Growth Suppression of the Hepatocellular Carcinoma Cell Line Hepa1-6 by an Activatable Interferon Regulatory Factor-1 in Mice

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ABSTRACT

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a poor prognosis and few therapeutic options. The aim of the study was to evaluate the potential of IFN regulatory factor-1 (IRF-1) for cytokine gene therapy of HCC using an IRF-1/human estrogen receptor fusion protein (IRF-1hER), which is reversibly activatable by β-estradiol (E2). IRF-1hER stably expressing murine Hepa-6 HCC cells (HepaRF-IhER) were characterized by high MHC I, low MHC II, and high CD54 phenotype of these cells. Furthermore, they were characterized by high MHC I, low MHC II, and high CD54, and lack of MHC II, CD80, and CD86 expression. Activation of HepaRF-IhER cells induced a high MHC I, low MHC II, and high CD54 phenotype. Furthermore, they were characterized by IFN-β secretion, decreased anchorage-independent growth in a soft agar assay, and diminished cell growth. Tumor growth in E2-treated syngeneic C57L/J mice, but not in E2-untreated mice, was suppressed. These E2-treated mice were protected against rechallenge with HepaRF-IhER and wild-type Hepa-6 tumors even in the absence of E2, suggesting induction of tumor specific immunity. In fact, significant CTL activity against Hepal-6 tumors and the endogenously expressed HCC-specific self antigen α-fetoprotein was observed. Antitumoral effects, however, were only partially dependent on both CD4+ and CD8+ T cells. IRF-1 treatment of mice bearing HepaRF-IhER tumors resulted in growth arrest of tumors, and a significant survival benefit was observed in comparison to E2-untreated mice. In conclusion, our data demonstrate that IRF-1 suppresses HCC growth through both a direct antitumor growth effect and enhanced immune cell recognition of the tumor and is a promising candidate for gene therapy of HCC.

INTRODUCTION

HCC ranks fifth in frequency among all of the malignancies in the world with an estimated number of 437,000 new cases in 1990 (1). Although various nonsurgical treatment modalities have been developed and the surgical techniques much improved, none of these therapies has significantly improved the extremely poor prognosis of patients with HCC. The overall 5-year survival rate worldwide is only 2% (2); therefore, novel gene and immunotherapeutic strategies for HCC are being developed. We attempted to use the broad role of IRF-1 (3) as a tumor suppressor and immune modulator for the treatment of HCC using an immunocompetent syngeneic HCC tumor model in mice.

IRF-1 expression leads to the induction of many ISGs (4–6) and thereby induces typical IFN functions including induction of histocompatibility antigens (7) and an antiviral state (5, 8). Because the IRF-1 gene per se is inducible by IFNs, it was suggested that it might be involved in IFN-mediated cellular responses (5, 9, 10). However, in mice and cells lacking functional IRF-1 genes, the IFN-induced induction of typical ISGs (e.g., 9-27, 1-8, PKR) is not affected (8, 11–13). Thus, IRF-1 seems to simulate the IFN-specific induction of ISGs by ISGF-3, which binds the ISG-promoter with the IRF-1-related subunit ISGF3 (14). A specific alteration in these mice is the lack of inducible NO synthase induction in response to IFN-β (15).

IRF-1 exerts an antiproliferative effect by DNA binding and transactivation (4). It is known to induce a number of genes that exert growth inhibitory effects. Among them are lysis oxidase (16), PKR (17), 2′,5′- OASE (18), indoleamine 2,3-dioxigenase (19), and angiotensin type II receptor (20). In established cell lines of fibroblast and epithelial origin, IRF-1 leads to cell growth arrest without signs of apoptosis (21). However, similar to the activity of the tumor suppressor p53 required for ras-induced apoptosis (22, 23), IRF-1 is able to exert oncogene-dependent apoptosis. We have shown that 3T3 cells that are growth inhibited by IRF-1 undergo apoptosis after conditional HER1 oncogene activation (24). Indeed, the promoter regions of certain caspase genes like ICE contain ISRE-like sequences (25). These genes might be targets for IRF-1 (26).

IRF-1 has been identified as a tumor suppressor (4, 17, 24, 27). Chromosomal deletions of the IRF-1 locus in humans are associated with myelodysplasia and certain leukemias (28). Primary embryonic fibroblasts with a null mutation in the IRF-1 gene are susceptible to transformation by the expression of a single oncogene (c-Ha-ras). These IRF-1-c/Ha-ras cells do not undergo apoptosis upon c-Ha-ras oncogene expression and serum starvation, whereas wild-type cells harboring IRF-1 genes undergo programmed cell death (21). IRF-1 expression also reverts the tumorigenic phenotype exerted by the c-myc and fosB oncogenes (29). Additional data indicated that mice lacking c-Ha-ras and IRF-1 exhibit a higher rate of tumorigenicity (30).

