REVIEW ARTICLE

ROLE OF CTLA-4 IN XENOTRANPLANTATION

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The replacement of diseased organs by transplantation is now an important medical therapy. However, transplantation is limited by organ shortage. One solution to this problem is xenotransplantation, in other words, the use of organs from animal donors. Although the problem of hyperacute rejection is now largely solved, there is evidence that xenografts suffer other forms of rejection, one of them being T cell response. Moreover, evidence suggests that the degree of immunosuppression required to prevent T cell mediated xenograft rejection will be higher than that needed to prevent T cell mediated allograft rejection. Thus, strategies for graft-specific immunosuppression and improved tolerance are needed, and are discussed in this review.

BACKGROUND

The CD28 molecule is a cell surface glycoprotein expressed predominantly on peripheral T cells and thymocytes.[1,2] Fifty percent of human $CD8^{+ve}$ and virtually all human $CD4^{+ve}$ T cell, as well as all murine T cells express CD28 constitutively. Following T cell activation, CD28 expression increases transiently and then decreases after its engagement with its natural ligands, the B7 molecules. This reduction in CD28 expression leads to a decreased the ability of the T cell to mobolise intracellular stores of calcium, a critical component of effective T cell signaling.[3]

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a structural homologue of CD28 (30% homologous). It was discovered amongst a set of T cell-specific, activation-induced genes.[4] CTLA-4 and CD28 are structurally related glycoproteins of the Immunoglobulin (Ig) super family. Both structures contain the conversed amino acid sequences MYPPPY, which represents a necessary sequence for binding to the B7 molecules.[5] However, there are a number of differences between CD28 and CTLA-4.[6] CD28 is expressed constitutively on resting T cells, whilst CTLA-4 is up regulated only after T cell activation. Although CTLA-4 glycoprotein is expressed on the surface of both activated $CD4^{+ve}$ and $CD8^{+ve}$ T cells, the majority of the protein remains intracellular.[7] The ligation of CD28 is essential for maximum CTLA-4 expression, since CD28-deficient T cells are not efficiently upregulated via CTLA-4

mRNA after activation unless exogenous IL-2 is added.[8] CD28 and CTLA-4 have different on/off rates for the B7 molecules, with CD28 displaying a fourfold faster dissociation rate from both B7 molecules than CTLA-4. Furthermore, CTLA-4 binds B7-2 with a lower affinity and a faster off rate than B7-1. Finally CTLA-4 has a dramatically higher affinity for the B7 molecules than CD28.

FUNCTION OF CTLA-4 AND CD28

Early studies demonstrated that ligation of CD28 by its natural ligands in the presence of TCR-engagement leads to cytokine production (such as IL-2, IL-9, IL-5, IL-13, IFN-γ and GM-CSF)[9] and T cell proliferation. CD28 engagement induced up to a 100-fold increase in IL-2 production, whereas T cell from CD28 knockout (KO) mice produced less than one-tenth the amount of IL-2 after activation.[10] The CD28-mediated increased in cytokine production reflects both an increased level of gene transcription and mRNA stability.[11] More importantly, stimulation of murine CD^{+ve} Th1 T cell clones in the absence of CD28 ligation induces T cell clonal anergy, which can be prevented by CD28 ligation.[12]

Other studies using CD28-deficient T cells or CTLA-4 Ig in primary T cell cultures demonstrated that CD28 engagement may regulate multiple T cell functions other than anergy-induction. First, in the presence of an effective CD28 signal, the degree of TCR engagement required for T cell activation is reduced significantly.[13] Second, CD28 co-stimulation induces bcl-xl, an intracellular factor

essential for long-term survival of activated T cells. Third, CD28 co-stimulation plays a role in the differentiation of Th1/Th2 subset.[14] Finally, CD28 appears to be essential for the development of CD8[+ve] T cells into cytolytic effectors.

