Acquired Erythropoietin Responsiveness of Interleukin-2–Dependent T Lymphocytes Retrovirally Transduced With Genes Encoding Chimeric Erythropoietin/Interleukin-2 Receptors

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Adoptive immunotherapy with tumor-infiltrating lymphocytes (TILs) causes regression of some human tumors. However, the sustained proliferation and antitumor activity of TILs requires the coadministration of potentially toxic amounts of interleukin-2 (IL-2). In an effort to overcome the requirement by T cells for IL-2, we have introduced alternative growth factor receptors that use the relatively nontoxic cytokine erythropoietin (Epo) as a ligand. In our model system, the coexpression of chimeric receptors consisting of the extracellular portion of the Epo receptor (EpoR) and the intracellular portions of the IL-2 receptor subunits, β and γ, conferred Epo responsiveness on a T-cell line. By contrast, cells expressing the wild-type EpoR did not proliferate in response to Epo. This suggested that Epo binding caused the activation of an IL-2 signal pathway mediated by the chimeric receptors. This approach can be used to minimize toxicity and potentially improve cancer immunotherapy with TILs.

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TUMOR-INfiltrATING lymphocytes (TILs) are derived from solid tumors by culturing single cell suspensions of tumor in vitro in recombinant human interleukin-2 (IL-2). The adoptive transfer of TILs plus the systemic administration of IL-2 can mediate tumor regression in selected patients with advanced cancer. A major limitation to the successful application of this treatment results from toxicity associated with IL-2 administration, which is required to stimulate the proliferation and antitumor activity of the adoptively transferred lymphocytes. We are studying ways to genetically modify TILs to alter their dependence on IL-2 and substitute it with reactivity to a molecule that can be systematically administered with little toxicity.

Recombinant human erythropoietin (Epo) has been well-characterized and is tolerated with minimal toxicity when administrated in vivo. Chemical cross-linking studies suggest that the Epo receptor (EpoR) exists as a multimeric complex with other membrane proteins that are actively involved in the transduction of the Epo signal through interaction with the extracellular region of EpoR. This suggested that the ligand-induced dimerization of wild-type EpoR itself is crucial for triggering the Epo signal. Similarly, dimerization of the β and γ subunits of the IL-2 receptor (IL-2R) may be essential for triggering the IL-2 signal. Therefore, the formation of chimeric molecules using the EpoR and the IL-2R subunits might allow triggering of the IL-2R using Epo as a ligand.

In this study, we attempted to confer the ability to respond to Epo on a mouse IL-2–dependent T-cell line, CTLD, as a model for human TILs, by introducing recombinant retroviruses expressing either human EpoR or human EpoR–IL-2R subunit chimeric receptors. Our studies showed that coexpression of the chimeric EpoR/IL-2R, but not the wild-type EpoR, in T cells could provide dependence on an alternative cytokine, Epo.

MATERIALS AND METHODS

Cell culture. CTLD-D cell (from Dr Z. Eshhar, Weizmann Institute, Rehovot, Israel), an IL-2–dependent murine T-cell line, was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) bovine calf serum and 50 U/mL of recombinant human IL-2 (IL-2 concentration is expressed as Cetus U/mL; Cetus, Emeryville, CA). D3 cell, (from Dr J.H. Pierce, National Institutes of Health, Bethesda, MD), was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) bovine calf serum and 10% Wehi 3B supernatant.

