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Annual Performance Report, Research Grant: "Radiation-induced Leukemia: Comparative Studies in Mouse and Man".
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We now have a clear understanding of the mechanism by which radiation-induced (T-cell) leukemia occurs. In irradiated mice (radiation-induced thymic leukemia) and in man (acute lymphoblastic T-cell leukemia, T-ALL) the mechanism of leukemogenesis is surprisingly similar. Expressed in the most elementary terms, T-cell leukemia occurs when T-cell differentiation is inhibited by a mutation, and pre-T cells attempt but fail to differentiate in the thymus. Instead of leaving the thymus for the periphery as functional T-cells they continue to proliferate in the thymus. The proliferating pre- (pro-) T-cells constitute the (early) acute T-cell leukemia (A-TCL). This model for the mechanism of T-cell leukemogenesis accounts for all the properties of both murine and human A-TCL. We have written extensive discussions of this model in mouse and in man in two papers that are currently in press: (1) "Induction of a fully tumorigenic phenotype in murine radiation-induced T-lymphoma cells is associated with the loss of differentiation antigens, gain of CD44, and alterations in p53 protein levels", by Gjerset et al., Molecular Carcinogenesis, In Press, and (2) "Role of the p53 tumor suppressor gene in the pathogenesis and in the suppression of acute lymphoblastic T-cell leukemia", by Yeargin et al., Leukemia, In Press. Both manuscripts are attached to this Report.

Important support for the model has recently come from work by Ilan Kirsch (Mol.Cell.Biol.10:6426,1990; MCB 11:5462,1991) and others (Oncogene 6:1477,1991; J. Exp. Med. 174:867, 1991), who have shown that mutations/ deletions in the genes SCL (TAL), SIL, and LCK constitute primary events in the development of T-ALL, by inhibiting differentiation of thymic pre- (pro-) T-cells.

This mechanism of T-cell leukemogenesis brings several specific questions into focus:

(A) How do early A-TCL cells progress to become potently tumorigenic and poorly treatable?
(B) Is it feasible to genetically suppress early and/or progressed A-TCL cells? and
(C) What is the mechanism by which the differentiation-inhibited (leukemic) pre-T cells proliferate?

During the first grant year we have worked on aspects of all three questions.

A. *Mutations in the p53 gene in T-ALL: Prevalence; Significance.* The presence of mutations in the p53 gene have been documented in >70% of T-ALL cell lines (Cheng and Haas, MCB 1990). All T-ALL lines studied were derived from relapse T-ALL cases. Are p53 mutations associated with the early, diagnosis phase of T-ALL, or with the relapse phase? Gaidano and coworkers (Proc. Natl. Acad. Sci. 88:5413, 1991) have recently reported that 7 out of 7 T-ALL cases that they had studied *lacked* mutations in p53; however these workers did not provide details on the origin (diagnosis, relapse) of the T-ALL samples they studied. Hence the questions concerning the role of p53 in acute leukemia still stand: (i) Do p53 mutations occur in T-ALL in vivo, (ii) at what phase of the leukemia do they occur, (iii) are such mutations hereditary or somatic, and what is their pathogenic significance? Furthermore, (iv) are p53 mutations induced during establishment of T-ALL lines? To study the role of p53 mutations in T-ALL we have initiated, and are currently doing four types of experiments:

1. *Are p53 mutations present in T-ALL cases in vivo?* A relapse T-ALL patient (HA) was found to possess a mutation at p53 codon 175. Leukemia cells from the peripheral blood of patient HA presented with a heterozygous mutation; one allele possessed a mutation at p53 codon 175, the other p53 allele carried a wild type p53 gene. To test whether the heterozygous mutation was due to a mixture of normal and mutated leukemia cells **a T-ALL cell line** was established from the peripheral blood (PB) of patient HA, as was an EBV-immortalized **B-cell line**. The in vivo tumor cells and the lines were sequenced, and their p53 proteins were also studied by immunoprecipitation with specific anti-p53 antibodies. The results of these experiments (Cheng et al., "An acute T-cell lymphoblastic leukemia with a mutation in the p53 tumor suppressor gene" submitted for publication, manuscript attached to this Report) are as follows: (i) Leukemia cells taken from T-ALL patient HA possessed a heterozygous *mutation* at p53 codon 175; (ii) The mutation was *stable* during the establishment of T-ALL lines from the peripheral blood of the patient; the cloned leukemia cell line, HATL, also possessed the heterozygous mutation. (iii) Establishment of the immortalized (normal) B-cell line HABL was *not associated* with the induction of mutations in p53; (iv) Establishment of the T-leukemia cell line HATL was *not associated* with the induction of *additional* p53 mutations; (v) The p53 mutation in relapse T-ALL patient HA was of *somatic* origin; (vi) Patient HA's leukemic T-cells resemble the T-leukemia lines that we have studied previously with respect to the heterozygous nature of the p53 mutations, as well as the absence of loss of heterozygosity.