The in vivo function of IRF-1 as a tumor suppressor is complex. Depending on the cell type, IRF-1 induces growth inhibition and apoptosis and effects the extracellular matrix as well as immunomodulatory functions. IRF-1 induces a number of immunomodulatory effects like MHC class I (31, 32), inducible NO synthase (15), and IFN-β (11) transcription, and it is also necessary for proper expression of IL-15 (33). Transient expression of IRF-1 leads to the activation of the IFN-β gene (9, 34, 35). Studies with IRF-1 knockout cells demonstrate that IRF-1 is involved in the differentiation and function of NK cells (33, 36), the generation of the TH1 type of T helper cells, and DNA damage (26, 37). IRF-1 is further involved in up-regulation of the antigen presentation by transcriptional induction of LMP2, TAP-1, and MECL1 (38, 39) as well as an induction of MHC class II (7, 40). These facts suggest that IRF-1 might act as a costimulator for presentation of antigens.

The aim of the present study was to evaluate the potential of IRF-1 for gene therapy of HCC. This report details the construction of plasmids and murine HCC cell lines encoding an IRF-1/human estrogen receptor fusion protein that becomes active in the presence of E2 and the detailed characterization of the in vitro phenotype of these HCC cell lines. Furthermore, we describe the protective and thera-
GROWTH SUPPRESSION OF THE HCC LINE HEPA1-6 BY IRF-1

MATERIALS AND METHODS

Establishment and Induction of Recombinant Mammalian Cells

The H-2<sup>b</sup>-restricted HCC cell line Hepa1-6 (92110305; European Collection of Animal Cell Cultures, Heidelberg, Germany) was stably cotransfected using an expression construct encoding IRF-1-hER (4) and a puromycin resistance-conferring plasmid (42). The cells were grown in RPMI 1640 medium supplemented with 10% estrogen-free, heat-inactivated FCS. Transfectants were selected with 1 µg/ml puromycin, and single clones were picked and expanded. For activation of IRF-1 in the IRF-1-hER fusion protein, E<sub>2</sub> (SERVA, Frankfurt, Germany) was added to the cell culture medium at the final concentration of 1 µM. Clones were subsequently screened by Western blotting for IRF-1-hER expression.

Western Blotting

Immunoblots derived from whole cell extracts were probed with antibodies directed against the hormone-binding domain of the human estrogen receptor (HC-30; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) according to manufacturer’s specifications.

IFN-Test

The IFN concentrations in the cell culture supernatants were determined by an antiviral assay using mouse L929 cells (43). To confirm the specificity of the antiviral activity, a neutralizing monoclonal antibody directed against mouse IFN-β was added to the supernatant before addition to the test cells.

Measurement of Cell Growth

For determination of cell growth, 2 × 10<sup>3</sup> cells/well were seeded into microtiter plates, and serial dilutions (1:1) were performed allowing several independent measurement points. Cells were treated with the indicated concentration of E<sub>2</sub>. Cell growth was determined using the WST kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instruction. Mean values of triplicates resulting in less than 10% deviation were plotted.

Assay for Anchorage-independent Growth

Anchorage-independent growth capability was determined by assessing the colony-formation efficiency of cells suspended in soft agar. Cells (1 × 10<sup>5</sup>) were seeded in 50 µl of 0.3% overlay agar in microtiter plates coated with 50 µl of 0.6% underlay agar. The induction medium was added to the top (50 µl/well). Colonies were counted 3 weeks after plating.

Mice

Male C57L/J (H-2<sup>b</sup>) mice were kept in the animal facility of the University Hospital Freiburg and used between the ages of 10 and 25 weeks.

Tumor Model

The Hepa 1-6 tumor model in C57L/J mice (44) was chosen because they show reliable growth in the syngeneic host. Hepa 6 cells are a derivative of the BW7756 mouse hepatoma that arose in a C57L mouse. MHC class I and II expression is identical between C57L and C57L/J mice, and Hepa-1-6 HCCs are characterized by AFP expression. It could be demonstrated that a reliable tumor growth of Hepa 1-6 murine HCC cells in 100% of mice was achieved using 1 × 10<sup>7</sup> Hepa-1-6 cells injected in 100 µl of serum-free MEM medium into the right flank of mice. After 6 days, tumors were visible and reached a size of about 2000 mm<sup>3</sup> after a mean time of 18 days. This tumor size was used as an end point in our study, and mice were subsequently sacrificed. Tumor incidence and volume were assessed every 2 days using calipers. Data are presented as mean volume ± SE.

