Comparative Activity of Cisplatin, Ifosfamide, Doxorubicin, Carboplatin, and Etoposide in Heterotransplanted Hepatoblastoma

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BACKGROUND. Hepatoblastoma is the most common primary malignant liver tumor affecting infants and young children. Recent clinical experience with advanced hepatoblastoma shows that a reliable in vivo model to study the tumor's response to drugs is needed urgently.

METHODS. Hepatoblastoma cell suspensions from three children were transplanted subcutaneously into NMRI nude mice (nu/nu). One of the primary tumors was a embryonal multifocal hepatoblastoma, whereas the other tumors were embryonal/fetal hepatoblastomas localized to one liver lobe. The xenograft tumors resembled their original tumors histologically and produced high levels of α-fetoprotein. The mice who received the tumors were given ifosfamide, cisplatin, doxorubicin, carboplatin, and etoposide as single agents. Thereafter, the tumor growth rate and α-fetoprotein levels in the animal sera were measured before and after chemotherapy and compared with the control group. After chemotherapy, the tumors were studied by conventional histology.

RESULTS. The tumors in the nude mice derived from the multifocal hepatoblastoma reacted minimally against four of the five cytotoxic agents, whereas cisplatin reduced the tumor volume significantly. There was a marked reduction in tumor volume in the other tumors after application of cisplatin and doxorubicin, respectively. The tumors displayed a moderate reduction in size after treatment with ifosfamide, etoposide, and carboplatin. The responses to the different cytostatic agents also corresponded with serum α-fetoprotein levels and mitotic rates in the tumor cells.

CONCLUSIONS. To the authors' knowledge, this is the first time an in vivo model for analyzing the effects of chemotherapy on hepatoblastoma has been established. The animal model may be suited for the evaluation of new drugs for the treatment of hepatoblastoma and for the investigation of multidrug resistance mechanisms in hepatoblastoma. ***Cancer* 1998;83:2400–7.** © *1998 American Cancer Society.*

KEYWORDS: hepatoblastoma, xenograft, immunodeficient mice, chemotherapy.

Hepatoblastoma (HB) is the most common primary malignant liver tumor affecting infants and young children. Therapeutic outcome has improved during recent years, largely through cooperative multicenter trials by defining chemotherapy regimens and surgical strategies. However, the prognosis for children with advanced or metastatic HB remains poor, which may be due, in part, to the development of drug resistance in nonresectable tumors. Evaluating the effectiveness of single cytostatic drugs is difficult. Since the first successful transplantation of human tumor tissue into athymic nude mice, the role of immunodeficient animals in oncologic research has increased continuously. Human tumor xenografts are used exten-

TABLE 1
Drugs, Dosage, and Schedule of the Two Alternative Chemotherapy Courses (I/II) in Study HB 89/94

Drugs	Dosage (mg/m^2/day)	Schedule (days)
I		
Cisplatin	20	4–8
Doxorubicin	60	9–10
Ifosfamide[a]	1000	1–3
II		
Carboplatin	200	1–4
Etoposide	100	1–4

HB 89/94: Cooperative German Pediatric Liver Tumor Study HB 89/94.
[a] Loading dose was 500 mg/m^2/day.

TABLE 2
Drugs, Dosage, Schedule, and Route of Application in NMHB

Drugs	Dosage (mg/kg/day)	Dosage (mg/m^2/day)	Route	Schedule (days)
Cisplatin	3	9	ip	1–4 /15–18
Doxorubicin	5	15	ip	1–3 /15–17
Ifosfamide[a]	600	1800	ip	1–4 /15–18
Carboplatin	15	45	ip	1–5 /15–19
Etoposide	10	30	i.v.	1,5,9/14,19,24

NMHB: nude mice hepatoblastoma; ip: intraperitoneal; i.v.: intravenous.
[a] Mesna was given at the same mg/kg dosage intraperitoneally with ifosfamide.

sively in the development of potential anticancer drugs and new antineoplastic strategies.[1–4] Only a few HB cell lines have been established and our own experience shows that long term cultures of HB cells are extremely cumbersome and often unsuccessful.[5–7] Therefore, we established an in vivo model of HB in nude mice and studied the tumors' response to those cytotoxic agents applied in the treatment strategies of International Society of Pediatric Oncology (SIOP) pediatric liver tumor trials, the German Cooperative Pediatric Liver Tumor Study HB 94 of the Society of Pediatric Oncology and Haematology (GPOH) and, partially, the Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) trials.