Retroviral vector construction. The pYM vector used for receptor gene expression was initially constructed with the IL-2Ra. pYM/IL-2Ra was constructed by replacing the cytomegalovirus promoter of pLNCX with human elongation factor α (Efa) enhancer-like and promoter regions and IL-2Ra cDNA. Briefly, pLXSNIIL-2Ra was constructed by inserting a 0.9-bp blunt-ended XhoI fragment encoding human IL-2Ra from the plasmid CD8/Tac into the Hpa I site of pLXSN. A 0.9-kb cDNA fragment for IL-2Ra was excised from pLXSNIIL-2Ra by EcoRI and DhoI digestion in which the Hpa I site was blunt-ended. A 0.4-kb Efa enhancer-like fragment was excised using Bgl II and Xho I from pEFα. A 0.5-kb Efa promoter fragment was obtained by the polymerase chain reaction using the oligonucleotides, 5'TTGGAGCTCGAGCAGCAATGG-CGGAGGAGAGATCTGTC3' and 5'TTTCTGGAATTCCACCGTGTTCCGAAAAAGAACGTTCACG3', followed by the Xho I and EcoRI digestion at the ends. A backbone fragment of pLNCX was obtained by BamHI and Hpa I digestion. Then, three fragments of the Bgl II-Xho I Efa enhancer-like fragment, Xho I-EcoRI Efa promoter fragment, and EcoRI–blunt-end IL-2Ra fragment were inserted into the Hpa I-BamHI backbone fragment. For pYM vector construction, a BamHI-Cla I fragment containing the IL-2Ra exon was removed from pYM/IL-2Ra, and then the backbone fragment was blunt-ended and recircularized to give a unique BamHI cloning site downstream of the Efa promoter in pYM vector.

Construction of EpoR–IL-2R subunit chimeric receptors. pYM1 EpoR was constructed by inserting a HindIII-Pvu fragment of pERHC into Dr D.M. Williams, Johns Hopkins School of Medi...
The chimeric EpoR/IL-2R subunit receptor constructs were made as follows. DNA fragments encoding the transmembrane and intracellular region of IL-2Rα or IL-2Rγ and the extracellular region of EpoR were isolated from pEFβ, pIL-2Rγ2, and pYM/EpoR, respectively, and were subcloned into pUC19 (pUCIL-2Rαint, pUCIL-2Rγint, and pUCEpoRext), and the extracellular region of EpoR was connected with the transmembrane and intracellular region of IL-2Rα or IL-2Rγ and inserted into the BamHI site of pYM. More precisely, cDNA for the transmembrane and intracellular region of IL-2Rγ was isolated in two fragments, a 163-bp EcoRI fragment and an 801-bp XhoI-BamHI fragment from pEFβ. The BamHI site was located in the 5′ end of the IL-2Rγ cDNA insert and was derived from pSP64 linker sequence, and these two fragments were then inserted into the KpnI-BamHI site of pUC19 (pUCIL-2Rαint) using pCGATACTCATTTCCGTTGCTC and pGCGACGGGAGAATGATATCGGTA, which create an EcoRV site at the S′ boundary of the transmembrane region of IL-2Rα. The BamHI fragment was first cut out of pIL-2Rγ2 with BglII, and a BamHI linker was attached, and again this fragment was digested with BamHI and XmnI. The resultant 430-bp XmnI-BamHI fragment was inserted into the KpnI-BamHI site of pUC19 (pUCIL-2Rγint) using the adaptors pCGATACTCC and pGGATATCCTTGGTAC, which create an EcoRI site at the 5′ boundary of the transmembrane region of IL-2Rγ. EpoR fragments encoding the extracellular region of EpoR (a 252-bp BamHI-XhoI fragment and a 497-bp XhoI-AvaI II fragment) were isolated from pYM/EpoR and ligated into the KpnI-BamHI sites of pUC19 (pUCEpoRext) using pGACCCCCAGCTGGGTAC and pCCAGCTGGGG, which create a PvuI site at the 3′ boundary of the extracellular region of EpoR. The chimeric receptors were then constructed by insertion of an XhoI-PvuI II fragment from pUCEpoRext and an EcoRV-BamHI fragment from pUCIL-2Rαint or pUCIL-2Rγint into the BamHI-XhoI 1 sites of pUC19 (pUCEpoR or pUCEpoRγ), finally, pYM/EpoRα and pYM/EpoRγ were constructed by inserting BamHI fragments from pUCEpoRα or pUCEpoRγ into the BamHI site of pYM, respectively (pYM/EpoRα and pYM/EpoRγ).