Flow Cytometry

MHC class I and CD80 expression in Hepa1-6 and HepaIRF-1hER cells were examined by FACS analysis using an antiserum H-2K<sup>b</sup>- H-2D<sup>b</sup> and antimouse CD80 specific antibody (clones 28-8-6 and 01940B, respectively) and a subsequent FITC-labeled antiserum (clone 02014D) or FITC-labeled antiserum (clone 10094D) antibody, respectively. Furthermore, expression of CD54 (clone 01544D), I-A/ I-E (clone 06355A), CD40 (clone 3123), and CD86 (clone 09274) was determined by PE- or FITC-labeled antibodies (all of the antibodies were derived from PharMingen, San Diego, CA).

Generation of Recombinant Vaccinia Viruses

To study CTL responses, an AFP-expressing virus and pSc111 (empty vector negative control) recombinant vaccinia virus were generated as described previously (44).

Cytotoxicity Assays

Spleen cells derived from tumor-challenged or control mice were suspended, and after 6 days of in vitro stimulation in 24-well plates, the spleen cells were analyzed for cytotoxic activity. In vitro stimulation was performed by incubating 4 × 10<sup>7</sup> of tumor-primed spleen cells with 1 × 10<sup>7</sup> wild-type Hepa1-6 cells or, as a negative control for specificity, syngeneic Lewis lung carcinoma cells (3LL), both irradiated with 8000 rad. To assess AFP-specific immune responses, spleen cells derived from tumor-challenged or control mice were stimulated in vitro with spleen cells of untreated syngeneic donor mice that had been infected by UV-inactivated (300 mJ) rVV-AFP or, as a negative control for specificity, rVV-pSc11 at a multiplicity of infection of five and then irradiated with 20 Gy (2000 rad) to prevent stimulator cells from proliferation. Subsequently, a 6-h 51Cr release assay was performed. As target cells, AFP-expressing syngeneic Hepa1-6 cells and AFP negative syngeneic Lewis lung carcinoma cells were used. Results were expressed according to the formula: % lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release). Experimental release represents the mean cpm released by target cells in the presence of effector cells. Total release represents the radioactivity released after total lysis of target cells with 5% Triton X-100. Spontaneous release represents the radioactivity present in medium derived from target cells only. Hepa1-6 tumor or murine AFP specific lysis was presented by subtraction of lysis values against 3LL from lysis values against Hepa1-6 targets.

IFN-γ and IL-4 ELISPOT Assays

Multiscreen-HA 96-well filter plates were coated with 4 µg/ml rat antimouse IFN-γ or rat antimouse IL-4 antibody (clone R46A2 or
1.5 mg of E2 every 2 days i.p., CD8 depletion (1.5 mg of E2 every 2 days until day 28. Subsequently, E2 treatment was stopped again in two mice, whereas the remaining four mice were maintained on E2 therapy. The mice of group 1 (n = 2); group 2, 1.5 mg of E2 every 2 days i.p. (n = 3); group 3, no treatment (n = 8); group 4, 1.5 mg of E2 every 2 days i.p. (n = 8); and group 5, 1.5 mg of E2 every 2 days i.p. (n = 2).

Protection against Rechallenge. Naive C57L/J mice or E2-treated animals that had been protected against HepaIRF-1hER tumor growth were rechallenged with 1 x 10^7 wild-type Hepa-6 (n = 3) or HepaIRF-1hER (n = 5) tumor cells 11 days after stopping E2 treatment (28 days after the initial tumor inoculation). Importantly, these mice did not receive any additional E2 treatment.

Tumor Therapy. C57L/J mice were inoculated with 1 x 10^7 HepaIRF-1hER (groups 1 and 2) cells into the right flank. Both groups did not receive any E2 treatment. At day 19, tumors had reached a size of about 2000 mm³ in each well at the start of the culture. As a control for specificity, spleen cells of tumor-challenged mice and the different irradiated stimulator cells were cultured alone.

Experimental Design of In Vivo Tumor Experiments

Tumor Protection Studies. C57L/J mice were inoculated with 1 x 10^7 Hepa-6 (groups 1 and 2) or HepaIRF-1hER (groups 3 and 4) cells into the right flank. Group 5 was not inoculated with any tumor. The different groups were treated as follows: group 1, no treatment (n = 3); group 2, 1.5 mg of E2 every 2 days i.p. (n = 3); group 3, no treatment (n = 8); group 4, 1.5 mg of E2 every 2 days i.p. (n = 8); and group 5, 1.5 mg of E2 every 2 days i.p. (n = 2).

Protection against Rechallenge. Naive C57L/J mice or E2-treated animals that had been protected against HepaIRF-1hER tumor growth were rechallenged with 1 x 10^7 wild-type Hepa-6 (n = 3) or HepaIRF-1hER (n = 5) tumor cells 11 days after stopping E2 treatment (28 days after the initial tumor inoculation). Importantly, these mice did not receive any additional E2 treatment.