MATERIALS AND METHODS

Patients

Three children, aged 7 months, 9 months, and 3 years, respectively, underwent laparotomy for resection of HB according to the protocol of the German Cooperative Pediatric Liver Tumor Study HB 89 and HB 94 (HB89/94) (Table 1).[8] Patient 1 had an embryonal multifocal HB without fetal differentiation but with areas of anaplasia, whereas Patients 2 and 3 had embryonal/fetal HBs localized to one liver lobe. Patient 1 received 4 courses of chemotherapy (3 courses with ifosfamide, cisplatin, and doxorubicin and 1 course with cisplatin [90 mg/m^2] and doxorubicin [80 mg/m^2]) as continuous infusion therapy after tumor biopsy, and 2 courses of cisplatin and doxorubicin as continuous infusion after extended left hemihepatectomy with complete tumor resection. This child had a tumor recurrence 4 months after surgery and died 6 months later. Primary chemotherapy and treatment for recurrence was ineffective because there was only a minimal reduction of tumor size in the liver. An HB with a viable embryonal tumor without fetal differentiation but with areas of anaplasia was found histologically in the tumor biopsy. After complete tumor resection, the histologic findings were identical to those in the initial tumor biopsy.

A primary complete tumor resection was performed by hemihepatectomy on Patients 2 and 3. Two courses of ifosfamide, cisplatin, and doxorubicin (Table 1) were given after surgical therapy. These patients were alive and well after 5 years of follow-up.

Animals

Female athymic (nu/nu) NMRI - mice, aged 6–8 weeks and weighing 20–25 g, were used in all experiments. The animals were obtained from the Central Breeding Laboratory of the Hannover Medical School and from Bromholdgarel Breeding and Research in Ry, Denmark. The mice were kept under pathogen free conditions, fed an autoclaved standard diet (Altromin; Altromin, Lage, Germany) and given free access to sterilized water.

Drugs

Commercially available drugs were used for the experiments: ifosfamide (Asta, Frankfurt/M, Germany); cisplatin, carboplatin, and etoposide (Bristol-Myers, München, Germany), and doxorubicin (Pharmacia, Erlangen, Germany). All drugs were prepared immediately before administration to the nude mice. The drugs were given in equitoxic doses for nude mice, which were determined from the literature.[9–11] The following formula was used to transform the equitoxic doses for the mouse from mg/kg to mg/m^2 (dose in mg/m^2) = $k_m \times$ (dose in mg/kg) [$k_m = 3$ for nude mice with body weight of 20 g].[12] Drugs, dosage, and route of application are shown in Table 2. The dosage of ifosfamide was higher and the dosage of etoposide and carboplatin were lower than in the patients, because no effect of ifosfamide with a mimic dose of 300 mg/m^2 was found in a pilot study. Greater than 50% of the animals with xenotransplanted tumors died after administration of etoposide or carboplatin with a higher dosage than in Table 2.

Xenotransplantation and Evaluation of Treatment Efficiency

The in vivo model for nude mice HB (NMHB) had been established before by us. Only HB cells with embryonal components were grafted and reproduced successfully in this model. The transplantation of HB cells with mesenchymal components was not possible.[13] Representative excisions from the tumors of Patients 1–3 were made immediately after resection. They were minced and suspended in RPMI 1640 medium (Gibco, Berlin, Germany) at 4 °C under sterile conditions. Approximately 250,000 tumor cells were injected subcutaneously into the paravertebral areas of nude mice under general anesthesia. After inoculation of tumor cells, a tumor growth was observed in approximately 90% of the animals. The animals were observed and the tumors measured. Tumor volume was calculated using the formula: volume = $a/2 \times b/2 \times c/2 \times 4/3\pi$, in which (a = length, b = width, and c = height).

