calculation two to three times as long as Epo (Table 1) (33). These data show that SEP both is a potent effector of red blood cell formation and has prolonged duration of action in vivo.

It is a long-standing goal of chemical protein synthesis to generate proteins with novel properties (13, 14). In the work reported here, we demonstrate that it is now possible to design and produce polymer-modified proteins that have full biological potency and increased in vivo lifetimes. The ability to construct homogeneous protein therapeutics such as SEP enables the systematic exploration of structure-function relationships, and consequent fine-tuning of the biological properties of the protein of interest. Such molecules are not accessible with current recombinant DNA–based protein expression or by post-expression protein modification with polyethylene glycol (6–9, 34, 35).

Chemical synthesis is free of inherent biological contamination (nucleic acids, viruses, prion proteins, etc.). It uses readily available building blocks (synthetic peptides, precision-length polymers) and is scalable. Also, it enables complete control over design, incorporation of noncoiled elements, and the precision modification of the protein of interest. Chemical protein synthesis thus addresses the known shortcomings of existing protein therapeutic procedures, see supporting material on www.sciencemag.org/cgi/content/full/299/5608/884/DCl Materials and Methods.

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Myc-Induced T Cell Leukemia in Transgenic Zebrafish
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The zebrafish is an attractive model organism for studying cancer development because of its genetic accessibility. Here we describe the induction of clonally derived T cell acute lymphoblastic leukemia in transgenic zebrafish expressing mouse c-myc under control of the zebrafish Rag2 promotor. Visualization of leukemic cells expressing a chimeric transgene encoding Myc fused to green fluorescent protein (GFP) revealed that leukemias arose in the thymus, spread locally into gill arches and retro-oral soft tissue, and then disseminated into skeletal muscle and abdominal organs. Leukemic cells homed back to the thymus in irradiated fish transplanted with GFP-labeled leukemic lymphoblasts. This transgenic model provides a platform for drug screens and for genetic screens aimed at identifying mutations that suppress or enhance c-myc–induced carcinogenesis.

The zebrafish (Danio rerio) is a potentially valuable vertebrate system in which to elucidate novel molecular pathways of oncogenesis. In particular, this model organism develops an array of benign and malignant tumors resembling those in humans (1, 2) and is amenable to large-scale forward genetic screens that could be targeted to conserved cancer pathways (3).

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21. This novel stratagem was dictated by the asymmetric distribution of the four Cys residues in the target 166-residue polypeptide chain, and necessitated differential protection of the other Cys side chains present at the elimination step: Cys16(C1-picolyl) and Cys28(acycteamidomethyl).
23. As designed, SEP had an artificially high apparent molecular mass. Measurement under nondenaturing conditions by size-exclusion chromatography gave an apparent effective hydrodynamic size of ~330 kD. These data show that the precision polymer increases the apparent molecular mass of SEP, presumably because of preferential hydration of the polymer portion of SEP.
24. SEP had a calculated mass of 50,821 daltons (average isotope composition).
25. F. Mao, unpublished observations.
27. This is in stark contrast to both typical glycoproteins, such as Epo, and typical polymer-modified proteins, all of which are highly heterogeneous in both polymer composition and sites of attachment.
with \textit{zRag2-mMyc}, 11 (5\%) developed tumors, as did 7 (6\%) of 122 injected with \textit{zRag2-EGFP-mMyc}. These results are similar to the proportion of embryos expressing green fluorescent protein (GFP) in developing thymocytes after injection of the control \textit{zRag2-GFP} reporter construct, suggesting that cancer develops in nearly every mosaic fish whose lymphoid progenitors carry a functional \textit{mMyc} transgene. The mean latencies of tumor development in \textit{zRag2-mMyc}+ and \textit{zRag2-EGFP-mMyc}+ fish were 44 and 52 days (range, 30 to 131 days), respectively.

The affected zebrafish had distended abdominal cavities and spayed eyes due to retro-orbital infiltration by malignant cells (compare Fig. 1, A and B, with Fig. 1, C and D). Some fish had growths protruding from the operculum adjacent to the thymus (fig. S1, A and B), whereas some had tumors at the base of the pectoral fin (Fig. 1, C and D). Many of the fish showed extensive subcutaneous infiltration by transformed cells (fig. 1F), resulting in progressive pallor.

To establish the extent of leukemic cell infiltration, we studied tissue sections from seven diseased fish. Lymphoblasts almost completely effaced the kidney marrow (the site of definitive hematopoiesis in the fish (Fig. 1, G and H)) and were found between the fibers of skeletal muscle (fig. S1, E and F). Leukemic cell invasion was also observed in the gut, gills, and fins, and it was especially prominent in the region adjacent to the olfactory bulb. Although zebrafish \textit{Rag} genes are expressed at the apical surface of the olfactory placode (6, 11), malignant cells in the olfactory region did not express \alpha-keratin and exhibited lymphoblast morphology, demonstrating that they were lymphoid and not epithelial in origin.

