

Immune and Cell Therapy of Hematologic Malignancies

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Abstract

Advances in molecular biology and immunology have identified means to activate the immune response against leukemia-associated antigens. Recent studies indicate that the stealth-like phenotype of leukemia cells can be reversed through transfer of genes encoding recombinant membrane-stabilized proteins of the tumor necrosis factor (TNF) family, such as the one encoding CD154, the ligand for CD40. A phase I clinical trial using autologous CD154-transduced leukemia cells as a cellular vaccine has provided encouraging results. Treatment not only appears capable of inducing a cellular anti-leukemia immunity, but also may have a direct effect on leukemia cells by inducing latent sensitivity to Fas (CD95)-dependent leukemia-cell apoptosis. Phase II studies currently are underway using multiple injections of autologous leukemia cells made to express recombinant CD154 via gene transfer. Conceivably, we may be entering an era of effective gene therapy for hematologic malignancies.

1. Immune and Cell Therapy of Hematologic Malignancies

The immune system may be capable of recognizing the changes that occur in transformed cells. Cancer is caused by mutations in genes encoding proteins that are critical for cell growth and/or survival. The proteins encoded by mutated genes can have structural alterations secondary to changes in the primary structure of the protein. Upon degradation via the proteasome, these proteins can generate "altered-self" peptides that may be presented by major-histocompatibility-complex (MHC) antigens. Alternatively, the genetic changes within the tumor cell can alter the expression levels of a particular self protein(s), allowing for presentation of greater numbers of the "self peptide" by the tumor cell's MHC antigens. In either case, the genetic changes that give rise to the malignant cell may be recognized by the patient's immune system, potentially inducing an immune response that is capable of destroying the neoplastic cells that possess such genetic changes.

However, the immune system of patients with cancer does not appear to react against autologous tumors. Studies have found that the T cells patients with hematologic malignancies, such as B cell chronic lymphocytic

leukemia (CLL), are not capable of responding to autologous leukemia cells in mixed lymphocyte reactions [1]. This is despite the fact that CLL cells express high levels of both class I and class II MHC antigens that are required for presentation of altered-self peptide antigens. Lack of immune reactivity could be due to an absence of T cells that can recognize such altered-self antigens, either initially or subsequent to transformation through a mechanism similar to that involved in the generation of central tolerance to self antigens. Alternatively, T cells may exist in patients with hematologic malignancies that are capable of recognizing altered-self leukemia-associated antigens. However, these cells may be unable to respond to such antigens as they are presented by CLL cells. Consistent with this notion, CLL cells are unable to stimulate even allogeneic T cells in lymphocyte mixed lymphocyte reactions. As such, CLL cells appear to be poor antigen presenting cells (APCs) despite having high-level expression of class I and class II MHC molecules.

Increasing evidence suggest that the latter picture of T cell anergy holds true for other hematologic malignancies, and for cancer in general. Tumor cells generally lack expression of immune co-stimulatory molecules, such as CD80 (B7-1) or CD86, that are necessary to

evoke a productive T cell response, even by T cells that bear T cell receptors for peptides presented by self-histocompatibility antigens on the surface of the tumor cell. This state has been coined "anergy" to indicate that some recognition can occur without leading to cellular proliferation, significant cytokine production, or protective immunity.

2. Tumor Necrosis Factor Family

The tumor necrosis factor (TNF) family of proteins could be highly useful for reversing anergy and inducing an anti-tumor immune response. The TNF family is comprised of several important molecules that play critical roles in the maturation, differentiation, activation, and/or clearance of cells within the immune system [2]. Many of the molecules of this family are important for regulating immune function and response to infectious and systemic disease. Controlling expression of key members of the TNF family could allow for control of immune function, allowing us to induce immunity against weak antigens, including those associated with the altered self antigens of the neoplastic clone.

The prototype molecule, TNF- α , probably was responsible for the active ingredient in Cooley's toxin, a preparation prepared from the response to bacteria infection that was used successfully at the turn of the last century to induce inflammatory anti-tumor immunity in patients with cancer. This molecule later was cloned and given the name tumor necrosis factor. However, we now know that this factor does not induce necrosis of cells, but rather can induce apoptosis of cells that bear the lower molecular weight form of the TNF receptor, namely CD120a (p55) [3]. Also, TNF can induce cellular activation and potential activation-induced cell death through interaction with the higher molecular weight form of the TNF receptor, namely CD120b (p75) [4].