Thereafter, each NMHB subsequently was transplanted into 50 mice for treatment groups. Treatment was initiated when the majority of the tumors reached a volume of 50–100 mm^3. The mice were stratified according to their tumor volume and randomly assigned to groups of ten animals each. The animals injected with tumor were given ifosfamide, cisplatin, doxorubicin, etoposide, and carboplatin as single agents in two blocks (Table 2). One group of ten animals for each original xenograft served as a control group. After initiation of treatment, the tumor growth was recorded at 5-day intervals for 25–30 days and the relative tumor volumes were calculated for each interval by the formula Vdx:Vd0, in which Vdx = tumor volume at any given time and Vd0 = tumor volume at initiation of treatment. Blood was drawn from the animals' retroorbital plexus 2 days before initiation of chemotherapy; after 30 days, when the animals were sacrificed, α-fetoprotein levels were measured using radioimmunoassay (CIS Biointernational, Gif-Sur-Yvettecedex, France, formerly Behring Institut, Dreieich, Germany). In the untreated control groups for each HB, α-fetoprotein levels were measured initially using a tumor volume of 100 mm^3 and after 30 days.

Twenty-four hours before the animals were sacrificed, bromodeoxyuridine (BrdU) was injected intraperitoneally for the semiquantitative determination of proliferation activity of the tumor cells (50 μg of BrdU/g body weight).

Analysis of Growth Curves and α-Fetoprotein Levels

The mean values of relative tumor volumes and α-fetoprotein levels in each group before and after chemotherapy were used to construct the growth curves and α-fetoprotein column. Differences in these measurements after chemotherapy compared with the control group were statistically analyzed with a two-sided student's t test on Day 25. Significance was assumed in case of P values <0.05.[14]

Immunoenzymatic Labeling of Tumor Cell Cytospins for Incorporated BrdU

Fresh tumor cells were separated from debris by density gradient centrifugation on Ficoll-Paque (Pharmacia, Freiburg, Germany) and washed 5 times in phosphate-buffered saline (56.8 mM Na_2HPO_4, 17.9 mM KH_2PO_4, 75 mM NaCl).[15] Cytospins were prepared on a Cytospin 2 centrifuge (Shandon, Astmore, UK). The slides were air-dried and fixed in ice-cold acetone for 8 minutes. Immunoenzymatic labeling for incorporated BrdU was performed using the APAAP method.[16] Three hundred cells of each cytospin were counted to distinguish BrdU positive and negative tumor cells. An average of 50 cytospins for each treatment group and control group was investigated. The data were demonstrated as proliferation activity in percents in comparison with the control group (= 100% proliferation activity). The limit for a sufficient reduction of proliferation activity by chemotherapy was fixed under 50%.

Histology

Representative sections from all tumors were fixed in 3.5% formaldehyde and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin, Giemsa, periodic acid–Schiff, stain, and Goldner and Bielschowsky's reticulin stains. The slides were examined for histologic type as proposed by Ishak and Glunz.[17] Occurrence of anaplasia or atypical mitoses and the mitotic rates were determined. The percentage of tumors with anaplastic components in each treatment and control group was defined as the rate of anaplasia. The amount of tumor regression of each tumor sample was determined as the area of intratumoral bleeding, necrosis, and cystic or fibrotic transformations in relation to the total tumor area in the slide.

In addition, the side effects of chemotherapy on the liver, kidney, and heart were investigated. Criteria of side effects were defined as myocardial inflammation, necrosis of tubuli in the kidney, and areas of regeneration or inflammation in the liver. Side effects were graded as mild, moderate, and severe in regard to the amount of changes.

All experiments were approved by the local official government's ethical committee for animal experiments in Hannover, Germany.