To analyze the gene expression patterns in transformed lymphoblasts, we applied RNA in situ hybridization to paraffin-embedded sections from seven leukemic \textit{F0} fish, testing for expression of the \textit{T} cell–specific gene \textit{zLck}, the \textit{B} cell–specific immunoglobulin heavy-chain (\textit{zIgM}) and light-chain (\textit{zIgLC}) genes (12), the early lymphoid genes \textit{zRag1} and \textit{zRag2}, and \textit{mMyc}. Each of the leukemias expressed \textit{mMyc} (fig. S3, A and E), \textit{zRag1} (fig. S3, B and F), \textit{zRag2} (13), and \textit{zLck} (fig. S3, C and G), but not \textit{zIgM} or \textit{zIgLC}, confirming expression of the transgene and the derivation of these leukemias from cells of the \textit{T} lymphoid lineage.

We further investigated the clonality and lineage of the lymphoblasts by Southern blot analysis of restriction enzyme–digested leukemic cell DNAs, using radiolabeled probes for \textit{zTcr-\alpha} (12) and \textit{zIgM} constant regions. Of the three leukemias analyzed, one had monoclonal and one had oligoclonal \textit{zTcr-\alpha} gene rearrangements (Fig. 2A). The third showed a germ line \textit{zTcr-\alpha} configuration, but very strong \textit{zLck} RNA expression, verifying the thymic origin of the lymphoblasts and suggesting that transformed thymocytes in this fish were developmentally arrested at a stage before \textit{zTcr-\alpha} gene rearrangement. None of the leukemias had rearranged \textit{zIgM} genes. In addition, flow cytometric measurements of the DNA content of tumor cells from a \textit{zRag2-EGFP-mMyc} fish showed that this \textit{Myc}-induced tumor was clonally aneuploid (Fig. 2B). Taken together, these data indicate that \textit{Myc}-induced leukemias in the zebrafish represent the clonal expansion of transformed \textit{T} lymphocyte precursors and suggest that additional mutations are needed to produce the malignant phenotype.

To quantify the extent of leukemic cell infiltration of the kidney and spleen, we analyzed hematopoietic cells from these tissues by fluorescence-activated cell sorting (FACS) (14). In wild-type zebrafish, the kidney (\(n = 7\)) contained \((8.4 \pm 3.7) \times 10^5\) (mean \pm SD) blood cells, and the spleen (\(n = 6\)) contained \((8.2 \pm 6.3) \times 10^4\) blood cells; these wild-type values were about one-sixth that of leukemic fish [kidney (\(n = 5\)), \((4.95 \pm 3.3) \times 10^5\) blood cells; spleen (\(n = 4\)), \((4.64 \pm 1.5) \times 10^5\) blood cells]. The leukemic blasts comprised \(\sim 87\%\) of the kidney marrow cells and had a granularity similar to that of normal lymphocytes (\(y\) axis in Fig. 3), although they were slightly larger in size (\(x\) axis in Fig. 3). This analysis of the kidney marrow, the equivalent of mammalian bone marrow (15–17), supports the histopathological classification of these \textit{Myc}-induced cancers as \textit{T} cell acute leukemia rather than disseminated lymphoma, in which the blast cells account for \(<30\%\) of the total cells in the marrow.

**Fig. 1.** External and histological features of leukemic \textit{zRag2-mMyc} \textit{F0} mosaic fish. (A and B) Wild-type fish and (C and D) \textit{zRag2-EGFP-mMyc} fish with leukemic infiltration into the retro-orbital soft tissue, olfactory region, and pectoral fins. Transverse sections of (E and G) wild-type fish and (F and H) leukemic fish with massive infiltration of lymphoblasts throughout the body [(E) and (F)] and into the kidney [(G) and (H)]. E, eye; F, fin; G, gut; K, kidney; M, muscle; O, olfactory region; and S, skin. Arrowheads indicate sites of leukemic cells. Scale bars in (E) and (F), 1 mm; scale bars in (G) and (H), 100 \(\mu\)m.

**Fig. 2.** Clonality of \textit{mMyc}-induced leukemias. (A) Southern analysis of the \textit{zTcr-\alpha} and \textit{zIgM} constant regions in leukemic (T1 through T3) and wild-type (WT1 through WT4) fish. The presence of either two or four restriction fragments hybridizing to the \textit{zIgM} probe results from a Bgl II polymorphic site within the gene, not gene rearrangement. (B) DNA content of leukemic cells from a \textit{zRag2-EGFP-mMyc} fish, as measured by flow cytometry. The DNA index (DI) of 1.15 for \textit{G1}-phase leukemic cells indicates clonal aneuploidy. 2N, DNA content of the normal diploid zebrafish genome. Bars indicate the position of modal DNA content peaks corresponding to the indicated \textit{G1}, or \textit{G2/M} cell populations.
To assess the transplantability of zebrafish leukemic cells, we first injected the zRag2-mMyc transgene into fertilized eggs from a stable transgenic zRag2-GFP line, so that the lymphoblasts would be GFP positive when leukemia developed. GFP-positive lymphoblasts from a leukemic 62-day-old fish were then transplanted intraperitoneally into eight irradiated wild-type adult zebrafish. Leukemic cells were apparent at the site of transplantation within 7 days after injection (Fig. 4A) and had begun to spread throughout the peritoneal cavity within 14 days after injection (Fig. 4B). Lymphoblast homing to the thymus occurred relatively early in the disease process (fig. S6B), with GFP-positive cells becoming evident at this site in one fish within 14 days after injection and in six fish within 26 days after injection. There was also prominent leukemic cell infiltration into the region adjacent to the olfactory bulb in two fish within 14 days after injection and in all eight within 26 days after injection (fig. S6, C and D). These experiments establish the transplantability of Myc-induced leukemias in zebrafish, indicate that the disseminated spread of leukemic cells proceeds along anatomically defined pathways, and suggest that the region adjacent to the olfactory bulb is a preferred site for the homing of immature T cells.

