 Immunotherapy and Gene Therapy of Cancer

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Abstract

In the past decade, immunotherapies have been developed that are capable of causing prolonged cancer regressions in selected patients with advanced metastatic disease. In the past year, attempts at the gene therapy of cancer have begun. These experimental cancer treatments deserve vigorous exploration.

The last decade has witnessed the rise of new biological treatments for cancer based on stimulating natural immune reactions against the disease. These treatments, collectively referred to as biological therapy, are beginning to join surgery, radiation therapy, and chemotherapy as effective means for treating cancer (1, 2). Biological therapy can be defined as cancer treatments that act primarily through natural host defense mechanisms or by the administration of natural mammalian substances. An increased understanding of the cellular immune system as well as new developments in biotechnology have made new approaches to biological therapy feasible. Using recombinant DNA technology, many biological compounds have become available in large amounts for the first time.

One approach to the development of biological therapies for the treatment of cancer has involved attempts to identify immune cells in tumor-bearing animals and patients that can distinguish cancer from normal cells and can be used in adoptive immunotherapy. Adoptive immunotherapy can be defined as the transfer to the tumor-bearing host of immunological reagents such as immune cells that have antitumor reactivity and can mediate, either directly or indirectly, antitumor effects. The major obstacle to the development of effective adoptive immunotherapies has been the inability to identify cells with specific antitumor reactivity that could be generated in large enough numbers for use in cancer treatment.

In the past decade, however, several cell types have been identified that can cause the regression of cancer in some patients. We began our work in this field with the description of TIL cells which were non-MHC-restricted killer cells capable of lysing fresh tumor but not fresh normal cells in culture. These studies led us to the description of more specific antitumor cells called TIL that were capable of recognizing unique antigens on tumor cells. More recently we have begun genetic modification of these TIL in an attempt to increase their antitumor reactivity.

Development of Interleukin 2-based Immunotherapies for Cancer

The difficulty in growing T-lymphocytes in culture for immunological studies represented a major impediment to the development of immunotherapies for the treatment of cancer. This difficulty was largely overcome by the description of a hormone, originally called T-cell growth factor, which was capable of causing the growth in culture of T-lymphocytes activated either by specific antigen or by lectins. This growth factor, subsequently renamed IL-2, opened new possibilities for expanding T-lymphocytes with antitumor activity for use in adoptive immunotherapy. In studies attempting to isolate T-cells from growing tumors we noted that lymphocytes exposed to IL-2 developed the ability to kill fresh tumor cells but not fresh normal cells in 4-h chromium release assays (3, 4). Further studies revealed that lymphocytes from tumor-bearing as well as normal hosts in both the mouse and the human developed this lymphokine-activated killing activity for fresh cancer cells. In vitro these LAK cells showed ubiquitous non-MHC-restricted killing of target cells altered either by malignant transformation or by growth in culture.

Shortly after the description of this in vitro activity we began extensive studies of the antitumor effects of the administration of IL-2 alone or IL-2 plus LAK cells (5-7). A summary of the findings in experimental animals using either IL-2 alone or LAK cells plus IL-2 is shown in Tables 1 and 2. These experimental studies guided the development of the clinical protocols that followed. In these murine studies we demonstrated that treatment with either high dose IL-2 alone or LAK cells plus IL-2 could mediate the regression of established liver and lung metastases in a variety of tumor models. Each of these effects was dose related and using each dose of IL-2 more extensive antitumor effects were seen with the combined administration of LAK cells. The mechanism of action of these immunotherapy treatments was shown to depend on the activation of endogenous CD8+ cytolytic T-cells and LAK cells, as well as the expansion in vivo of adoptively transferred lymphocytes. Thus a basic principle of adoptive immunotherapy was established in which IL-2-dependent lymphocytes with antitumor reactivity were used to treat the tumor-bearing host. Continued administration of IL-2 led to the in vivo expansion of these lymphocytes which persisted as long as IL-2 administration continued. Following the discontinuance of IL-2 administration these IL-2-dependent antitumor cells died in vivo.