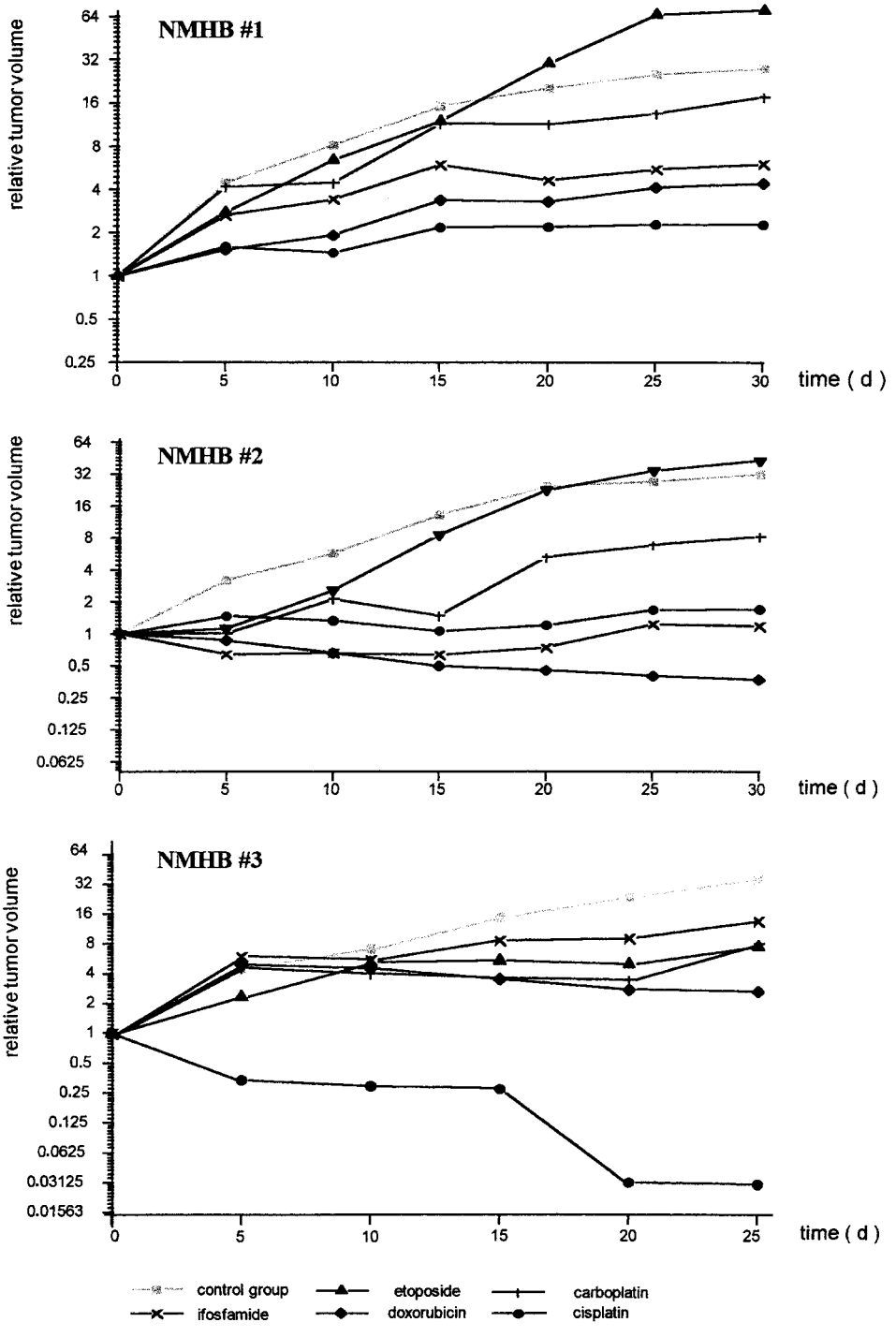

FIGURE 1. Antitumor activity of five drugs against nude mice hepatoblastoma (NMHB) 1–3 (n = 10, mean).