In Vivo Monoclonal Antibody Ablation. CD4 and CD8 T-cell subpopulations were depleted by i.p. injection of purified hybridoma supernatant. A total of 1 mg/mouse/injection of anti-CD8 (clone YTS 191.1; Refs. 45, 46) was injected on days 5, 3, and 1 before HepaIRF-1hER tumor inoculation and every 5 days thereafter. The different groups were treated as follows: (a) 1.5 mg of E2 every 2 days i.p., no depletion (n = 2); (b) 1.5 mg of E2 every 2 days i.p., CD8 depletion (n = 2); (c) 1.5 mg of E2 every 2 days i.p., CD4 depletion (n = 2); and (d) no treatment, no depletion (n = 2).

Statistical Analysis

All of the data were analyzed by Wilcoxon’s signed rank test. A two-sided P of less than 0.05 was considered statistically significant. Tumor appearance and growth to 2500 mm³ was calculated by the Kaplan-Meier method, presented as SD of the mean for each group, and differences between immunized and control mice were calculated by the Mantel-Haenszel test.

RESULTS

In Vitro Analysis

IRF-1 Inhibits Cell Growth of the HCC Cell Line Hepa-6. Constitutive expression of IRF-1 imposes a strong cell growth inhibition to several cell lines (4, 24, 40). This results in stable transfectants that are selected for very low, often unstable expression of the heliologic IRF-1. Therefore, to determine the activity of IRF-1 as a growth inhibitor of the HCC cell line Hepa-6, we used a conditionally active IRF-1-hER fusion protein. It has been demonstrated that in the absence of hormone stimulation, constitutively expressed chimeric proteins are inactive but can change to an active conformation upon binding of E2 to the hER part of the protein (4, 17, 24). IRF-1-hER was stably transfected into Hepa-6 cells. Different levels of IRF-1-hER expression were observed in the transfectants (Fig. 1A, top panel) and normalized to actin expression (Fig. 1A, bottom panel). Three cell clones with different strengths of IRF-1-hER expression were selected (c4, c9, and c22). Cell growth of these clones was determined 7 days after IRF-1 activation. As shown in Fig. 1B, cells derived from the three cell clones were sensitive to IRF-1 activity with respect to growth inhibition. The extent of proliferation inhibition varied from 40 to 80% between the different cell clones and correlated with the different expression amounts of IRF-1. Wild-type Hepa-6 cells not expressing IRF-1-hER were used as a control. The nontransfected cell line did not respond to E2 with alterations in cell growth. This indicates that all of the three cell clones expressed activatable IRF-1 and responded to the typical growth inhibitory properties of the IRF-1 phenotype. However, it should be noted that growth inhibition of the Hepa-6 cells was not very strong when compared with other cell lines (24). Despite the reduction in cell proliferation, the cells could be cultivated for a considerable time in this state. Furthermore, these cells did not show any signs of apoptosis upon IRF-1 activation by E2. This was confirmed by examination of subdiploid DNA (Ref. 47 and data not shown) and annexin staining (Ref. 48 and data not shown).

Activated IRF-1 Induces IFN Secretion. The induction of IFN-β is a typical property observed after IRF-1 activation. The amount of

Fig. 1. IRF-1 mediates cell growth inhibition in Hepa-6 cells. A, Western blot analysis of IRF-1-hER fusion protein in Hepa-6 cells transfected with IRF-1-hER. Lysates (50 μg of protein) derived from these cell clones were subjected to SDS-PAGE and analyzed using an antireceptor antibody (top panel). The membrane was stripped and reprobed with an anti-actin antibody to control protein loading of the samples (bottom panel). B, for determination of cell growth, cells derived from the same clones were seeded in wells of microtiter plates and grown with (black bars) or without (white bars) 1 μM E2. The metabolic activity of the cultures was measured after 7 days of treatment. Because the growth characteristics of individual Hepa-6 cell clones differ slightly, the metabolic activity of untreated cells was set as 100%.
secreted IFN-β can be taken as a measure of IRF-1 activity (17). Because IFNs are relevant for immunomodulation, the secretion of IFN-β was determined by an antiviral assay (Table 1). IRF-1 was shown to be activated by E₂ in all of the three cell clones. The highest IFN secretion was shown by clone 22, which is in agreement with the strength of IRF-1 expression (Fig. 1A) and proliferation inhibition (Fig. 1B). Using neutralizing antibodies directed against IFN-β, we confirmed that the secreted antiviral activity was exclusively IFN-β. IFNs are known to inhibit cell proliferation. However, the amount of IFN-β secreted by the Hepa1-6 cell clones is not sufficient to mediate the observed effect on cell growth as determined by the treatment of control cells with comparable amounts of recombinant murine IFN-β (data not shown).