Our first studies of the in vivo administration of IL-2-dependent cells were performed in three patients with advanced sarcomas who received cells labeled with either 51Cr or 111In to monitor their in vivo distribution (4). It was found that these adoptively transferred IL-2-dependent cells distributed first to the lung followed by slow clearance to the liver and spleen. Although murine studies demonstrated that the administration of both IL-2 and LAK cells was necessary for maximal antitumor effects, substantial practical difficulties existed in the application of these findings to humans with advanced cancer. The very tiny amounts of IL-2 that were available precluded the administration of significant amounts to humans and also precluded the generation of LAK cells in sufficiently large amounts for use in human treatment. We thus began a series of Phase I studies in which naturally derived IL-2 from a high producer Jurkat cell line was administered to patients (8, 9). We also generated killer cells using exposure to phytohemagglutinin and administered these cells to patients in Phase I studies (10).
The immunotherapeutic effect of IL-2 on 10-day macrometastases is mediated by asialo-GM1-positive LAK cells. In immunogenic tumors, Lyt-2-positive cells also participate.

High dose IL-2 administration leads to in vivo lymphoid proliferation in visceral organs. These cells have LAK activity in vitro.

A direct relationship exists between the dosage of IL-2 and therapeutic effect.

The immunotherapeutic effect of IL-2 on 3-day micrometastases is mediated by Lyt-2-positive cells.

The immunotherapeutic effect of IL-2 on 10-day macrometastases is mediated by Lyt-2-positive cells.

Inmunosuppression with irradiation or cyclophosphamide can inhibit IL-2 therapy against 3-day metastases but can enhance the effects of IL-2 on 10-day macrometastases.

The sensitivity of macrometastases to therapy involving IL-2 appears to be directly related to the expression of MHC antigens (Class I) on the tumor.

The administration of IL-2 can enhance the therapeutic effect of concomitantly administered LAK cells, TIL, and specifically sensitized T-lymphocytes.

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Table 1 Immunotherapy of murine tumors with IL-2 alone

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>IL-2 alone</th>
<th>IL-2 + LAK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>CR + PR (%)</td>
<td>CR + PR (%)</td>
</tr>
<tr>
<td>Renal</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Melanoma</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>Colorectal</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Breast</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>16</td>
</tr>
</tbody>
</table>

* Includes all treated patients except one lost to follow-up and one who died of therapy.

** CR, complete response; PR, partial response.

† Includes all treated patients except one lost to follow-up and one who died of therapy.

‡ Two patients each with hepatoma and brain cancer; one each with ovary, pancreas, and uterus cancer.

§ One patient each with cancer of brain, esophagus, ovary, testes, thyroid, gastrinoma, unknown primary and two patients with Hodgkin’s lymphoma.

The cloning of the gene for IL-2, its insertion into bacteria, and the availability of very large amounts of purified recombinant IL-2, however, opened many new possibilities for the treatment of patients with these approaches. Phase I studies were conducted using either natural or recombinant IL-2 alone in 39 patients and using either phytohemagglutinin-activated killer cells or LAK cells alone in 27 patients with advanced cancer to determine the safe tolerable doses of each of these reagents (8–10). These Phase I studies have been reported in detail and served as the basis for the use of combinations of IL-2 and LAK cells in patients with advanced cancer. None of the 66 patients with advanced cancer treated in these Phase I trials responded to treatment.

