granzyme B are all involved in other inflammatory skin conditions such as infection, psoriasis, and other auto-immune dermatoses, as well as being expressed in syngeneic rat limb transplant models without any rejection processes, thereby limiting their usefulness as specific indicators for rejection.^{40–44}

Wolfram *et al* sought to differentiate rejection from contact hypersensitivity and, using a rat model, identified that an expression profile consisting of C-C Motif chemokine ligand 7 (CCL7), interleukin-1b (IL-1b), interleukin-18 (IL-18), and tumour necrosis factor (TNF) is strongly suggestive of acute rejection, but only on a pairs-wise comparison and not absolute values. However interleukin-1 (IL-1) remains a subject of possible interest to help differentiate rejection on histology.²⁷ Transcriptional profiling shows that the coordinated activation of interferon stimulated genes and immune effector functions (granzymes) is a final common immune mediated tissue destruction pathway no matter the instigation be it infection, cancer, auto-immunity or allograft rejection.⁴⁵ A number of probe-based assays now exist for in depth and technically straightforward assessment of tissue biopsy molecular markers based on consensus derived from robust microarray studies of SOT in the literature, although it is not yet clear whether these will also translate to skin biopsies.¹²

Serum markers indicative of rejection

The search for blood markers has been pursued with vigour in the quest to find a relatively non-invasive method of immune monitoring in SOT. However, the intermittent nature of blood tests means that their main utility would be diagnostic rather than premonitory, though the latter would be ideal and perhaps could be deduced from trending changes in immunological cell sub types.

Utilising multi-parametric flow cytometry to analyse peripheral blood lymphocyte subsets is a useful method to monitor for alterations after transplantation. The problem is while general activation markers may be increased in rejection they are not specific to allograft activation of the immune system and cannot differentiate from other causes of immune activation such as infection or autoimmunity. Findings in one organ do not necessarily extrapolate to other organ types. For example, the T-cell population rejection- related genes (CD8, interleukin-10 (IL-10), NOTCH1, PDCD1, TNF) are found to be upregulated and expressed at higher levels in the blood of VCA recipients compared to kidney

transplant recipients and patients without transplants.⁴⁶ Specifically, in VCA there have not been many longitudinal studies of blood cell types with only one study reporting a shift in T helper cells from Th2 to Th1 and Th17 subtypes associated with rejection.³³ The shift to Th1 is seen in other organ transplant rejection but frustratingly the Th1/Th17 shift is also seen in inflammatory skin conditions.⁴⁷

Lymphocyte stimulation assays

Lymphocyte proliferation or stimulation assays indicate the level of immunosuppression such that low adenosine triphosphate (ATP) levels or a low proliferation index means the lymphocytes are less likely to respond to an immune stimulus. A commercially available assay, ImmuKnowTM, has been evaluated in kidney, liver, heart and intestinal transplants and identified high ATP levels in CD4⁺ T cells as associated with acute cellular rejection, whereas low levels are associated with infection.⁴⁸ However, other studies have been inconsistent and no studies in VCA rejection have been performed to date.

Cell effector markers in blood

Downstream markers of T-cell activation such as IFN- γ and interferon signalling such as the CXC chemokine ligands 9 and 10 have been studied only twice in VCA. One study of 24 cytokines in the peripheral blood of six face transplant recipients showed only one cytokine, monocyte chemoattractant protein-1 (MCP-1) to be raised during rejection.³³ The same patients were studied for proteomic changes during rejection and found to have elevated matrix metalloproteinase 3 (MMP3) levels in severe rejection compared to non-severe rejection or no rejection.²⁹ Kollar *et al* (2019) went on to examine 140 samples in nine face transplant and ten upper extremity transplant recipients.⁴⁹ MMP3 levels were found to increase after transplantation and during severe rejection, although there was no specific association between MMP3 levels and the grade of rejection.

Other blood targets of cell effector function to be studied by gene expression profiling during rejection include perforin, granzyme, and fas ligand (FasL) that are upregulated in acute cellular rejection in the blood of kidney transplant recipients, but also in infections. These have not yet been studied in the blood of VCA recipients during episodes of rejection.

MicroRNAs (miRNAs) are small molecules of RNA that exert their effects by interfering with translation or degradation of larger RNA molecules.⁵⁰ In recent years it has been recognised that they have wide-ranging effects on many systems, including both the innate and adaptive immune responses.⁵¹ This has prompted investigation into their use as a potential biomarker for monitoring in organ transplantation.⁵¹ In a rat hindlimb VCA model elevated levels of miRNA146a, miRNA155 and miRNA-182 were found during acute rejection episodes.^{51,52} Of note these were raised before either clinical or histological rejection was recognised.⁵¹ These findings suggest miRNAs may have a potential role for monitoring in VCA transplantation, however there is not yet any evidence for their use in human VCA patients. This is an area which requires further investigation.