The concept that CD28 functions as a co-stimulatory molecule has been widely accepted. In contrast, the function of CTLA-4 remains controversial. Some studies suggest that CTLA-4, like CD28, functions as an additional co-stimulatory molecule on activated T cells.[15,16] However, other in vitro and vivo studies using anti-CTLA-4 Monoclonal antibodies (mAbs) clearly demonstrate that CTLA-4 acts as a negative regulator for T cell activation, For example, in vivo blockade of CTLA-4 leads to the exacerbation of experimental autoimmune encephalomyelitis,[17] the enhancement of an antitumour response, and the augmentation of a T cell response in an adoptive transfer model. Data from CTLA-4 knockout mice provides further evidence that CTLA-4 has an inhibitory function.[18] Several groups have reported that in vivo blockade of CTLA-4 results in the breakdown of T cell anergy-induced by a soluble peptide or superantigen, suggesting that CTLA-4 ligation is required for the induction of peripheral T cell tolerance.[19,20] Turka and colleagues observed that anti-CTLA-4 treatment could prevent CTLA-4 Ig-induced tolerance to alloantigen in a murine heart transplantation model.[21] Recent studies have begun to explain the mechanisms by which CTLA-4 could downregulate T cell activation: CTLA-4 could inhibit T cell responses, by out-competing CD28 for binding to B7 ligands, by antagonizing CD28-mediated signaling and/or, by antagonizing TCR-mediated signalling. These three proposed mechanisms are not thought to be mutually exclusive. Taken together, the studies mentioned among others provide strong evidence that CD28 and CTLA-4 have opposing effects on T cell activation. It is generally accepted that CTLA-4 is a negative regulator and may actively be involved in the induction of peripheral T tolerance.

T CELL ACTIVATION, CD28 AND CTLA4

Xenograft rejection depends on T cell activation.[22] T cell activation results from specific interactions with the TCR/CD3 complex and co stimulation via other T cell surface receptors. Prevention of co-stimulation can result in clonal anergy.[23,24] The best characterized co-stimulatory pathway is transduced through the CD28 surface molecule. CD28 is a receptor for B7. CTLA-4 is a gene closely related to CD28 which also serves as a B7-ligand.[15,25] CTLA-4 shares many features with CD28 including a common counter-receptor B7 that is present on Antigen presenting cells (APCs).[26] Also, the amino acid sequences of CTLA-4 and CD28 are very similar.[27] Both CD28 and CTLA-4 exist as disulfide-linked homodimeric glycoprotein[9,15,28] and both are members of the immunoglobulin super family that contains single V-like domains.[1] Furthermore, an unpaired cysteine residue at a position just proximal to the transmembrane domain is present in both.[27] CTLA-4 and CD28 both possess a distinctive hexapeptide motif, MYPPPY, conserved across virtually all species, which is essential for binding to B7, as determined by mutagenesis.[5,29]

Although CTLA-4 and CD28 share many structural similarities, they differ in many aspects also. The outcome of the CD28 ligation in the presence of TCR occupancy is T cell proliferation, enhanced survival and cytokine production, all of which are essential for normal T cell function.[30,31] Based on early studies, CTLA-4 was believed to play a similar role to CD28 in the regulation of T cell responses, due to the amino acid sequence homology and ligand binding specificity. However, recent functional studies in mice and humans, suggest a different role for CTLA-4. These studies support a role for CTLA-4 as a downregulatory molecule in T cell activation.[25] Consistent with this view, are experiments where Fab' fragments of anti-CTLA-4 Abs have been observed to augment T cell proliferation.[25] This is in turn consistent with the blocking of a negative regulatory function, whereas agonistic cross-linking of intact Abs has revealed potent inhibition of T cell proliferation.[32] Direct evidence for a physiologic role for CTLA-4 in the downregulation of activated T cells comes from studies with CTLA-4 deficient mice.[18,32] Data from these studies are consistent with a role for CTLA-4 signalling in the negative regulation of activated T cells.[25] Reports have shown that CTLA-4 deficient mice rapidly develop lymphoproliferative disease and suffer from fatal multiorgan tissue destruction.[33]

ADVANCES IN ORGAN TRANSPLANTATION

Over the last 40 years, remarkable results have been achieved in the field of organ transplantation. Some of the major factors that have contributed to these results are better immunosuppression, matching for HLA, better preservation, and resolution of most of the earlier technical problems associated with organ transplantation. One of the biggest problems is chronic rejection and the achievement of tolerance to an organ graft represents the best solution to this problem.[34]

Moreover, a solution to the problem of allograft shortage is xenotransplantation, i.e. the use of organs from animal donors.[35] However, in order for

xenotransplantation to be an effective therapy, the host immune response (leading to xenograft rejection) must be solved. Partially inbred miniature swine are attractive as donors for clinical xenotransplantation due to their size, breeding characteristics, physiology, and availability of major histocompatability complex (termed SLA in swine) homozygous lines.[36] Until now, phenomenon of hyperacute rejection was a barrier in xenotransplantation and resulted in rapid destruction of the xenograft.[37] However, human complement transgenic pigs (DAF) have been generated and organs from such animals are resistant to rejection.[38] These organs do however suffer other types of rejection, including T cell responses.[39,40]