In late 1984, we began studies combining treatment with LAK cells plus IL-2 in patients with advanced cancer and it was with this treatment that we observed our first antitumor responses in humans (11–14). In accord with lessons learned in animal models, LAK cells incubated for 3 or 4 days in IL-2 were administered in patients along with IL-2 given by bolus administration every 8 h. An update of the results of 180 patients treated as of 1990 with IL-2 plus LAK cells is shown in Table 3. Antitumor responses were seen in patients with advanced melanoma, renal cell cancer, colon cancer, and non-Hodgkin’s lymphoma. Approximately 10% of patients with advanced melanoma and renal cell cancer experienced a complete remission of their cancer. As experience accumulated with the use of high dose IL-2 we began to see responses with IL-2 administration alone and these results are summarized in Table 3.

These studies demonstrated that it was indeed possible to use strictly immune manipulations to mediate the regression of advanced cancer in humans. Prior studies with α-interferon had demonstrated tumor regression in selected patients with renal cell cancer although the direct antiproliferative effect of α-interferon made it unclear as to whether the antitumor effects were seen because of an alteration in host immune reactivity or the direct impact of α-interferon on the growing tumor (1, 2). IL-2 itself has no direct antitumor activity in any in vitro model and thus treatment with IL-2 or IL-2 plus LAK cells represented a true result of biological therapy in that it mediated its antitumor effects through natural host defense mechanisms.

Tumor-infiltrating Lymphocytes

Although LAK cells could mediate antitumor effects, both in vitro and in vivo, we continued our search for more potent...
lymphocytes that might be used in adoptive immunotherapy. Our specific interest was the identification of T-cells that could recognize specific tumor antigens in a MHC-restricted fashion. Techniques were developed for isolating TIL from resected tumors and these cells provided evidence of specific tumor antigen recognition by lymphocytes obtained from a tumor-bearing host (15–18). These cells were isolated by culturing single cell suspensions from tumors in IL-2. Infiltrating lymphocytes that bore IL-2 receptors, presumably because of their reactivity to tumor, grew in the presence of IL-2 and destroyed tumor cells simultaneously growing in the culture. Thus, 2 to 3 weeks after initiating cultures pure populations of TIL could be established without the presence of contaminating tumor cells. We established techniques for isolating TIL from both mouse and human tumors and extensively studied these cells in vitro as well as in tumor models in mice. In the mouse, TIL cells were exclusively CD8+ although in the human both CD4+ and CD8+ T-cells grew in culture. In the mouse, specific recognition of tumor antigens could be identified on the basis of specific lysis of target cells as well as by specific cytokine secretion when TIL were cocultivated with the type of tumor cells from which they were derived (19–21). These reactivities were MHC restricted and could be inhibited by monoclonal antibodies against Class I antigens.

Extensive studies were conducted in animal models of the antitumor effects of tumor-infiltrating lymphocytes. These studies are summarized in Table 4. Titration of TIL in mice with established lung and liver metastases indicated that these cells were from 50 to 100 times more potent than LAK cells in conjunction with IL-2. In advanced tumor models, TIL plus IL-2, but not LAK cells plus IL-2 in conjunction with cyclophosphamide, was effective in eliminating established metastases from the lung and liver.

Following these early studies with TIL cells, attempts were made to find ways to improve their antitumor efficacy in mice. Because of our finding that TIL might not be effective against tumors that expressed very low levels of Class I antigens we transfected genes coding for Class I antigens into tumors and showed that TIL raised from these tumors could mediate their regression as well as the regression of the parental non-Class I expressing tumor after treatment of the mouse with γ-interferon. Gamma interferon was shown to up-regulate Class I MHC antigens in vivo and thus the vital role played by MHC expression in these tumor immunotherapies was established (22–26). We further showed that local tumor irradiation could substitute for cyclophosphamide in synergizing with TIL and IL-2 (27). The reason for the need for this local tumor treatment, either with cyclophosphamide or irradiation is not clear. It is possible that these treatments result in elimination of suppressor cells or increase the traffic of immune cells to the tumor site. The cytoadherent effect of these treatments may also play a role in their effectiveness.