**Decreased Anchorage-independent Growth During IRF-1 Activation.** The most definitive in vitro characteristics distinguishing tumorigenic cells from nontumorigenic cells is anchorage-independent growth. To determine whether IRF-1 reverses the transformed phenotype of this HCC cell line in vitro, we tested its ability to form anchorage-independent colonies in the presence of inactive or activated IRF-1 (Fig. 2). The untransfected tumor cell clones grew well in soft agar. The colony formation of the transformed wild-type Hepa1-6 cell line not expressing IRF-1hER was not influenced by E₂. In contrast, the ability of soft agar growth was significantly decreased by the activated IRF-1 in the different cell clones. In contrast to untransfected cells, the IRF-1hER-bearing cells formed fewer but somewhat bigger colonies. In the presence of E₂, clone 22 formed the lowest number of colonies in soft agar, which inversely correlates to the strength of IRF-1hER expression. Clone 9 showed the highest ratio of soft agar colony formation from untreated over E₂-treated cells. Therefore, clone c9, in following simply designated HepaIRF-1hER, was used for further in vitro characterization and for the in vivo tumor model.

**Activated IRF-1hER Modulates Immunologically Relevant Cell Surface Protein Expression.** IRF-1 expression has been shown previously (7, 31) to increase expression of MHC genes. We examined the levels of MHC class I, MHC class II, CD54, CD40, CD80, and CD86 expression on the cell surface before and after IRF-1 activation with E₂ by FACS analysis. Wild-type Hepa-1-6 and E₂-un-treated HepaIRF-1hER cells were characterized by the lack of MHC class II, CD40, CD80, and CD86 expression. MHC class I (H-2Kb/H-2Db) was expressed at low levels, and CD54 was expressed at high levels. E₂ treatment of HepaIRF-1hER cells resulted in a strong up-regulation of H-2Kb/H-2Db. CD54 expression remained unchanged, and MHC class II expression was weakly up-regulated (Fig. 3), CD40, CD80, and CD86 expression remained negative (data not shown).

**In Vivo Analysis**

**IRF-1 Activation Inhibits HCC Growth.** To determine the antitumoral efficacy of IRF-1hER expression against murine Hepa1-6 HCCs growing s.c. in C57L/J mice, different treatment groups were randomly designed. The Hepa1-6 HCC cell line used for the tumor model in syngeneic C57L/J mice was characterized by moderately fast tumor growth, and 100% of implanted animals developed a tumor. Both wild-type Hepa1-6 and HepaIRF-1hER cells exhibited nearly identical tumorigenicity after s.c. injection in vivo, suggesting the presence of an inactive IRF-1hER fusion protein in E₂-un-treated HepaIRF-1hER cells (Fig. 4A; P > 0.5). Within 17 days, large tumors developed with an average size of 1500 mm³. If mice inoculated with wild-type Hepa1-6 cells were treated with E₂, no effect on tumor development was observed in comparison with mice challenged with Hepa1-6 or HepaIRF-1hER cells without E₂ treatment. These results demonstrate that the E₂ treatment itself has no negative effect on tumor growth and animal health. However, if C57L/J mice inoculated with HepaIRF-1hER received two daily i.p. injections with E₂ starting at the time of tumor inoculation, tumor growth was significantly

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**Table 1**  
IRF-1 activation leads to IFN secretion

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**Fig. 2.** Activation of IRF-1 reverts the tumorigenic phenotype of Hepa1-6 cells. A, anchorage-independent growth of IRF-1-transfected cell clones in soft agar. B, the number of colonies of the indicated cell clones was determined after 3-week culture without (black bars) and with (white bars) 1 μM E₂.

**Fig. 3.** Activation of IRF-1 leads to increased MHC class I and MHC class II expression. FACS analysis of surface proteins of Hepa1-6 cells expressing IRF-1-hER (clone 9) was done using antibodies directed against H2Kb/Db (A), 1-AI-B (B), CD54 (C), and CD40 (D). HepaIRF-1hER cells were cultured for 3–4 days in estrogen-free medium or in medium containing a 1:1000 dilution of 1 mM E₂. Subsequently, cells were stained with the appropriate FITC- or PE-labeled antibodies. As controls for specificity, cells were stained with FITC- or PE-labeled unspecific isotype controls. Data are derived from three independent experiments.
suppressed. It was an important finding that six of eight animals were completely protected against tumor growth, and two animals developed only very small tumors that were characterized by a slow growth rate (Fig. 4A). After 40 days, the tumor size was only 450 mm³, and stopping E₂ treatment at day 42 did not result in a faster growth rate of the tumor.

