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Paclitaxel: An Effective Antineoplastic Agent in the Treatment of Xenotransplanted Hepatoblastoma

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Background. Hepatoblastoma is an uncommon liver tumor of infancy and early childhood. Though most patients with nonmetastatic hepatoblastomas can be cured by defining surgical strategies and chemotherapy regimes, new drugs are needed for children with advanced hepatoblastomas. The activity of paclitaxel as a new antineoplastic agent with limited experience in pediatric oncology was studied in a xenograft model. ***Procedure.*** Hepatoblastoma cell suspensions from three children were transplanted subcutaneously into nude mice NMRI (nu/nu). One of the primary tumors was an embryonal multifocal hepatoblastoma, whereas the other tumors were embryonal/fetal hepatoblastomas localized on a liver lobe. After 4 weeks, xenografted tumor sizes reached 50–100 mm^3. The xenografted tumors resembled their originals histologically and produced high levels of α-fetoprotein. The efficiency of paclitaxel at equitoxic doses was analyzed. ***Results.*** Paclitaxel produced an effect in all three hepatoblastomas. There was a significant reduction of tumor volume ($P < 0.001$) and α-fetoprotein levels after chemotherapy ($P < 0.0001$). The proliferation activity of the tumor cells corresponded with these results. Histologically, after treatment with paclitaxel the tumor regression was 35%–49%. The mechanism of paclitaxel action could be demonstrated by light microscopy immunohistochemistry and electron microscopy. ***Conclusions.*** The preliminary results in phase I trials of solid tumors in children and the results of this study suggest that paclitaxel in phase II studies can now be entertained for patients with hepatoblastoma. Med. Pediatr. Oncol. 32:209–215, 1999.

Key words: hepatoblastoma; xenografts; immunodeficient mice; paclitaxel

INTRODUCTION

Paclitaxel (TAXOL), the first organic compound with a taxane ring demonstrated to possess antineoplastic activity was isolated in 1967 from the bark of the Western yew, *Taxus brevifolia* [1]. Clinical results from completed phase II trials in ovarian, breast, and non–small-cell lung cancers suggest that TAXOL may become an important and widely used antineoplastic drug [2]. Experience with TAXOL in the treatment of pediatric tumors is very limited [3–5]. Our study investigated the cytotoxic activity of paclitaxel against heterotransplanted hepatoblastomas (HB).

Long-term survival for children with hepatoblastoma is only realistic if the primary tumor can be completely excised [6–10]. At the time of diagnosis, complete tumor resection is only possible in fewer than 50% of pediatric patients with hepatic cancers [11]. Although chemotherapy can reduce extension of nonresectable tumors, cytostatic drugs alone can rarely eradicate HB [12,13]. Therapy of preliminary nonresectable or advanced HB can be improved with a rational approach using different strategies for identifying new active agents and refined surgical procedures. Therefore, a reliable in vivo model to study the response to new drugs is urgently needed. As yet, only few hepatoblastoma cell lines have been established and long-term cultures of this tumor are extremely cumbersome and often unsuccessful [14,15].

MATERIALS AND METHODS

Patients

The original tumors were removed from the livers of 7-, 9-, and 34-month-old children. The children underwent laparotomy according to the protocols of the German Cooperative Pediatric Liver Tumor Studies HB 89 and HB 94 [16,17]. Patient 1 had an embryonal multifo-

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cal HB, whereas patients 2 and 3 had embryonal/fetal HBs localized in one liver lobe. A primary complete tumor resection was performed by hemihepatectomy on patients 2 and 3. These children were given two courses of ifosfamide (IFO), cisplatin (CDDP), and doxorubicin (DOXO). They are alive, without evidence of disease, after 5-year follow-up. Patient 1 received four courses of chemotherapy. An extended left hemihepatectomy with complete tumor resection was performed after two courses of chemotherapy. This child had a tumor relapse 4 months after operation and died 6 months later. The chemotherapy was ineffective, there was minimal reduction of tumor size in the liver, and histologically an HB was found with viable embryonal tumor and areas of anaplasia, but without fetal differentiation. These were present in the tumor biopsy, as well as after complete tumor resection.