Measurable markers of VCA tissue injury in blood

Donor derived cell free DNA (dd-cfDNA) is a non-specific marker of organ injury, although there is no strong evidence supporting its use as a rejection biomarker.⁵³ However it is important to note that there is a contemporaneous association between dd-cfDNA levels with rejection and its eventual resolution.

To date, a single study has assessed dd-cfDNA in VCA transplantation. Haug *et al* prospectively measured dd-cfDNA levels in one face and one bilateral arm transplant recipient but did not detect raised plasma dd-cfDNA in either case during acute rejection.⁵⁴

FUTURE CHALLENGES OF VCA BIOMARKER AND SURROGATE DEVELOPMENT

In contrast to SOT, no unifying definition or quantification of function or loss of function exists in the field of VCA. This can largely be attributed to the variability of components and functions that exist between different VCA types. The only reliable end points common to all VCAs are graft survival, morbidity associated with immunosuppression (e.g. renal failure, hypertension, diabetes, and malignancy), and patient mortality. So, what are VCA biomarkers intending to mark, and what will they mean?

Biomarker discovery in SOT has been directed toward the non-invasive diagnosis of acute rejection, and the development of a predictive biomarkers that would then allow guided tailoring of immunosuppression. This is because there is a known association between graft injury and loss of function. However, in VCA, despite limited pre-clinical evidence, the clinical association between acute

rejection and loss of function, graft loss or survival is yet to be established.^{55–58} Therefore, it is not possible in VCA to identify or validate biomarkers that are diagnostic or predictive of graft injury and loss of function. Although we may identify biomarkers of VCA rejection, the interpretation and clinical relevance of the presence of rejection is unknown. General biomarkers of immunosuppression-related morbidity and mortality may however remain interpretable.

The need for biomarkers of rejection, graft injury and immunological status is obvious in SOT as the organs are not visible, and clinical symptoms and signs may occur late and only once injury is established and damage irreversible. In VCA such a need is less clear, as the transplant is continuously visible, and the skin component demonstrates rejection changes early before significant graft injury has occurred. What is unclear is whether the skin component is always indicative of the deeper hidden VCA components and whether by relying on skin changes deeper rejection is being missed, and if so, whether “missed” rejection is relevant to longer-term outcomes.⁵⁹ If the skin component is not indicative of the deeper VCA structures, and rejection is being missed, and such rejection does prove to be related to loss of graft function or loss of the graft itself, then biomarkers of rejection of the deeper tissues may be useful. Another useful related question would be, does clinically significant VCA rejection occur without skin changes? If so, biomarkers may prove useful for this scenario. Unfortunately, we do not know the answers to these questions. Studying the known biomarkers from other transplant organs may go some way to determining these answers.

The real challenge is to determine what the significance of rejection in VCA is. Despite the very high rates of acute rejection experienced in VCA compared to SOTs, graft survival remains high longer term, therefore the relevance of detecting and treating rejection to the overall function and survival of the VCA compared to SOT is unknown.^{15,60–62} Despite the lack of association between rejection and functional or graft loss, VCA biomarkers have focussed on rejection detection either by measuring markers indicative of organ injury or of immune system activation. Further questions surround the lack of evidence of the optimal treatment of rejection in VCA with some rejection being successfully treated by topical immunosuppression alone, and most by a combination of topical and steroid treatment, with only a small proportion being resistant. All these questions require clarity in order to test the validity and fulfil criteria of successful biomarkers.

A number of challenges remain for researchers in the field of VCA, including how to gather protocol-based serum or tissue samples preceding rejection, the lack of a diagnostic gold standard for

rejection, the lack of validation that rejection is a surrogate for transplant function or survival, the lack of measure of VCA function, and the insensitivity of using graft survival as the defined measure. In addition, VCAs are uncommon, extremely variable in size and composition, but also vary greatly in the management and immunosuppressive protocols. On the plus side, VCAs with their easily visible signs of rejection should permit early detection and collection of samples for analysis.

CONCLUSIONS

Currently, other than histological assessment, no validated biomarkers or surrogates of VCA rejection exist. However, VCAs with their unique ability to telegraph rejection may prove to be a fruitful vehicle to collect early rejection samples on which to study biomarkers of the very early stages and of mild rejection. Biopsies are easily and readily performed and will permit high throughput unbiased screening for potential candidates. It is highly likely that a pattern of biomarkers will be necessary to identify rejection and differentiate it from other causes of immune-mediated inflammation. However, perhaps research priorities in this area should also focus on defining how rejection affects different components of the VCA and what is to be gained by early identification of rejection, relating findings to clinical outcomes in these patients.

CONFLICT OF INTEREST

None

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