**IFR-1 Activation in Tumor Cells Induces T-cell Memory.** We were interested to investigate the presence of tumor-specific memory T-cells in E₂-treated mice protected against challenge with HepaIRF-1hER. Therefore, tumor-free mice were inoculated with 1 × 10⁷ wild-type Hepa-1-6 (n = 3) or HepaIRF-1hER cells (n = 3) 28 days after tumor challenge and 11 days after stopping E₂ treatment. Importantly, these mice did not receive any additional E₂ treatment. As demonstrated in Fig. 4B, all of the mice in both groups were protected against tumor growth but not after CD8⁺ T-cell in vivo depletion (n = 2; data not shown). In contrast, naive C57L/J mice without E₂ treatment were characterized by rapid Hepa-1-6 and HepaIRF-1hER tumor growth. These results suggest the presence of tumor-specific T-cell memory after primary priming of tumor-specific immunity by the expression of active IFR-1hER.

**Induction of CTL Activity through IFR-1-activated Tumor Cells.** In fact, strong CTL activity against Hepa-1-6 target cells after in vitro stimulation using irradiated Hepa-1-6 cells was observed in mice challenged with HepaIRF-1hER cells using E₂ (Fig. 5A). This CTL activity was specific against Hepa-1-6 tumor cells because spleen cells derived from mice that were not tumor challenged or from E₂-treated mice challenged with HepaIRF-1hER cells alone or using effectors alone did not result in increased background killing activity (Fig. 5A). Primary T-cell responses were evaluated by monitoring cytokine-producing cells in vivo. A significant increase in the number of spleen cells secreting IFN-γ (1 in 5,000) and IL-4 (1 in 10,000; Fig. 5B) upon stimulation with irradiated Hepa-1-6 cells was observed in E₂-treated mice inoculated with HepaIRF-1hER tumors in comparison with E₂-untreated/HepaIRF-1hER, E₂-untreated/Hepa-1-6, or E₂-treated/Hepa-1-6-challenged mice (1 in 20,000 IFN-γ and 1 in 40,000 IL-4 secreting T cells; P = 0.01), which suggested significant development of both TH1 and TH2 tumor immunity. In contrast to the in vivo results obtained by ELISPOT analysis, the differences of CTL in vitro killing activity in the 5¹Cr release assay (Fig. 5A) were not statistically significant between the different groups. This may be the result of in vitro expansion of tumor-specific T cells and the lower sensitivity of the 5¹Cr release assay in comparison with the ELISPOT technique.

**IFR-1 Activation Breaks Ignorance to Tumor-specific Self Antigens.** To determine immune responses against HCCs in more detail and to find out if expression of activated IFR-1 was able to prime immune responses against a tumor-specific antigen, we chose the HCC-specific self antigen AFP, which is frequently expressed at high levels in HCC cells as a target. Intermediate CTL activity against Hepa-1-6 HCCs endogenously expressing AFP at high levels was observed in E₂-treated/HepaIRF-1hER-challenged mice (Fig. 6A). CTL activity was significantly stronger (P = 0.02) and the number of AFP-specific IFN-γ (Fig. 6B)-producing spleen cells (1 in 11,000) was higher (P = 0.001) in these mice in comparison with the other groups (1 in 1,000,000). In control animals without tumors, no specific CTL activity or enhanced background lysis against Hepa-1-6 or 3LL target cells was observed. In addition, no increased lysis of these target cells was seen after in vitro stimulation of effector cells with rVV-pSc11 derived from E₂-treated mice challenged with HepaIRF-1hER cells, which suggested specificity of CTL activity against AFP (Fig. 6A). Performing ELISPOTs using rVV-AFP-infected spleen cells alone without effectors or using effectors alone did not result in increased background spot formation, additionally suggesting AFP specificity (data not shown). These data demonstrate that tolerance to the self antigen AFP can be broken by intratumoral expression of activated IFR-1, a mechanism presumably participating in HCC tumor growth control.

**Factors other than Host Immune Response Participate in Rejection of IFR-1-activated Tumor Cells.** To determine whether the host antitumoral immune response is the only parameter responsible for tumor rejection, in vivo T-cell depletion experiments were performed in E₂-treated mice challenged with HepaIRF-1hER tumors (Fig. 7A). Undepleted mice were again protected against tumor growth. Both CD4 and CD8 T-cell depletion resulted in tumor growth in all of the mice, which, however, was significantly delayed in comparison to undepleted mice that did not receive E₂ treatment. This finding implies that the host immune response is an important factor in tumor protection but seems to be only partial in the initial control of tumor growth.