We think of TNF- α as a soluble molecule or factor. Indeed the mature form of the molecule is thought to be the factor that is released from cells that express this protein. However, TNF starts out as a surface molecule that is later cleaved to produce the soluble molecule. It is not necessary for the molecule to be cleaved from the membrane for it to have activity. In fact, the membrane bound form of TNF can interact most effectively with cells that bear either of the TNF receptors, CD120a or CD120b. A structural model for TNF-TNF-receptor interactions can explain why.

The TNF receptors lack tyrosine kinase domains or interaction with other cytosolic proteins capable of inducing phosphorylation. The TNF receptors can be made to signal when aligned in the appropriate conformation through interaction with more than one TNF molecule. The minimal functional unit of TNF is that of a trimer, which brings three TNF receptor molecules together in the proper stereo-configuration on the plasma cell membrane. This allows cytosolic proteins called TNF receptor adaptor factors (or TRAFs) to bind to the cytosolic portions of the receptors and initiate different signaling cascades, depending upon the composition of the TRAFs

present in the cytosol [5].

Because of this, TNF appears more active when attached to the plasma membrane than the released TNF cytokine. Expression of membrane TNF can assemble the TNF receptors into a matrix of trimeric multimers that have greater signaling capacity than the single trimer that can be assembled through interaction with the active trimeric form of soluble TNF. For this reason, the signaling potential of TNF attached to the plasma membrane has a higher specific activity than that of the soluble molecule [6].

We have classified the membrane bound form of TNF into four different domains. The first domain is in the cytosol and has no apparent signaling activity. The second domain is the transmembrane domain comprised of highly hydrophobic amino acids. This domain also serves no apparent signaling function. The third domain is the extracellular domain that is most proximal to the plasma membrane. This domain serves like a stalk that supports the fourth domain, which is the functional part of the TNF molecule. Cleavage of TNF from the membrane occurs in the third domain at selective sites that are acted upon by metalloproteinases. A specific metalloproteinase involved in the cleavage of TNF has been identified and designated as TNF- α activating converting enzyme (or TACE). TACE cleaves the full membrane bound TNF molecule, releasing the soluble cytokine that is primarily comprised of the fourth domain.

Production of too much soluble TNF cytokine could lead to systemic toxicity, and even death. This condition, termed "toxic shock", is due to the release of soluble active TNF into the systemic circulation, inducing capillary leak, vasodilation, and apoptosis of cells bearing the appropriate TNF receptors. Although this reaction can be lethal and may be of no advantage to the host animal, it may have had some selective advantage in herd immunity, acting to purge seriously infected animals from the population.

Nevertheless, because TNF has this property, the molecule has a relatively limited therapeutic index. There was much excitement when TNF initially was cloned as it was felt to be of potential value in the treatment of patients with cancer. Although early clinical trials with TNF did reveal that this molecule could result in shrinkage of tumors, especially lymphomas, dose escalation of TNF was complicated by unacceptable systemic toxicity. This has precluded the use of soluble TNF in modern day cancer therapy.

It is possible to provide for membrane stabilized members of the TNF family. Identification of the domains in which metalloproteinases cleave membrane TNF allowed for generation of the recombinant molecule lacking this MMP site (termed Δ TNF). This molecule is expressed on the plasma membrane and can effect signaling in contiguous cells that bear one or both of the TNF receptors. Because enzymes other than TACE may act on the surface-bound TNF, even Δ TNF can be released from the plasma membrane, albeit at levels that are significantly less than that of wild-type TNF.

We have generated families of recombinant forms

TNF that have potential MMP sites deleted. These molecules, term IGX-TNF, are highly stable cell surface molecules. To examine the relative stability of each of the TNF molecules on cells transfected to express these molecules, we generated adenovirus vectors that encode wild type TNF, Δ TNF, or IGX-TNF under the control of the strong human cytomegalovirus promoter/enhancer. Transfection of cells with different multiplicity of infection ratios of these vectors generates cells that produce high levels the TNF transcripts. However, the amount of surface TNF varies depending on the type TNF molecule used. Cells transfected with IGX-TNF had the highest surface expression of TNF at any one MOI, whereas cells transfected with wild-type TNF had the lowest. Cells transfected with the Δ TNF had intermediate levels of surface TNF expression. In contrast the amount of soluble TNF generated by each of these transfected was the inverse as that noted for surface TNF expression. Although cells transfected with Δ TNF had ten-fold less soluble TNF in the culture supernatant, the cells transfected with IGX-TNF had negligible amounts of the free cytokine that were one-hundred fold lower than that observed in supernatants of cells transfected with Δ TNF. Functional studies involving these transfectants revealed that the cells with highest levels of surface membrane TNF had the highest activity on cells bearing the TNF receptors.