T-CELL MEDIATED REJECTION

Cellular responses to xenoantigens in grafted organs are mediated by two mechanisms. One is the 'direct' xenotransplantation mechanism that is mediated by T lymphocytes whose receptors have specificity for the xenogeneric Major Histocompatibility Complex (MHC) class I or class II molecule in combination with the peptide. Such responses are characterized by a high precursor frequency of responding T cells. Their receptors are specific for xenogeneric peptides that are derived from the grafted organ and mediate indirect recognition of the graft. Proteins from the graft are processed by self antigen presenting cells and therefore presented by self MHC class I or class II molecules.[34,41]

IMMUNO-SUPPRESSSIVE TREATMENTS

At present, major therapies to prevent the rejection of xenogenic organ transplants rely on immunosuppressive drugs, such as cyclosporine A and FK 506, or monoclonal antibodies (mAbs) to CD3. Immunosuppressive drugs (also called anti-rejection drugs) are important because they prevent the body rejecting the transplanted organ. If this damage occurs within days it is known as acute rejection. If the time course is longer than this it is defined as chronic rejection. The risk of rejection is greatly reduced by immunosuppressive drugs that protect the transplanted organ and preserve its functionality. These drugs aim to suppress unwanted immuno-responses and thus make reactions against the transplanted organ less likely. A variety of drugs achieves this same basic aim but work in different ways to reduce the immune response. However, immunosuppressive drugs may also exhibit side effects. First, their use increases the risk of infections. The body's immune system protects against bacterial and viral infections and, when the immune system is compromised, the chance of infection increases accordingly. Furthermore, in this context, pharmaceutical use increases the risk of cancer as the immune system also plays a role in protecting the body against some forms of cancer. When immunosuppressive drugs are used for a long period of time there is, by comparison with the general population, an increased risk of developing skin cancer caused by a combination of the drugs and exposure to sunlight. However, in xenotransplantation, relying on drugs alone to prevent rejection may not be effective. Thomas et al[42] showed that cyclosporine was only effective in xenograft models when it was used in very high, toxic doses. They also found that FK 506 was toxic at doses above 1.5–2 mg/Kg with a significant high rate of animal death. Surprisingly, Todo's work[43] in higher primates suggested very little toxicity with FK 506, even when it was used with cyclosporine-A. Thomas's group also observed that the combination of these two drugs was particularly toxic and suggested that this combination was unlikely to produce good results, whether in allografting or xenografting, because of its inherent toxicity.[42] Thus, an alternative approach to using immunosuppressive drugs needs to be developed in order to prolong xenograft survival. Many novel treatments are in development, particularly antibodies blocking surface molecules critical in T cell activation as B7.1 (CTLA4-lg) and anti-CD40L.

CTLA-4 AND CANCER THERAPY

Very recently, there have been studies shown encouraging results and may appear to be a promising strategy for cancer immunotherapy. Numerous clinical trials testing the blockade of CTLA-4 prompted efforts to target the signalling molecule for a variety of cancers including: malignant glioma, colorectal carcinoma and melanoma.[44–51] Recent developments in the understanding of CTLA-4 signalling pathways are expected to provide new opportunities for safer chemotherapy. We expect many of these new developments will now be rapidly implemented and will soon play an important role within chemoprevention strategies in the treatment of cancer patients. It was also made clear that several important new pharmacological strategies and innovative approaches in this area are under active current development, and that these are soon likely radically to change our management of cancer patients.

CHARACTERISTICS OF CTLA4-LG

CTLA-4lg is a soluble recombinant protein, which contains the extracellular domain of human CTLA4-lg fused to a human Ig C γ-chain. CTLA4-Ig binds efficiently to murine and rat B7.[52] CTLA4-lg inhibits B7 – dependent immune responses in vitro, while in vivo CTLA4-Ig blocks T cell –dependent B cell antibody production and prevents the rejection of xenogeneic islet and allogeneic islet and allogeneic cardiac allografts.[21,53] It has been observed in studies of cardiac allograft

rejection that animal treated with daily injections of CTLA4-Ig for seven days, initiated at the time of transplantation, had greatly prolonged graft survival, although most animals eventually rejected the graft.[21] A key characteristic, which distinguishes CTLA4-Ig from other approaches to immune suppression, is its ability to induce tolerance to specific immune responses. Induction of tolerance may allow for the treatment of immune disorders without globally compromising the ability of the immune system to mount a response to an infection.[23]