2. *Is the relapse of T-ALL associated with the induction of p53 mutations?* We have collected a series of peripheral blood (PB) and bone marrow (BM) samples from patients with T-ALL. *Twin samples* were collected from the same patients, samples from the early, diagnosis phase, and samples from one or more relapses. [Anticipating this type of experiments, we have been collecting, saving, and freezing down live T-ALL cells for the last 6 years]. The twin samples are tested by SSCP (single-strand conformation polymorphism), cloning and sequencing to definitively answer the question whether the relapse phase of T-ALL is associated with the induction of p53 mutations. These experiments are in progress.

3. *Are p53 mutations responsible for the refractoriness of T-ALL cases to the induction of remission by chemotherapy?* We have observed that some T-ALL patients whose relapse disease was *refractory to the induction of remission* by chemotherapy possessed mutated p53 genes. In some of these patients no relapse was ever induced, i.e. at the time of first presentation the leukemia cells possessed mutated p53, while in other cases the failure to induce remission occurred following several relapse episodes. This would imply that p53 mutation in T-ALL is associated with a poor prognosis. Such may well be the case, as we have seen (not yet published) that the introduction into T-ALL cells of a retroviral construct encoding a mutant p53 gene increased the growth rate and the in vivo tumorigenicity of the cells (see below). To answer the question whether mutation of p53 is associated with refractoriness to remission induction, we have collected a group of T-ALL cell samples from patients whose leukemia was resistant to the induction of remission[1]. P53 mutation in these samples is being studied by SSCP, cloning and sequencing of the p53 gene. These experiments will answer the question whether p53 mutations render T-ALL cells refractory to the induction of remission.

4. *Is p53 mutation associated with the expression of the MDR1 (multiple drug resistance) gene and the induction of drug resistance?* The presence in T-ALL cells of a mutated p53 gene appears to be associated with the generation of MDR1-associated drug resistance. This would, in part, explain the failure to induce remission in the group of relapse T-ALL patients in whom remission could not any more be induced[2]. Our working hypothesis that refractoriness to chemotherapy is associated with p53 mutation results from experiments in which retroviral constructs encoding

[1] This is of course a euphemism to indicate that the patients died in spite of the attempted chemotherapeutic intervention.
[2] Evidence to date suggests that mutation of the p53 gene acts on three levels: (i) through the loss of the tumor suppressor function, (ii) by the acquisition of a "dominantly-acting" oncogenic function, and (iii) by affecting the expression of the multiple drug resistence gene MDR1.

mutant or wild type p53 cDNAs were used to infect a series of T-ALL cell lines. Description of the experimental findings and the arguments resulting from these experiments are complex and beyond the scope of this Report. The experiments suggest that the refractoriness to chemotherapy of T-ALL cells, and the consequent loss of the patients in question, appear to be related to the state of the p53 gene. Other genes may, of course, also be involved. Using different approaches we are studying the role of mutant p53 in the induction of multiple drug resistance in T-ALL cells, and the possibility of *inducing drug sensitivity by retroviral constructs encoding wild type p53.*

B. *Suppression of the tumorigenic (leukemic) phenotype of T-ALL cells by the introduction of a retrovirus-encoded wild type p53 gene.*

1. *Experiments with human leukemia cells.* The finding of p53 mutations in a significant fraction of T-ALL lines suggested that it might be possible to suppress the leukemic phenotype of T-ALL cells by the introduction and expression of a suitably-promoted wild type p53 tumor suppressor gene. We have now shown the feasibility of "gene therapy of T-ALL" by constructing a helper-free retrovirus encoding the human wild type p53 gene. Initially we used a T-ALL cell line (Be-13) that lacks detectible expression of p53. The results show that introduction of the p53 gene into the Be-13 T-ALL cells slowed their proliferation in vitro, inhibited their colony formation in methylcellulose cultures (an in vitro correlate of in vivo tumorigenicity), and abolished their tumorigenicity in vivo in nude mice. The results of these rather dramatic experiments are in press: Cheng et al., "Suppression of acute lymphoblastic leukemia by the human wild type p53 gene", Cancer Research, Jan 1, 1992 issue. The manuscript is attached to this Report.