Production of retroviral producer line. The retroviral vectors were transfected into PA31772 in the presence of 8 μg/mL of polybrene (Aldrich, Milwaukee, WI) using virus transiently expressed in an ecotropic packaging line GP + E8660 that was transfected with the corresponding plasmid vector by calcium-phosphate method. The transfected PA317 cells were selected in 400 μg/mL of Geneticin (Life Technologies, Gaithersburg, MD), and the amphotropic virus producer clones were chosen on the basis of high virus titer. To get the ecotropic virus producer lines, GP+E86" that was prepared by the transduction mouse IL-3-dependent Epo-dependence (data not shown).29 We initially evaluated if expression of EpoR in T cells would confer Epo dependence. Although several groups reported that EpoR was not functional in IL-2Rα cDNA as a reporter gene and was detected by anti-IL-2Rα immunostaining. Consequently, EpoR was expressed using a human Epo R receptor.20 First, we constructed a pLXS construct, which is broadly used in studies of gene therapy. The fluorescence-activated cell sorter (FACS; Becton Dickinson, Mountain View, CA) profiles showed that the expression of IL-2Ra gene was greater in the cells infected with virus from pYM construct as compared with those from pLXS construct in CTL-D cells (data not shown).

Expression of EpoR in IL-2-dependent T cells. We initially evaluated if expression of EpoR in T cells would confer Epo dependence. Although several groups reported that EpoR was not functional in IL-2Rα or IL-2Rγ cDNA as a reporter gene and was detected by anti-IL-2Rα immunostaining. Consequently, EpoR was expressed using a human Epo R receptor.20 First, we constructed a pYM/EpoR and showed that it could direct the expression of functional EpoR by converting transduced mouse IL-3-dependent 3D cells43 to Epo dependence (data not shown).25 Subsequently, the EpoR virus was used to infect T-cell D cells [termed T-cell (D) (EpoR)], and the cells were analyzed for expression of EpoR by FACS (Fig 2a) using the antihuman Epo R monoclonal antibody, 16.5.1.24 The responsiveness to Epo was measured by [3H]thymidine uptake (see Fig 4). Despite surface expression of EpoR, proliferative response to Epo was not observed in
Fig 1. Structures of the retroviral vector pYM and EpoR-IL-2Rβ or γ chimeric receptors. The retroviral vector pYM was constructed by replacing the cytomegalovirus promoter segment of pLNCX with human EFα enhancer-like sequence and promoter segment. The pYM EβR and pYM EγR vectors were made by insertion of the extracellular portion of EpoR and transmembrane and intracellular portions of either IL-2Rβ or IL-2Rγ into the BamH1 site of pYM just downstream of the EFα promoter. Construction creates an extra sequence, CAGATC (Gln, Ile), at the junction between the extracellular region of EpoR and the transmembrane region of IL-2Rβ or IL-2Rγ.

Fig 2. Cell-surface expression of receptors is shown. Cells were stained with anti-EpoR antibody 7.9.2 as a first step and with phycoerythrin-labeled goat anti-mouse IgG as a second step and were analyzed using FACS. Dotted line shows second-step staining alone.
Epo selection and growth profile of the CTL-D infectants.