Lanschow was the first investigator to demonstrate that CTLA4-Ig was effective in an animal model of disease. Lenschow[53] transplanted insulin-producing cells from humans into diabetic mice and monitored their ability to control blood sugar. Untreated mice rejected the transplant within one week, however, mice treated with CTLA4-Ig for two weeks showed no sign of rejection even after the CTLA4-Ig treatment was discontinued. These data demonstrated for the first time that CTLA4-Ig has the potential to be used as an immunosuppressive drug and that it may thus induce tolerance. Lin et al found that donor – specific cell transfusion at the time of transplantation, followed by a single dose of CTLA4-Ig two days later, is enough to lead to prolonged, often indefinite, cardiac allograft survival. Since no treatment is required before transplantation, these results may be clinically applicable for cadaveric organ and tissue transplantation in humans.[54]

Furthermore, Tang et al[55] in his study, using a strategy that induces transplantation tolerance, demonstrated that the effect of CTLA4-Ig administration in vivo leads to two alterations in normal T cell priming. Following immunization, pigeon chromosome c (PCC) reactive T cells from animal treated with a single dose of CTLA4-Ig expanded to 50% of the level achieved in control animals. These results are consistent with those of previous studies. The second effect observed was the induction of anergy in the residual population.[21]

Moreover, based on Perico et al, CTLA4-Ig given for seven days at the dose of 0.2 mg/day as the sole immunosuppressive therapy, considerably prolonged rat renal allograft survival.[56] Also, humoral responses and allograft and xenograft rejection have been suppressed in rodents treated with CTLA4-Ig. These findings have supported the potential for human CTLA4-Ig (hCTLA4-Ig) therapy in humans. Levisetti et al[57] investigated how hCLTA4-Ig affected a non-human primate model of allogeneic pancreatic islet transplantation. Their results demonstrated that a short course of hCTLA4-Ig therapy is safe and suppresses transplant-specific humoral responses in the absence of any other immunosuppressive drug. Moreover, two of five treated monkeys showed prolonged graft survival

and evidence of donor-specific cellular hyporesponsiveness in vitro.[57]

Following these initial observations, CTLA4-Ig has been shown by numerous research groups to exhibit activity in animal models of solid organ transplantation (heart and kidney), bone marrow transplantation and autoimmune diseases such as lupus and multiple sclerosis.

USE OF CTLA4-IG IN CLINICAL XENOTRANSPLANTATION

In order to prolong the survival of xenografts in humans, the T cell anti-graft response must be successfully suppressed. Two doses of CTLA4-Ig peri-transplantation are enough to lead to inhibition of mouse responses against the graft, since direct immunogenicity is lost. However, unlike allografts, pig organs appear to be especially immunogenic in man because they provoke particularly vigorous 'direct' and 'indirect' xenoresponses. Furthermore, pig endothelial cells (EC) that express porcine B7 molecules (CD80 and CD86) stimulate strong 'direct' human T cell response. The implication of this is that the 'direct' immunogenicity of the graft will persist long after transplantation implying that any immunosuppressive treatment, including CTLA4-Ig, may have to be administered for prolonged periods of time. In order to avoid generalized systemic immunosuppression it is therefore desirable to develop reagents with graft- specificity.[58]

Vaughan *et al* described the cloning and sequencing of the pig homologue of CTLA-4 (pCTLA-4) and the characterization of a derived soluble fusion protein, pCTLA-4 Ig. When pCTLA-4 was compared to human, a high degree of conservation was found in the predicted protein sequence, although a leucine residue replaced the methionine at position 97. A fusion protein was constructed from the extracellular regions of pCTLA-4 and the constant regions of human IgG1 and it was observed that it could bind pCD86 with the same affinity to that of human CTLA4-Ig. Nonetheless, pCTLA4-Ig bound inadequately to human B7 molecules expressed on fibroblast transfectants and EBV- transformed human B cell lines. In functional assays, with MHC class II expressing porcine EC and human B cells, pCTLA4-Ig blocked human CD4^{+ve} cell responses to pig but not human cells whereas control human CTLA4-Ig inhibited both. Based on these outcomes pCTLA-Ig, by being unable to inhibit the delivery of co-stimulatory signals provided by human B7, may prove to be a relatively specific reagent for inhibiting the direct human T cell responses to immunogenic pig tissue. Based on the finding of this difference between the donor and host species, Vaughan et al suggested

that pCTLA4-Ig may be a reagent that should have graft-specific immunosuppressive properties.[59]

CONCLUSION

There have been huge advances in research into CTLA-4. New approaches, based on cell death techniques, in treating several malignancies and transplantation are highly promising. We are currently in a phase where these techniques are now being refined ever more precisely towards the successful attainment of effective therapies to increase the cure rate of cancer and successful transplantation.

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