**IFR-1 Activation Stops Increase of Actively Growing Tumors.** To assess therapeutic efficacy of IFR-1hER activation against HepaIRF-1hER HCCs growing s.c. in E₂-untreated C57L/J mice, E₂ treatment was started at day 19 after tumor challenge. At this point in time, tumors had reached an average size of about 2000 mm³. As demonstrated in Fig. 7B, tumor growth was permanently arrested as early as 4 days after starting of E₂ treatment, demon-
strating a significant therapeutic potential of IRF-1 activation against HCCs. By contrast, HepaIRF-1hER tumors grew rapidly in E\textsubscript{2}-untreated mice. E\textsubscript{2} treatment for 9 days was sufficient to induce long-term tumor growth control. This was readily demonstrated in two of six E\textsubscript{2}-treated mice in which E\textsubscript{2} treatment was stopped at day 28 again. Tumor growth in these mice did not differ as compared with mice that continued to receive two daily E\textsubscript{2} injections.

**DISCUSSION**

HCC is a highly malignant tumor with a poor prognosis and few therapeutic options. Therefore, we have examined a new immunotherapeutic approach aimed at the activation of IRF-1. A murine HCC cell line (HepaIRF-1hER) encoding an IRF-1/hER, which becomes active in the presence of E\textsubscript{2}, was constructed. The in vitro phenotype, cell growth, anchorage-independent growth in vitro, immunogenicity in vivo, and the therapeutic potential of IRF-1 were all examined. Stable constitutive IRF-1 expression has the disadvantage to select for low expressing clones. The actual transgene expression in such cell lines does not much override endogenous IRF-1 expression. Furthermore, constitutive expression induces a selection toward loss of IRF-1 responsiveness over time (4, 40). In contrast, the activatable IRF-1hER system used in the present study allows the tight regulation of IRF-1 activity and the expression of rather high levels of IRF-1 in the tumor cells. The E\textsubscript{2} activatable system has been extensively studied and also compared with a tetracycline-regulated transcription activa-

Fig. 5. CTL activity and T-cell precursor frequency against Hepa1-6 tumors. A, spleen cells derived from tumor-challenged or control mice (n = 5 in each group) were restimulated using irradiated Hepa1-6 cells and subsequently analyzed for cytotoxic activity against syngeneic Hepa1-6 and Lewis lung carcinoma cells at the E:T ratios indicated. For control of specificity, E\textsubscript{2}-treated mice challenged with HepaIRF-1hER cells were in vitro stimulated with 3LL cells and subsequently analyzed for cytotoxic activity against syngeneic Hepa1-6 and 3LL carcinoma cells. Hepa1-6 tumor-specific lysis was presented by subtraction of lysis values against 3LL from lysis values against Hepa1-6 targets. Values represent means of triplicate determinations. B, spleen cells derived from tumor-challenged or unchallenged mice (n = 5 in each group) were stimulated for 20 h with Hepa1-6 or 3LL cells. Subsequently, IFN-\gamma and IL-4 ELIS-POT assays were performed. The spots in each well were counted under a microscope, and the values are expressed as numbers of spot-forming cells relative to the number of spleen cells added to each well at the start of the culture.

Fig. 6. AFP-specific CTL responses and CTL-p frequencies. A, spleen cells derived from tumor-challenged or control mice (n = 5 in each group) were restimulated using irradiated autologous spleen cells infected with UV-inactivated rVV-murine AFP and subsequently analyzed for cytotoxic activity against syngeneic Hepa1-6 and Lewis lung carcinoma cells at the E:T ratios indicated. For control of specificity, E\textsubscript{2}-treated mice challenged with HepaIRF-1hER cells were in vitro stimulated with spleen cells infected with rVV-pSC11 and subsequently analyzed for cytotoxic activity against syngeneic Hepa1-6 and Lewis lung carcinoma cells. AFP-specific lysis was presented by subtraction of lysis values against 3LL from lysis values against Hepa1-6 targets. Values represent means of triplicate determinations. B, spleen cells derived from tumor-challenged or unchallenged mice (n = 5 in each group) were stimulated for 20 h with UV-inactivated rVV-AFP or rVV-pSC11-infected syngeneic irradiated spleen cells. Subsequently, IFN-\gamma ELIS-POT assays were performed.
of the wild-type IRF-1 gene. No differences were found. Because of the use of a mutant estradiol receptor gene (49), the fusion protein is insensitive to low estradiol concentrations and, thus, can be used in mice without being activated by endogenous estrogen levels. A respective tamoxifen-specific mutant of IRF-1 hER (50), however, did not show tight regulation of IRF-1 activity. Therefore, using the E2-inducible system, we could address the antitumoral effects of IRF-1 in more detail and dissect the activation of its different antitumoral effector arms.