RESULTS

Growth Curves

The curves of relative tumor volumes in HB 1-3 represent the differences of the growth kinetics in the treatment groups compared with the control group (Fig. 1). Compared with the untreated control group, cisplatin and doxorubicin produced a significant retardation of the mean tumor volume in all three HB (*P* values in Table 3). The complete disappearance of a visible tumor in 5 of 10 mice after 25 days could be obtained by cisplatin treatment in NMHB 3. Carboplatin achieved a significant moderate reduction of

TABLE 3
***P* Values for Statistically Significant Differences in Growth Curves between Treatment and Control Groups Using the Two-Sided Student's *t* test**

	NMHB 1	NMHB 2	NMHB 3
Cisplatin	< 0.001	< 0.001	< 0.001
Doxorubicin	< 0.01	< 0.001	< 0.01
Ifosfamide	< 0.02	< 0.001	< 0.05
Carboplatin	> 0.14	< 0.03	< 0.01
Etoposide	> 0.23	> 0.19	< 0.01

NMHB: nude mice hepatoblastoma.

tumor volume in NMHB 2–3 ($P < 0.05$), which were fetal/embryonal HBs, but had no effect on NMHB 1, which was a purely embryonal HB. Etoposide was ineffective in NMHB 1 and 2 and demonstrated only a moderate response in NMHB 3 (Table 3).

α-Fetoprotein Levels

The initial serum levels in α-fetoprotein were elevated in all tumor-bearing mice before chemotherapy (Fig. 2). There was a relatively wide distribution of the α-fetoprotein levels for each HB. The purely embryonal NMHB 1 had lower levels of α-fetoprotein than the more differentiated NMHB 2–3.

An increase of α-fetoprotein levels in nearly all three HBs could be observed during chemotherapy. In relation to the individual animal's pretreatment α-fetoprotein levels after treatment with cisplatin and doxorubicin, an insignificant decrease in α-fetoprotein levels could be measured, except in NMHB 2. A significant reduction of α-fetoprotein after treatment with cisplatin (from 15457 [± 5199] KIE/L to 1307 [± 685] KIE/L) resulted only in NMHB 3 ($P < 0.001$). The increase of α-fetoprotein levels was related to the increase of the tumor size during chemotherapy. Nevertheless, the comparison of α-fetoprotein levels between the treatment and control groups demonstrated a significant reduction of α-fetoprotein values after cisplatin in all three HBs ($P < 0.05$ in NMHB 1 and $P < 0.0001$ in NMHB 2 and 3). In NMHB 1, the other drugs induced only an insignificant delay of increase of the tumor marker in the treatment groups compared with the control group. In NMHB 2 and 3, the measurements also showed a significant reduction of α-fetoprotein levels in the treatment groups versus control groups after the administration of doxorubicin, ifosfamide, and carboplatin ($P < 0.001$). Alpha-fetoprotein levels in NMHB 2 also showed a significant response to etoposide versus the control group ($P < 0.01$).

There was no significant reduction of serum α-fetoprotein levels within the treatment groups after chemotherapy, except in the treatment of NMHB 3 with cisplatin.

Proliferation Activity with BrdU/Anti-BrdU Labeling

In comparison with the control group, the proliferation activity of the tumor cells was reduced as much as 32% (range, 8–32%) in all 3 xenotransplanted HBs after treatment with cisplatin (Table 4). The decrease of proliferating cells to 8% in the cisplatin group correlated with the disappearance of visible tumors in nearly 50% of cases after chemotherapy, especially in NMHB 3. Doxorubicin and ifosfamide showed a reduction in the proliferating activity of >50% in NMHB 1 and 2 versus the control groups. Etoposide reduced the tumor cell proliferation in NMHB 3 to 43%. The other agents tested generally showed weak effects in the reduction of proliferating activity in the three HBs.