Therefore, two chimeric receptors (EPR or EyR) were constructed using the extracellular region of human EpoR and the transmembrane and intracellular region of human IL-2RP or IL-2Rγ alone can not bind IL-2,24,35,36 but coexpression of both subunits forms a functional intermediate affinity IL-2R.24 The heterodimerization of IL-2βR and IL-2Rγ appears to be essential for IL-2 binding and IL-2-induced signal transduction. Because EpoR dimerization probably occurs on binding of the ligand, we speculated that chimeric receptors bearing EpoR on the outside and either IL-2β or IL-2Rγ on the inside might dimerize in the presence of Epo to trigger an IL-2 signal. Therefore, two chimeric receptors (EβR or EyR) were constructed using the extracellular region of human EpoR and the transmembrane and intracellular region of human IL-2β or IL-2Rγ. Genes encoding these chimeric receptors were inserted into pYM (Fig 1), and the virus-producer lines were obtained. First, we infected CTL-D cells with each of the amphotropic EPR and EyR viruses (termed CTL-D(EβR) and CTL-D(EyR)) and analyzed the expression of chimeric receptors (Fig 2b). The expression of EβR was observed at marginally detectable levels. The low expression of EβR seemed to be related to the nature of the protein, because the levels of mRNA were almost the same when compared with those for EpoR or EyR (data not shown). Next, we infected the second virus into each of CTL-D (EβR) and CTL-D (EyR) and maintained them in the presence or absence of 5 U/mL of Epo or 50 U/mL of IL-2 (Fig 3). After selection in Epo, the only proliferating CTL-D cells were those resulting from sequential infection of both EβR virus and EyR virus, although the appearance rate of the Epo-dependent cells was much slower than that of G418-resistant cells from the parental CTL-D cells infected with the same virus supernatant. The other single virus-infected CTL-D cells died in Epo selection medium (Fig 3). This experiment was repeated

Fig 3. Infection of second virus expressing EβR or EyR into the first virus-infected CTL-D cells and rescue of Epo-dependent cells. CTL-D(EβR) and CTL-D(EyR) cells were reinfected with either EβR or EyR virus, respectively, and divided into three cultures. Each culture was maintained in RPMI medium containing no ligand, Epo, or IL-2. (+), the culture in which growing cells were observed; (−), the culture in which all cells were dead. The recovered cell lines from the (+) culture were designated CTL-D(EβR), EβR, (1); CTL-D(EyR), EγR, (2); CTL-D(EyR, EβR), (3); CTL-D(EyR, EγR), (4); CTL-D(EβR, EγR, Epo-selected), (5); CTL-D(EyR, EβR, Epo-selected), (6).
twice, and the same results were obtained. Furthermore, we repeatedly tried to obtain Epo-dependent cells from CTL-D cells infected with either EβR or EyR virus alone. Even when cells were transduced by cocultivation with the corresponding virus-producer cells, all attempts failed. The infection with both EβR and EyR viruses was required to induce Epo dependency. In addition, the expression of the chimeric receptors were greatly enhanced by Epo selection (Fig 2b) when examined by FACs analysis. It is probable that the cells expressing higher levels of chimeric receptors would be selected in Epo selection medium preferentially.

To further analyze the response to Epo, the proliferative response of cells in varying concentrations of Epo or IL-2 was examined using [3H]-thymidine uptake (Fig 4). There was a clear dose-dependent response to Epo by Epo-selected CTL-D cells. Additionally, these cells showed long-term growth in response to Epo (data not shown).

**Southern blot analysis of the CTL-D infectants.** The infected CTL-D cells were evaluated for retroviral vector integration by Southern blot analysis. Selection of the CTL-D cells infected first with EβR followed by EyR viruses (CTL-D EβR/EyR) in Epo resulted in the dramatic enrichment of the cells integrating the second virus (Fig 5). There was a similarly dramatic enrichment of cells integrating the second virus in the CTL-D EyR/EβR after Epo selection. Integration of the second virus was undetectable in the unselected cells. We also examined two other Epo-dependent CTL-D cell lines resulting from the sequential infection of EβR virus and EyR virus, and the same result was obtained. (data not shown). The lack of detectable integration of the second virus in the unselected cells and the enrichment after selection for growth in Epo further supported the supposition that cells growing in response to Epo required both EβR and EyR genes. In addition, using quantitation with the β-scope (Betagen Corp, Waltham, MA), the band representing the second virus was 1.6-fold denser than that of the first virus in the Epo-dependent CTL-D cells, implying that about 65% of the cells have integrated two copies of the second virus.