By demonstrating growth inhibition and transformation of Hepa-1-6 cells in vitro, we have confirmed a property that has to be attributed to the innate immune activity of IRF-1. Ectopic IRF-1 expression suppresses cellular transformation properties in vitro induced by different oncogenes, such as myc, fosB (29), IRF-2 (51, 52), EGFR, and Ela/β (24). In accordance with results obtained earlier with embryonic fibroblasts from IRF-1+/− mice, in which expression of c-Ha-ras oncogene in wild-type cells but not in IRF-1−/− cells forces the cells to undergo apoptosis under growth-restricted conditions (29), the combined activity of epidermal growth factor receptor and IRF-1 in NIH3T3 cells was shown to induce significant cell death by apoptosis (24). However, the data presented in this report indicate that oncogene-dependent tumor suppression in vitro is not necessary to be mediated by apoptosis. Thus, other mechanisms of transformation inhibition are expected to act. IRF-1 exerts its effects by DNA-binding and transactivation of a number of genes that might contribute to transformation inhibition. Among them are lysyl oxidase (16), PKR (17, 53), 2′-5′ OASA (18), indoleamine 2,3-dioxygenase (19), and angiotensin type II receptor (20). The role of IFN-β secretion in this context is not clear but might act as a feedback enhancer of these genes.

IRF-1 has several other relevant in vivo antitumor activities. These are attributable to the immunomodulatory effects of IRF-1, such as the stimulation of helper T and NK cells (54, 55) and the transcriptional enhancement of MHC genes (4, 31, 56, 57) and of genes involved in antigen presentation (38, 58). Thus, most events or drugs that enhance the expression or activity of IRF-1 might be useful in cancer therapy by inducing specific killing of transformed cells. Indeed, experiments in mice have been described that expression of IRF-1 in aggressive nonimmunogenic sarcoma cells suppresses the malignant phenotype (40). The most important findings of our study are the suppression and control of a highly tumorigenic HCC cell line in vivo. E2 treatment protected 75% of mice against challenge with the HepaIRF-1 hER tumor, and mice developing tumors were characterized by a significant suppression of tumor growth and enhanced survival as compared with E2-untreated animals. Similar results were observed in a recent study (40) where the constitutive expression of IRF-1 in a sarcoma cell line resulted in partial tumor control. Our T-cell depletion experiments demonstrated that CD4+ and CD8+ T cells play an important role in control of tumor growth confirming the known importance of IRF-1 in induction of TH1 differentiation (55). In addition, we observed a significant activation of tumor-specific TH2 cells that may synergistically act against tumor growth (59, 60). We cannot exclude the possibility that NK cells contribute to tumor control. However, the rapid initial tumor growth argues against a significant NK cell-mediated antitumoral effect. In fact, strong CTL responses against the Hepa-1-6 HCC cell line were observed in E2-treated mice challenged with HepaIRF-1 hER tumors.

More importantly, for the first time we were able to demonstrate that intratumoral expression of IRF-1 induces significant T-cell responses against a tumor-associated antigen such as the HCC-specific self antigen AFP, which suggests that tolerance toward AFP can be broken by this approach. AFP-specific CTL activity was low as compared with highly immunogenic viral antigens, such as hepatitis B virus or hepatitis C virus structural proteins (61, 62). This may be the result of the low CTL precursor frequencies and/or low affinity T-cell receptors. However, recent studies demonstrated that AFP-specific T cells after DNA- or dendritic cell-based immunization are functional in vivo against AFP-expressing murine HCCs (44, 63), which suggests that AFP-specific T cells contributed to the antitumoral effects as presented in this study. Although cellular immunity alone was not able to completely control tumor growth as demonstrated by the in vivo depletion experiments, significant T-cell memory against the tumor was induced, which protected mice against a rechallenge with HepaIRF-1 hER and even wild-type Hepa-1-6 cells without additional E2 treatment. More importantly, we tested a potential therapeutic efficacy of IRF-1 expression reflecting the clinical situation after HCC diagnosis. Treatment of mice bearing large HepaIRF-1 hER tumors with E2...

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M. Köster and H. Hanser, unpublished observations.

A. Kröger, unpublished observations.
resulted in a growth arrest of the tumor within 4 days. E2 treatment over a period of 9 days was sufficient to control tumor growth long-term without any additional E2 treatment. According to the tumor protection studies described above, this long-term tumor control may be primarily immune mediated.

Enhanced immunogenicity of HCC tumors expressing IRF-1 may be mediated by up-regulation of MHC class I and II molecules as described previously, though MHC class II induction was low in HCC cells. The costimulatory molecules CD80 and CD86 could not be detected, suggesting that priming of antitumoral immune responses must have occurred in the draining lymph nodes by professional antigen-presenting cells. Additional factors involved in IRF-1-mediated tumor growth control may be the increased generation of MHC class I-restricted antigenic peptides for presentation to the immune system by proteasomes (38, 39, 58).

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