The power of the zebrafish model lies in its ability to accommodate “forward-genetic” screens to identify modifier genes that influence the development of Myc-induced leukemia, an application requiring a stable transgenic zebrafish line that expresses the mMyc transgene. Thus, mosaic zRag2-EGFP-mMyc F₀ fish injected as single-cell embryos were mated, and the F₁ offspring of one fish developed leukemia with a mean latency of 32 days (range of 21 to 44 days). GFP fluorescence was readily detectable in the thymic lymphoblasts of these fish (Fig. 4, D through F), demonstrating germline transmission and expression of the chimeric transgene.

The rapid onset of leukemia after germline transmission of the transgene made it necessary to propagate this stable line by in vitro fertilization (IVF). Hence, we killed 90-day-old leukemic males and used their sperm to fertilize eggs from normal females. The resultant progeny showed expansion of GFP-positive leukemic cells from the thymus by 1 month of age. Technology to render the expression of transgenic myc alleles that are regulatable with estrogen has been developed for in vivo use in the mouse (18), and we are currently testing this approach for its capacity to generate large numbers of transgenic fish for further genetic manipulations, thus eliminating the need for IVF procedures.

Modifier screens in the mouse have identified retrovirally activated genes that accelerate Myc-induced oncogenesis (19), and breeding studies with genetically manipulated mice have identified other critical interact-
The vascular endothelium was once thought to function primarily in nutrient and oxygen delivery, but recent evidence suggests that it may play a broader role in tissue homeostasis. To explore the role of sinusoidal endothelial cells (SECs) in the adult liver, we studied the effects of vascular endothelial growth factor (VEGF) receptor activation on mouse hepatocyte growth. Delivery of VEGF-A increased liver mass in mice but did not stimulate growth of hepatocytes in vitro, unless SECs were also present in the culture. Culture hepatocyte growth factor (HGF) was identified as one of the SEC-derived paracrine mediators promoting hepatocyte growth. Selective activation of VEGF receptor–1 (VEGFR-1) stimulated hepatocyte but not endothelial proliferation in vivo and reduced liver damage in mice exposed to a hepatotoxin. Thus, VEGFR-1 agonists may have therapeutic potential for preservation of organ function in certain liver disorders.

The vascular endothelium is a highly versatile system and, in addition to its well-established function of nutrient and gas exchange between tissues and blood, it plays multiple homeostatic roles (7). Furthermore, the endothelium has an inductive effect on liver (2) and pancreas (3) development before the establishment of a blood flow. Vascular endothelial growth factor–A (VEGF-A) (4), a major regulator of normal and pathological angiogenesis, binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) (5) and VEGFR-2 (KDR/Flk-1) (6, 7). VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF-A (8). However, many conflicting reports about the function of VEGF-R-1 exist. This receptor has been implicated in the inhibition of VEGF-dependent endothelial mitogenesis and chemotaxis by several mechanisms (9–11). Other studies have shown that VEGF-R-1 mediates monocyte chemotaxis (12), recruitment of endothelial cell progenitors (13), and survival of hematopoietic stem cells (14). VEGF-R-1 activation also has been reported to result in collateral vessel growth through recruitment of bone marrow–derived cells (15). Thus, the importance of VEGF-1 signaling in the vascular endothelium is largely unclear.

We sought to investigate the effects of VEGF activation on parenchymal cell proliferation and survival. To achieve sustained systemic levels of VEGF, we injected Chinese hamster ovary (CHO) cells expressing VEGF165 or control CHO cells into the legs of nude mice (16). We observed substantially increased liver sizes in the CHO-VEGF groups. The liver/brain ratio (that is, the relative liver mass) of the CHO-VEGF group (4.73 ± 0.39) was significantly increased compared with that of the CHO–dihydrorotate dehydrogenase (CHO-DHFR) (3.18 ± 0.25; P < 0.0001) and CHO–Hakata antigen (CHO-HA) (3.00 ± 0.45; P < 0.0001) controls. This reflects an increase in relative liver masses of 49% and 59%, respectively.

Histological analysis of the livers of

References and Notes

5. X. Yin et al., Oncogene 20, 4650 (2001).
9. Materials and methods are available as supporting material on Science Online.
13. D. M. Langenau et al., data not shown.
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