**Chimeric receptors expression in Epo dependent CTL-D infectants.** To further examine the chimeric receptor expression in CTL-D cells, immunoprecipitation of cell lysates was performed using an antihuman EpoR antibody. The Epo-selected CTL-D cells were used, because they appeared, by FACs, to express the highest levels of receptor surface expression (Fig 2b). As shown in Fig 6, the levels of expression of the two chimeric receptors differed substantially in two Epo-dependent CTL-D cells. The level of EβR was higher in the CTL-D EβR/EyR (lane 3), whereas the level of the EyR was higher in the CTL-D EyR/EβR (lane 4). Thus, it appeared that the level of protein coded for by the first virus may have been preferentially expressed.

**DISCUSSION**

A major problem in immunotherapy is the toxicity associated with the administration of IL-2, and we have been seeking less toxic alternatives to IL-2 for sustaining the growth of TILs in vivo. In this study, we have conferred Epo dependence on the IL-2-dependent murine T-cell line, CTL-D, by expressing chimeric EpoR/IL-2R subunits using retroviral vectors.

Previous studies using epidermal growth factor receptor (EGFR)/EpoR or kit/EpoR chimeric receptors in mouse IL-3-dependent BaF3 or 2D cells suggested that the dimerization of the extracellular regions of the EpoR produced a mitogenic signal. Studies using the EpoR/fms and EpoR/EGFR chimeric receptors in BaF3 suggested that the binding of Epo would cause the dimerization of the extracellular regions of the EpoR. However, this must be interpreted carefully, because wild-type EpoR is functional in BaF3 cells. Potentially, BaF3 cells expressed the putative additional membrane component(s) that interacts with the extracellular region of EpoR and that results in Epo-specific signal. It is likely that these putative additional molecule(s) triggered a mitogenic signal induced by Epo through the extracellular region of EpoR in the EpoR/fms and EpoR/EGFR chimeric receptors. Thus, it remains unclear whether Epo could actu-
ally induce the homodimerization of EpoR by binding to the extracellular region.

In the current study, we used the interaction of intracellular regions of IL-2Rβ and IL-2Rγ that has been reported to cause a mitogenic signal in CTLL-2 cells after heterodimerization. ⁴⁻¹⁻⁴⁰⁻¹⁻⁴ Our data showed that coexpression of EβR and EγR chimeric receptors resulted in Epo-dependent growth of CTL-D cells. In addition, wild-type EpoR was not functional, further suggesting that the mitogenic signal by Epo in CTL-D(EβR, EγR, Epo-selected) and CTL-D(EγR, EβR, Epo-selected) was induced by the interaction of intracellular regions of IL-2Rβ and IL-2Rγ but not by the interaction of extracellular region of EpoR and the putative additional molecules. Thus, although we did not directly show the dimerization of the chimeric receptors containing the extracellular portion of EpoR, our data further support the concept that EpoR binding of the ligand results in the formation of EpoR homodimers. In addition, in studying the signaling, we have looked at the tyrosine phosphorylation of proteins after stimulation through the chimeric receptor proteins. Although preliminary, the studies have suggested that the Epo-induced signal via both EβR and EγR is similar to that induced by IL-2 (data not shown). Further study will be required to show the direct association of EpoRs after Epo binding and to further elucidate the signaling pathways involved in mitogenesis.

A major problem in immunotherapy is the toxicity associated with the administration of IL-2, which may interrupt treatment. Epo is a potential alternative ligand, because recombinant human Epo has been safely used for the therapy of anemia on a clinical basis. Our initial attempt to confer Epo dependency on CTL-D cells by introducing wild-type EpoR failed. However, by making chimeric receptors consisting of the extracellular region of EpoR and the intracellular region of IL-2R subunits, we succeeded in making Epo-dependent CTL-D cells. Furthermore, the mitogenic signaling by Epo appeared to be caused by the activation of IL-2 signaling machinery after Epo binding. Thus, this study offers the possibility that immunotherapy could be made safer and more effective by allowing the administration of Epo, rather than the more toxic cytokine IL-2, to sustain the in vivo proliferation and antitumor activity of transferred TILs.

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