3. CD40-ligand (CD154)

We observed several years ago that we could modify the phenotype of normal or leukemia B cells via co-culture with highly activated T cells that had been treated with immobilized mAbs specific for CD3 [1]. This activation required cell-cell contact and resulted in the de novo or enhanced expression of several important immune co-stimulatory molecules on the B cell surface. We added mAbs to various cell surface antigens on the T cell or B cell to block this effect. We found that mAb specific for CD40 could interfere with the ability of activated T cells to induce such changes in normal or leukemia B cells.

A critical component to this reaction is the ligand for CD40 (CD154), a member of the TNF family that is expressed on the activated T cell surface. This, along with signals derived from other members of the TNF family, triggers a cascade of events that ultimately results in the leukemia cell expressing significantly higher levels of immune co-stimulatory surface accessory molecules, such as CD54 (ICAM-1), CD80, and CD86. These molecules are critical for inducing a proliferative T cell response to presented antigens. Moreover, these changes allow the leukemic cell to engage non-activated autologous T cells to respond productively to present leukemia-associated antigens.

Like TNF, CD154 can exist as a surface membrane-bound or soluble molecule with activity on cells that bear its receptor, CD40. Also like TNF, membrane MMP can cleave CD154 within domain 3, releasing a molecule that may have agonist activity. High levels of

soluble agonist CD154 can be observed in the setting of certain diseases associated with systemic autoimmune activation or vasculitis, such as systemic lupus erythematosus or rheumatoid arthritis [7,8]. In studies on patients with SLE, we found that the level of soluble CD154 agonist correlated with the extent of disease activity, as assessed using the SLE disease activity index. Although causal data are lacking, there appears to be a strong association between the amount of soluble CD154 and disease activity in SLE.

Clinical trials have been conducted using the soluble active trimeric form of CD154. However, systemic inflammatory changes were noted in patients treated with this molecule that was administered as a subcutaneous injection. At the lowest dose tested, the patients experienced unacceptable hepatic inflammation and other inflammatory effects [9]. Thus, like TNF, soluble CD154 may have limited utility in the treatment of patients with cancer, owing to its low therapeutic index. This is unfortunate, as CD154 is an important molecule for activating APC.

To induce expression of a membrane-bound form of CD154, we generated a crippled adenovirus that carried the gene encoding a recombinant form of CD40-ligand, designated Ad-CD154. Infection of CLL B cells with Ad-CD154 caused dramatic changes in the leukemia-cell phenotype. Within 18 hours of infection, the CLL B cells started expressing immune co-stimulatory molecules that are critical for inducing a vigorous immune response. Also, factors that render the leukemia B cells tolerogenic were down-modulated by infection with Ad-CD154. Such modified leukemia cells became highly effective stimulators in autologous mixed lymphocyte reactions and could induce generation of cytotoxic T lymphocytes (CTL) specific for autologous non-infected leukemia cells *in vitro* [10].

4. Phase I Clinical Trial in Immune Cell Therapy

Because Ad-CD154-infected CLL B cells could induce autologous T cells to generate CTL against the patient's leukemia cells *in vitro*, we conducted a phase I clinical trial for gene therapy of CLL [11]. Leukemia cells were harvested by leukapheresis and then infected *ex vivo* with Ad-CD154 in a good-manufacturing practice (GMP) facility. The cells were examined for expression of the CD154 and immune-co-stimulatory molecules. After sterility testing, some of the modified leukemia cells were administered back to the same patient as a single intravenous injection given over a few minutes. This strategy allowed us to conduct a dose-escalation study, in which we could infuse defined numbers of leukemia cells that expressed defined amounts of the CD154-transgene.