Histology: Mitotic Rate and Tumor Regression

NMHB 1 was an embryonal HB with a rate of anaplasia between 50–75% in both treatment and control groups. The mean mitotic rate was 24 mitoses/10 high-power fields (10 HPF) in the control group. A reduction of the mean mitotic rate occurred only after treatment with cisplatin. No difference was observed in the other groups compared with the control group (Table 5). The range of the tumor regression in all treatment groups was 40–50%, with the main component of regression being tumor bleeding. Necrosis or cystic and fibrotic transformation were not found. NMHB 2 was a fetal/embryonal HB with a predominance of embryonal differentiation. The mean mitotic rate was clearly reduced after treatment with cisplatin, ifosfamide, and doxorubicin (Table 5). The tumor regression in these groups was 40–50% for all 3 drugs. The regression in the carboplatin group was only 22% and 31% in the etoposide group. The rate of anaplasia was <20% in the treatment groups. No anaplasia was found in the control group. It is interesting to note that no fetal components were found in tumor biopsies of NMHB 2 after chemotherapy.

NMHB 3 was a fetal/embryonal HB with no signs of anaplasia. The mean mitotic rate in the untreated group was high (60 mitoses/10 HPF). After chemotherapy, a reduction in the mitotic rate of >50% was found in all treatment groups. Tumor regression was good after application of cisplatin, doxorubicin, and ifosfamide (38–51%). Regression was <30% after treatment with carboplatin and etoposide.

After chemotherapy, NMHB 3 also showed only embryonal areas in the tumors and no fetal components after investigation by light microscopy.