To further investigate the potential of using a "gene therapy" approach in the treatment of acute T-cell leukemia we have studied the suppression of T-ALL cells expressing wild type, and cells expressing mutant p53 genes. We have been able to show that the tumorigenic phenotype can be suppressed in T-ALL cells that possess wild type p53 genes, and in cells possessing mutated p53 alleles, in addition to cells lacking p53. These experiments have not yet been concluded, and will be prepared for publication during the next grant period.

The importance of studying the feasibility of suppressing the tumorigenic phenotype of T-ALL cells lies in the possibility of purging leukemic cells from bone marrow during autologous bone marrow transplantation. Though many questions must still be answered, it appears to be distinctly possible that one may be

able to apply p53 gene therapy during BMT in the future. Please see the discussion in attached paper (Cheng et al., "Suppression of Acute..." etc.) for additional details.

2. *Experiments with murine radiation-induced leukemia cells.* Though many of us like to do work that relates to the human condition, and prefer to work on human (leukemia) systems, in many instances the use of animal systems has clear advantages. Study of the suppression of T-leukemia in radiation-induced *murine cells* may enable us to answer the following questions:

(i) Can the leukemic phenotype of murine radiation-induced thymic leukemia (PXTL) cells be suppressed by the introduction of a wild-type p53 gene?
(ii) Are early (non-progressed) PXTL cells and their fully progressed progeny similarly suppressible by the introduction of a wild type p53 gene? In other words, does the exact state of progression of the leukemia determine its susceptibility to suppression by the p53 gene?
(iii) Is *in vivo* radiation-induced thymic leukemia suppressible by the introduction of a retrovirus encoding a suitably-promoted wild type p53 tumor suppressor gene?

[This last question relates directly to the problem of in vivo suppression of human acute lymphoblastic leukemia by gene therapy. We can use the murine system to study the parameters of suppression **during** the leukemogenesis process and in autologous bone marrow transplantation experiments.]

(i) *Suppression of murine PXTL cells by wild type p53 virus.* These experiments are in progress. We have infected 4 clonal lines of murine PXTL cells of different pre-T phenotype with wild type p53 virus. We also have infected these PXTL cells with a mutant p53 virus construct. Wild type p53-infected and drug-selected cells proliferated with a significantly *reduced* growth rate, while mutant p53 virus-infected cells proliferated with a significantly *increased* growth rate. (For similar representative data please see Fig. 2 in Yeargin et al., "Role..." etc). Mice have just been inoculated with the infected cells. At the time of writing, the results of these experiments are being collected. We expect to see that the tumorigenic phenotype of PXTL cells can be suppressed by the introduction and expression of an exogenous wild type p53 gene, and can be potentiated by the introduction of the mutant p53 virus.

(ii) *Suppression of oncogene-induced, fully progressed PXTL cells by wild type p53.* Suppression experiments with early PXTL cells will be followed by experiments designed to study the suppression of oncogene-induced, fully progressed progeny of PXTL cells

(Gjerset et al., "Induction.." etc). The murine system enables us to compare the degree of suppression that can be reached in early radiation-induced leukemia cells and in their oncogene-induced, fully tumorigenic progeny. Following infection and drug selection of the cells, in vitro and in vivo tumorigenicity tests will be used (Cheng et al., "Suppression ..." etc). Results with the genetic suppression of fully tumorigenic *human* T-ALL cells suggest that genetic suppression of *murine* radiation-induced leukemia cells with retrovirus encoding the wild type p53 gene will also succeed.

(iii) *Is radiation-induced **in vivo** leukemia suppressible by the introduction of a retrovirus encoding a suitably-promoted wild type p53 tumor suppressor gene?* One intermediate goal of these experiments is to determine the feasibility of suppressing *in vivo* radiation-induced leukemia in mice. Thus far our experiments suggest that it may be possible to "cure" a mouse of radiation-induced leukemia. It is not the purpose of this Report to discuss the full extent of these questions, but to show the approaches we will follow.