More recently, improved techniques for generating TIL with antitumor activity in vivo have been established. The use of low dose IL-2 (60 to 120 IU/ml) generates cells with more specific cytolytic activity and more effectiveness in vivo than the use of high dose IL-2 (6000 IU/ml) (21). The long term persistence of TIL in mice cured by the in vivo injection of TIL has been demonstrated using congenic Thy-1.1 TIL in Thy-1.2 mice bearing syngeneic tumors (28). Extensive efforts to identify the characteristics of TIL in mice that correlate with antitumor reactivity have shown that specific cytokine secretion rather than specific lysis is most critical (20). Thus these studies of the in vivo antitumor activity of TIL in murine models have played an important role in guiding the design and conduct of human trials with TIL in patients with advanced cancer.

Based on these animal models, pilot studies have begun the treatment of patients with advanced metastatic melanoma using tumor-infiltrating lymphocytes (29). The results of our first 50 patients with advanced melanoma treated with TIL are shown in Table 5. Thirty-eight % of patients have responded to treatment. Interestingly, similar response rates were seen in patients previously failing high dose IL-2-based therapy compared to those that had previously not been treated with IL-2. TIL have been valuable for studying the nature of the immune response of humans to their cancers. From approximately one-third of patients with melanoma, TIL with specific cytolytic activity for the autologous tumor and not autologous normal tissues can be isolated (16–18). More recently we have shown that specific cytokine secretion can also identify immune responses in patients with growing malignancy. Specific cytokine secretion has identified immune cells in patients with melanoma and breast cancer.

TIL have been valuable in studying the nature of shared antigens among patients with cancers of similar histology. Extensive studies of TIL from patients with melanoma tested against a panel of allogeneic melanomas with known HLA subtypes have demonstrated that melanomas from different individuals appear to share common tumor antigens that are restricted by a specific HLA specificity (30). The HLA-A2 genotype appears to be particularly common as a restriction element in the recognition of melanoma antigens. To further demonstrate the nature of the shared antigens in patients with melanoma, we have transfected the gene for HLA-A2 into

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**Table 5 Immunotherapy of murine tumors with TIL and IL-2**

<table>
<thead>
<tr>
<th>Objective response</th>
<th>PR + CR (number of patients)</th>
<th>%</th>
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<tr>
<td>No previous IL-2</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>IL-2 + CY</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>IL-2 (No CY)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Previous IL-2</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

* Excludes patients with brain metastases at start of treatment (all NR).

† NR, no response; PR, partial response; CR, complete response; CY, cyclophosphamide.

‡ Excludes two patients who received IL-2 at 30,000/kg; both were NR.

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* NR, no response; PR, partial response; CR, complete response; CY, cyclophosphamide.
melanomas and have shown that 6 of 6 different melanomas transfected with the gene for HLA-A2 are recognized by a single HLA-A2-restricted TIL (31). It thus appears that antigens shared among melanomas can be recognized by TIL and possibilities for the use of these antigens for immunization of patients against cancer appear feasible. Using TIL as a recognition system we are currently in the process of attempting to clone the gene that codes for tumor antigens in both the mouse and human by transfection of genes from sensitive to resistant cell lines. Should such genes be cloned they could be inserted into vaccinia or other viruses for use in the immunization of patients against cancer antigens.

Finally, studies with TIL have demonstrated that these cells recirculate and specifically localize in cancer deposits. Up to 0.015% of all injected TIL localized per g of tumor based on in vivo studies in humans using TIL labeled with $^{111}$In (32, 33).

Thus, TIL cells have proved to be a valuable resource not only for the study of the immune reaction of patients against their tumors but also for use in immunotherapy. Extensive efforts under way to improve upon immunotherapy with TIL are listed in Table 6. Additional efforts include: enhancing in vivo effectiveness of existing TIL or to generate more effective TIL either by growing more effective cells, by genetically modifying TIL, or by raising TIL from genetically modified tumor. In addition studies are under way to study these IL-2-based immunotherapies in conjunction with other treatments such as chemotherapy and radiation therapy. New recombinant cytokines are being tested for antitumor reactivity. We have recently published our results showing that IL-6 has antitumor activity when administered to tumor-bearing mice (34) and clinical trials with IL-6 will be undertaken shortly.