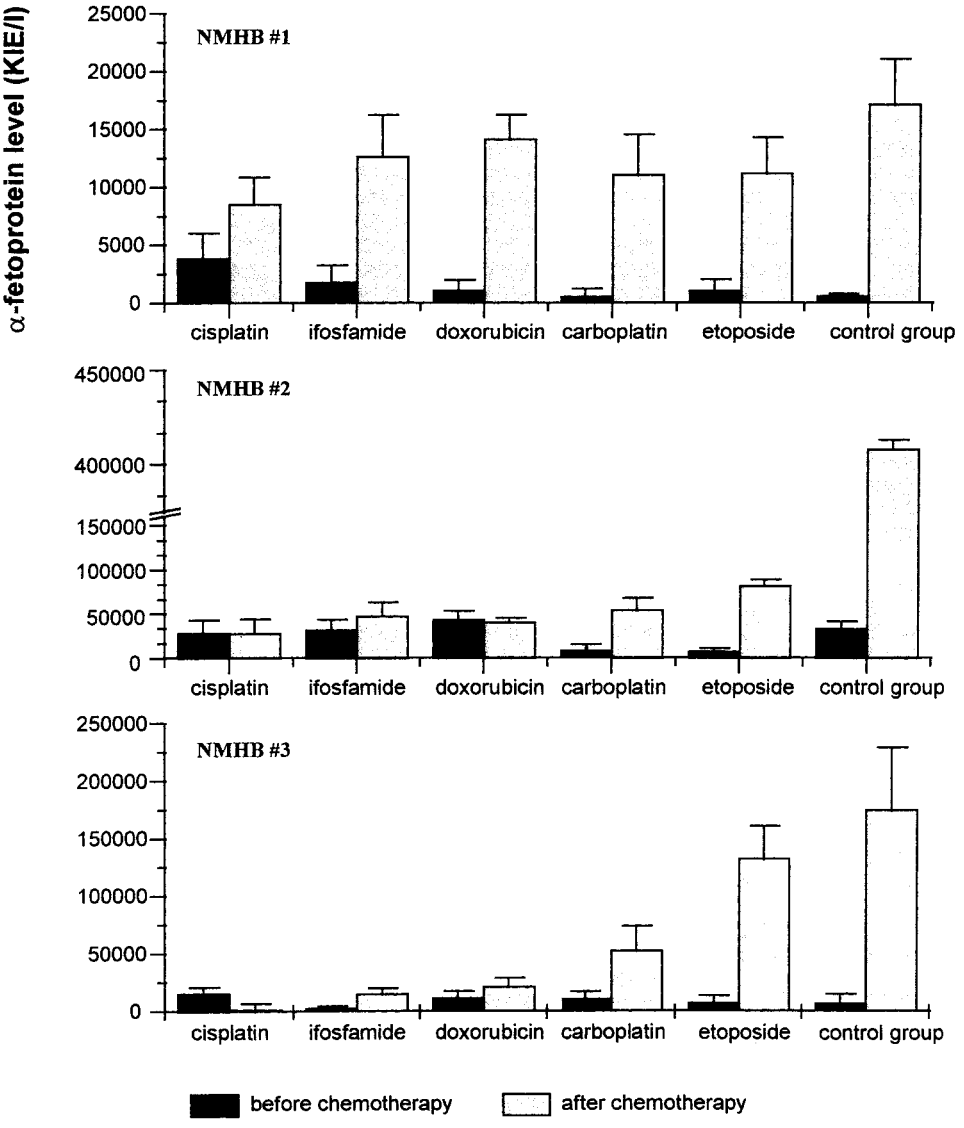

FIGURE 2. α-fetoprotein levels before and after chemotherapy in nude mice hepatoblastoma (NMHB) 1–3 versus the untreated control group (n = 10 for each drug; mean, standard deviation).

Chemotherapy Side Effects

Chemotherapy side effects were observed in only 10% of the mice, without any lethal consequences. Doxorubicin induced necrosis of the renal tubuli in two nude mice. A moderate infiltration of the heart muscle with eosinophilic leukocytes and monocytes was observed in one animal. In 6%, the liver tissue showed areas of regeneration and inflammation with infiltration of lymphocytes, especially after the application of doxorubicin and ifosfamide.

DISCUSSION

A major problem in the treatment of cancer is the development of resistance to chemotherapy and subsequent recurrence. Recent clinical experience with advanced HB shows that a reliable in vivo model for studies on the response of this embryonal tumor to drugs is urgently needed.[18–21] Athymic nude mice have become an important model in experimental oncology. The majority of biologic properties of the tumors are maintained and remain stable during several passages in this model. This has been confirmed for the histologic appearance, the expression of typical tumor markers and, especially, the responsiveness of the tumors to cytostatic drugs.[22] The treatment of xenotransplanted tumors with cytostatic agents in pediatric tumors is also a well known model.[23] Although HB is the most frequent malignant liver tumor in

TABLE 4
Proliferation Activity after Chemotherapy versus Control Group in Percent (mean, n = 50)

	NMHB 1	NMHB 2	NMHB 3
Cisplatin	32	24	8
Doxorubicin	38	32	52
Ifosfamide	44	43	57
Carboplatin	49	72	52
Etoposide	51	88	43
Control group	100	100	100

NMHB: nude mice hepatoblastoma.

TABLE 5
Mitotic rate per 10 High-Power Fields after Chemotherapy (mean, n = 10)

	NMHB 1	NMHB 2	NMHB 3
Cisplatin	12	8	11
Doxorubicin	20	13	11
Ifosfamide	23	10	27
Carboplatin	20	18	15
Etoposide	21	23	16
Control group	24	23	60

NMHB: nude mice hepatoblastoma.

childhood, only a few in vitro and in vivo models are available.[5,6,13,24,25] Our current study demonstrated that it is possible to transplant fresh HB cells into nude mice and achieve stable growth over multiple generations without major histologic and functional alterations.[13] To our knowledge, the current study is the first to describe the systematic evaluation of the effectiveness of single drugs in heterotransplanted HB using the clinical treatment strategies of the SIOP and GPOH. The results demonstrate that evaluation of the effectiveness of chemotherapy in nude mice HB is only possible by analysis of multiple parameters such as decrease of serum α-fetoprotein, decrease of proliferation activity, and histologic regression of the tumor under chemotherapy.