The biologic effects of this treatment were encouraging. Within 24-48 hours after receiving the modified cells, nearly all patients had measurable increases in plasma cytokines, such as IL-12, IFN- γ , and/or IL-6. All the CLL B cells in the blood of the treated patients started expressing low levels of immune co-stimulatory

molecules, similar to what we had observed with the bystander effect noted *in vitro*. This was noted one to two days after treatment, and lasted for several days, if not longer. Such immune co-stimulatory molecules were not induced on non-infected CLL B cells that were incubated in plasma from the treated patients, indicating that a soluble factor was not responsible for this effect.

The clinical effects of this treatment were encouraging. Most of the patients experienced acute falls in the blood leukemia cell counts within the first few days after treatment. Subsequently the lymphocyte count returned to approximately 60% of pre-treatment levels. However, not all the blood lymphocytes that returned were CLL B cells. In nearly all treated patients we noted significant increases in the absolute numbers of both CD4⁺ T cells and CD8⁺ T cells at 1 week after treatment, sometimes to more than 4 times that of pre-treatment levels. Moreover, T cells reactive against autologous leukemia cells were increased after therapy, as assessed via mixed lymphocyte reactions and ELISPOT analyses. In addition, circulating bystander, non-infected CLL cells were induced to express Fas (CD95), a member of the tumor necrosis factor (TNF) receptor family that can induce apoptosis via a procaspase 8-dependent pathway.

Although the mechanism(s) responsible for the noted reductions in tumor cell burden were not resolved, it is conceivable that the sustained expression of CD95 induced on the entire leukemia cell population rendered it susceptible to Fas-mediated apoptosis by cells bearing the Fas-ligand (FasL), such as activated natural killer (NK) cells or activated T cells. Indeed, the noted clearance of leukemia cells in the days following infusion of autologous CD154-expressing CLL cells was more acute than what we would have anticipated following infusion of a vaccine intended to induce anti-leukemia immunity. We did find evidence for CD4⁺ T cells that could induce apoptosis of CD40-activated, but not resting, autologous or allogeneic CLL cells. CTL-mediated apoptosis was not inhibited by monoclonal antibodies (mAb) specific for class I antigens of the major histocompatibility complex (MHC), but could be inhibited by mAbs specific for FasL. This was surprising in view of the previously noted resistance of CD40-activated CLL cells to Fas-mediated apoptosis.

To investigate whether expression of FasL was sufficient to induce cytolysis of CD40-activated CLL cells, we used Chinese Hamster Ovary (CHO) cells transfected with FasL as cytotoxic effector cells [12]. This also allowed us to explore the kinetics of acquired sensitivity to Fas-mediated apoptosis in CLL following CD40-activation. CD40-activated CLL cells were initially resistant to CD95-mediated apoptosis despite high-level expression of CD95. However, after 72 hours, CLL cells became increasingly sensitive to CD95-mediated apoptosis. This correlated with a progressive decline in FLIP and TRAF1 protein levels, which were induced within 24 hours after CD40-ligation. Down-regulation of FLIP with an anti-sense oligonucleotide or a pharmacologic agent, however, was not sufficient to render CLL cells sensi-

tive to CD95-mediated apoptosis in the 24-72 hours after CD40-activation. Although the levels of pro-Caspase-8 appeared sufficient, inadequate levels of FADD and DAP3 may preclude assembly of the death-inducing signaling complex (DISC). Seventy-two hours after CD40-ligation, sensitivity to CD95 and a progressive increase in FADD and DAP3 were associated with the acquired ability of FADD and FLIP to co-immunoprecipitate with the DISC following CD95-ligation. This demonstrates CD40-ligation on CLL B cells induces a programmed series of events in which the cells initially are protected and then sensitized to CD95-mediated apoptosis through shifts in the balance of the anti-apoptotic and pro-apoptotic proteins FLIP and FADD. Such a mechanism may account for some of the noted clearance of bystander CLL cells that are activated through contact with CD154-expressing CLL cells *in vivo*.

The CLL B cell counts of many of the treated patients remained at or below treatment levels for several weeks, if not longer. One to two weeks after gene therapy, nearly all of the treated patients experienced significant reductions in lymph node size lasting for more than several weeks. Several of the treated patients still have required additional therapy for their CLL now two years later. As such, this strategy may have activity even in patients who have advanced disease with high leukemia cell counts and diffuse adenopathy. More pronounced clinical effects are anticipated with repeat dosing, which is being examined in a current phase II clinical study being conducted at the University of California, San Diego and Harvard Medical School.

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