**Gene Therapy of Cancer**

The ability to introduce and express foreign genes in eukaryotic cells has opened new possibilities for the therapy of cancer. Our first attempts at the gene therapy of cancer involved modification of TIL by the insertion of marker genes (35, 36). More recently genes coding for cytokines have been introduced into TIL.

In the first phase of these studies we inserted into TIL a bacterial gene coding for neomycin phosphotransferase which could induce resistance to the antibiotic neomycin and enable us to differentiate adaptively transfected TIL from endogenous host lymphocytes (35, 36). The goal of these studies was to demonstrate the feasibility and safety of using retroviral-mediated gene transfer to introduce genes into humans and to study the long term distribution and survival of autologous TIL.

Because of the practical, safety, and ethical issues involved with human gene transfer and the possible consequences of using a modified retrovirus derived from the Moloney murine leukemia retrovirus randomly integrating a new gene into the human genome, our clinical studies utilizing human TIL transduced with the gene coding for neomycin resistance (NeoR) were preceded by extensive in vitro studies and were extensively reviewed by a variety of review groups composed of clinicians, scientists, ethicists, and lay people. In these studies we demonstrated that the gene could be successfully inserted into human TIL, that the properties of human TIL were not altered, that the gene was expressed in TIL, and that these procedures could be performed with little risk to the patient and no risk to health care personnel and the public. Retroviral mediated gene transduction of TIL was selected because of the high efficiency of gene transfer using this technique (37, 38).

Studies performed on TIL from multiple cancer patients as well as the 10 patients who have now received NeoR gene-modified TIL demonstrated that the NeoR gene could be inserted into human TIL, that it was satisfactorily expressed, and that the general properties of the TIL were not changed (35, 36). Semiquantitative Southern blots indicated that approximately one viral genome copy was present in the gene-transduced TIL. The phenotype and cytotoxicity of the transduced and nontransduced cells were similar. Analyses of T-cell receptor gene rearrangements revealed the oligoclonal nature of the TIL population and no major changes in the DNA rearrangement patterns or the level of mRNA expression of the $\beta$ and $\gamma$ chains following transduction and selection of TIL in the neomycin analogue G418 (35). Similarly, cytokine mRNA expression was not significantly altered following the transduction of TIL.

Transduced TIL consistently showed resistance to G418, a neomycin analogue lethal to all eukaryotic cells. The polymerase chain reaction technique was utilized to detect the NeoR genome. Approximately one transduced cell in $10^6$ normal cells could be consistently detected (36).

Safety considerations were paramount in our consideration of the use of this gene transduction technique for introducing genes into TIL for use in patient therapy. Extensive safety studies were performed on all transduced TIL including tests for aerobic and anaerobic bacteria, fungi, and *Mycoplasma* and tests using sarcoma-positive, leukemia-negative assays for ecotropic, xenotropic, and amphotropic infectious viruses. Amplification tests using NIH 3T3 cells were performed and were shown to be capable of detecting a single viral particle per ml of solution. All safety tests performed on TIL in preclinical studies as well as on TIL from the patients who received the gene-modified cells were negative and no safety problems of any kind were detected (36). Gene-modified cells could be detected in the circulation up to 189 days and in tumor deposits up to 64 days after the infusion of gene-modified TIL (36).

Laboratory and clinical studies using TIL transduced with the gene for neomycin resistance demonstrated that retroviral mediated gene transduction was a feasible and safe means for introducing genes into humans. Because of the accumulation of TIL at tumor deposits we have conducted studies attempting to modify human TIL with genes that can improve the antitumor effectiveness of these cells. The first gene that we have selected for these studies is the gene for TNF.