The possibility of intervening genetically to suppress radiation-induced leukemia *in vivo* will be tested. The basic experiment planned is to infect C57Bl/6 mice with one of several retroviral constructs encoding wild type p53. We will use the Mo-LTR-based vector pLp53RNL (Cheng et al., "Suppression..."), and also two other retroviral vectors that are being constructed in our laboratory, pLNCMVp53L (in which the p53 gene is under transcriptional control of the cytomegalovirus promoter), and pLLCKp53NL (a dicystronic viral construct in which the p53 gene and the neo gene are under transcriptional control of the LCK promoter which is specifically expressed at a high level in pre-T cells!).

The effect of these (helper-free) retroviruses will be tested on (i) normal development of newborn Bl/6 mice, on (ii) normal development of young adult Bl/6 mice which are susceptible to leukemia induction by radiation, and on (iii) the development of radiation-induced leukemia in mice injected with the viruses as newborns or as young adults. P53 virus will be injected prior to the induction of leukemia by radiation, immediately following the application of the split-dose radiation series, and just prior to the development on the disease (2 months post-radiation). In these experiments, the choice of the specific promoters used in the viral constructs is of utmost importance; this question is not discussed here.

We will also produce p53-encoding *pseudotype* retroviruses using the innocuous dualtropic virus MCF/BM5 (Haas et al., Cancer Research 49:2184,1989). These cloned MCF viral isolates grow to high titers in BL/6 mice but lack any pathogenicity. p53-MCF/BM5 -pseudotype viruses should be expressed in many cells and organs of the infected mice, and should be useful in the suppression and /or the delay of onset of leukemia in irradiated mice. Pseudotype virus between the MCF/BM5 helper virus and the LCK p53 dicystronic viral construct should be of particular interest due to its (projected) pre-T cell specificity of expression. Again, the proper choice of the viral promoter used is crucial.

The aim is to prevent and/or reverse the onset of radiation induced T-cell leukemia. Obviously, these types of experiments can only be done in mice. However, our early success with suppression of the tumorigenic phenotype of human acute T-leukemia cells suggests that it may become possible to apply similar techniques to human disease.

C. *What drives the proliferation of leukemic pre- (pro-) T-cells?* Since our early publications concerning the probable nature of the autocrine growth factor in radiation leukemia (Journal of Immunology 145:3497, 1990; Leukemia 4:230, 1990), and our success at growing T-ALL cells with human recombinant IGF-I as growth factor, several manuscripts have come to our attention in which the authors confirm our early findings. It is thus reasonable to assume that autocrine (pre-T) PXTL cells proliferate in response to an IGF-I-*like* factor. In the coming year we will continue to invest time and effort into the isolation and characterization of the murine autocrine factor that drives radiation leukemia cell proliferation, in an effort to eventually clone the gene encoding this factor.

D. *Graduate Students working on this Grant, for different periods, during its first year:*

Jian Cheng, Program in Molecular Pathology, Department of Pathology, University of California, San Diego.

Brian Strauss, Program in Molecular Pathology, Department of Pathology, University of California, San Diego.

Sriram Padmanabhan, Department of Biology, University of California, San Diego.

Huyen Pham, Department of Medicine, University of California, San Diego.

In addition we are supporting a particularly gifted Senior in Biology, as a student doing an undergraduate thesis at UCSD, by the name of David Ku.

E. *Publications*:

Publications In Press:

1. Ruth A. Gjerset, Jyoti Arya, Sarah Volkman, and Martin Haas: "Induction of a Fully Tumorigenic Phenotype in Murine Radiation-Induced T-lymphoma Cells is Associated with the Loss of Differentiation Antigens, Gain of CD44, and Alterations in p53 Protein Levels". Molecular Cancinogenesis, In Press.

2. Jian Cheng, Jiing-Kuan Yee, Jo Yeargin, Theodore Friedmann, Martin Haas: "Suppression of Acute Lymphoblastic Leukemia by the Human Wild-Type p53 Gene". Cancer Research, In Press.

3. Jo Yeargin, Jian Cheng, and Martin Haas*. "Role of the p53 Tumor Suppressor Gene in the Pathogenesis and in the Suppression of Acute Lymphoblastic T-Cell Leukemia". Leukemia, In Press.

4. Jian Cheng and Martin Haas: "Sensitivity of detection of heterozygous point mutations in p53 cDNAs by direct PCR sequencing". PCR, Methods and Applications. In Press.

Publications Submitted:

5. Jian Cheng, Jo Yeargin, Alice Yu, Ruth Gjerset, Mark Bogart, and Martin Haas: "An Acute T-Cell Lymphoblastic Leukemia with a mutation in the p53 Tumor Suppressor Gene". Submitted.

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