NMHB 1 is an example of a tumor with a poor response to the majority of cytotoxic agents. Cisplatin, ifosfamide, and doxorubicin caused a reduction in the tumor growth, but the α-fetoprotein levels did not decrease accordingly. Analysis of the proliferation activity and the histologic investigations clarify the contradictions. Mitotic activity was high in nearly all treatment groups and the rate of anaplasia was decreased only slightly in comparison with control groups, but anaplasia persisted in >50% of tumors. The mitotic activity was reduced only in animals treated with cisplatin. However, in this HB, cisplatin is moderately active. The experimental results are comparable to the clinical course of the patient. After chemotherapy, this patient showed insufficient shrinkage of the tumor volume. Histologically, there was high grade anaplasia and insignificant regression of the tumor determined after complete resection. The child suffered from recurrent tumor in the liver 4 months after complete remission and died 6 months later.

NMHB 2 and 3 were similar in their biologic behavior during chemotherapy. There was a good correlation between reduction of tumor volume, α-fetoprotein levels, proliferation activity, and histologic findings. Cisplatin, ifosfamide, and doxorubicin appeared to be the most effective for the treatment of these heterotransplanted HBs. Cisplatin showed the strongest effect. This fact was supported by the complete disappearance of visible tumor in NMHB 3 after cisplatin treatment in nearly 50% of the mice. All NMHB had the lowest levels of α-fetoprotein and the lowest rate of proliferating tumor cells after treatment with cisplatin. However, histologic tumor regression after cisplatin application (range, 40–50%) was not higher than that occurring after doxorubicin or ifosfamide (range, 38–40%). Possible explanations of this phenomenon may include: 1) HB in this model can show spontaneous regression without chemotherapy,[26] 2) we applied a semiquantitative laboratory method with the histologic investigation, and 3) these tumors can be heterogeneous.

The xenografted HB responded only moderately to carboplatin. Etoposide alone was ineffective in the treatment of NMHB. Only NMHB 3 showed a moderate response to etoposide in comparison with the control group.

The synergistic effect of the combination of etoposide and carboplatin in clinical trials is known.[27] The use of combinations of cytoxic drugs in nude mice is problematic. During monotherapy, the average body weight lost was 10% and the rate of side effects in the animals with regard to the kidney, heart, and liver was <10%. In a limited pilot experiment using combination treatment, body weight loss of >25% was induced in the animals and >50% of the animals died a few days after drug application. However, a similar investigation of the tumor material after the application of etoposide and carboplatin correlated well with the growth curves of tumor volume and α-fetoprotein levels in all NMHB. The histologic tumor regression was <30%, in contrast to the cisplatin, ifosfamide, and doxorubicin group. Intratumoral bleeding alone was the major component in tumor regression after the application of etoposide and carboplatin. Necrosis or cystic and fibrotic transformation were observed infrequently.

It is interesting to note that the fetal/embryonal

HBs showed a tendency of the fetal tumor components to disappear under chemotherapy. This is difficult to explain and opposes the theory that HB arise from pluripotent cells and differentiate under chemotherapy.[26,28] Perhaps this phenomenon is caused by the different dynamics of the proliferation activity in the animal model versus human HBs. Further investigations with HB cell lines are necessary. Recently, our group established a cell line, HepT1, from HB cells from Patient 1.[29]

HB is a transplantable tumor in nude mice that resembles the original human tumor with respect to histologic and functional phenotype. Therefore, the xenografts are a feasible in vivo model for studies of growth characteristics and response to cytotoxic drugs. Cisplatin and doxorubicin are the most effective agents for treatment of xenotransplanted HB. Ifosfamide is effective against some xenotransplanted HB. Carboplatin shows only moderate activity and etoposide is, as a single substance, insufficient for the treatment of NMHB. However, combinations of drugs should be the mainstay of chemotherapy in the treatment of patients with HB. This model can contribute to an improvement in therapy regimens for children with HBs and may be a good basis for analyzing the drug resistant mechanisms in HB.

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