Extensive animal research in the Surgery Branch, National Cancer Institute, and in many other groups have demonstrated...
that the injection of recombinant TNF can mediate the necrosis and regression of a variety of established murine cancers. However, tumor-bearing mice can tolerate up to 400 µg/kg TNF and these doses are required to mediate tumor regression; the administration of less TNF is far less effective. In contrast, the maximum tolerated dose of TNF in humans in both Surgery Branch, National Cancer Institute, and other studies is approximately 8 µg/kg day (1). Thus when given i.v. injections humans can tolerate only 2% of the TNF dose required to mediate antitumor effects in the mouse and this appears to explain why antitumor effects have not been seen when TNF was administered systemically to humans.

We have thus sought means to selectively increase the local concentration of TNF at the tumor site. Because TIL can traffic directly to tumor deposits and concentrate at those sites (32, 33) we have hypothesized that TIL that are genetically modified to produce large amounts of TNF may generate high TNF concentrations in the local tumor microenvironment and thus exhibit an increased antitumor effect compared to normal TIL.

The retroviral vector construct selected for insertion of the TNF gene into human TIL contains the TNF gene promoted by the murine long terminal repeat and the neomycin resistance gene promoted by the SV40 early promoter region. The two-gene retroviral construct was introduced into the PA317 producer cell line and the supernatant from this line was used to transduce the TIL from patients with advanced metastatic melanoma.

Although it has been relatively easy to express cytokine genes in tumor cells and demonstrate production of large amounts of TNF, difficulties exist in the expression of cytokine genes in lymphocytes. It has been difficult to achieve consistently high levels of cytokine production in lymphocytes using these retroviral vectors probably because regulatory mechanisms exist for cytokine transcription, translation, and/or secretion in lymphocytes that do not exist in other cell types. We have, however, been able to achieve the expression of TNF genes in lymphocytes from selected patients and have achieved cytokine secretion in excess of 100 pg/10^6 cells/24 h.

We have begun clinical studies using these TNF gene-modified TIL in patients with advanced cancer. Increasing numbers of TNF-transduced TIL are being administered, first in the absence of IL-2 and then when safely tolerated doses are achieved, in conjunction with IL-2. To the present time four patients with advanced melanoma have been treated with the TNF gene-modified TIL. No side effects have been seen at cell doses which have ranged up to 10^11 cells in a single infusion. A large series of patients treated with gene-modified TIL plus IL-2 will be required to know whether or not this approach gives improved results compared to the use of unmodified TIL. We currently have permission to treat up to 50 patients with TNF gene-modified TIL utilizing this approach.

Additional genes being studied for insertion into TIL to improve their antitumor activity include γ-interferon, IL-2, IL-6, and genes for chimeric T-cell receptors.

More recently we have begun studies investigating the use of genetic modification of tumor cells to increase their immunogenicity. We and others have demonstrated that insertion of cytokine genes can increase the immune recognition of tumor cells and can lead to the production by the host of cytolytic cells that are not produced in response to the parental non-modified tumor (39-43). These experiments have been conducted by a variety of groups and include insertion of genes for IL-4, IL-2, tumor necrosis factor, γ-interferon, and granulocyte-macrophage-colony-stimulating factor. In related experiments we have shown that insertion of the gene for Class I major histocompatibility antigens can also increase the immunogenicity of tumors and lead to the generation of TIL that cannot be produced from low MHC-expressing tumors (26).

This approach to gene therapy, by genetically modifying tumors for use in immunization, holds promise not only for active immunotherapy but also for the development of immune cells that might be used in adoptive immunotherapy.

In the last decade biological therapy has been shown to be capable of mediating the regression of established cancers in some patients with advanced malignancy. The continued development of these biological approaches offers the hope that they can lead to the development of effective, safe, and practical treatments for patients with cancer.

References


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