Xenotransplantation

Female athymic (nu/nu) NMRI-mice, 6–8 weeks old and weighing 20 to 25 g, were used in all experiments. The mice were kept under pathogen-free conditions, fed with an autoclaved standard diet (''Altromin'') and given free access to sterilized water. The animals were obtained from Bromhold Breeding and Research in Denmark.

The in vivo model for nude mice HB had been established before by the authors [14]. Approximately 250,000 tumor cells from patients 1–3 were injected subcutaneously into paravertebral areas of nude mice immediately after tumor resection. Nude mice with subcutaneous tumors were sacrificed when the tumors reached a volume of 200–300 mm^3. Tumor volume was calculated with the formula $V = a/2 \times b/2 \times c/2 \times 4/3\ \pi$. Thereafter, each nude mouse HB was subsequently transplanted into 60 mice for treatment and control 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 10 animals each. One group of 10 animals for each original xenograft served as a control group.

Evaluation of Drug Response and Effects on Tumor Tissue

In the treatment groups, each animal was injected i.v. with 12 mg/kg KG TAXOL (Bristol-Myers Squibb, Princeton, NJ) on days 5 to 8 and 15 to 18. Equitoxic doses of TAXOL were determined from the literature [18].

Parameters for judgment of drug response of TAXOL against heterotransplanted hepatoblastomas were reduction of tumor volume and serum levels of the tumor marker α-fetoprotein. The effects of TAXOL on the tumor tissue were determined with the proliferation activity of the tumor cells in immunocytologic investigation, the data on the postchemotherapy hematoxilin/eosin (H&E) findings, and transmission electron microscopy.

Tumor growth after i.v. drugs was recorded at 5-day intervals for 30 days and the relative tumor volume was calculated for each interval using the formula Vdx/Vd0, where Vdx is the tumor volume at any given time and Vd0 is the tumor volume at initiation of treatment.

The α-fetoprotein levels were measured using radioimmunoassay (CIS Biointernational, France). Blood was drawn from each animal's retroorbital plexus 2 days before and after chemotherapy or, in the untreated control groups, when the tumor reached a volume of 100 mm^3 or more, and after 30 days.

The mean values of relative tumor volumes and α-fetoprotein levels in each group, before and after chemotherapy, were used to construct growth and α-fetoprotein columns. Different tumor volumes and various α-fetoprotein concentrations after chemotherapy against untreated control group were statistically analyzed on day 30 with a two-sided *t*-test [19].

Immunoenzymatic Labeling of Tumor Cell Cytospins for Incorporated BrdU

Twenty-four hours before the animals were sacrificed, bromodeoxyuridine (BrdU) was injected intraperitoneally for semiquantitative determination of the proliferation activity of the tumor cells (50-μg BrdU/g body weight). Fresh isolated tumor cells from different nude mice hepatoblastomas (NMHBs) were separated from debris by density gradient centrifugation on Ficoll-Paque (Pharmacia Freiburg, Germany) and washed five times in PBS (56.8-mM Na_2HPO_4, 17.9-mM KH_2PO_4, 75-mM NaCl). Cytospins were prepared on a cytospin 2 centrifuge (Shandon, Astmore, U.K.). The slides were air-dried and fixed in ice-cold acetone for 8 min. Immunoenzymatic labeling for incorporated BrdU was performed using the APAAP-method [20,21]. 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 made.

Histology

Representative sections from all tumors were fixed in 3.5% formaldehyde and embedded in paraffin. Paraffin sections (5 μm) were stained with H&E. The slides were examined for histological type as proposed by Ishak and Glunz [22]; presence or absence of anaplasia was determined. 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.

Immunostaining on Cryostat Sections Against Tubulin

Rabbit antitubulin polyclonal antibody (Chemicon, USA) was used for identification of tubulin assembly

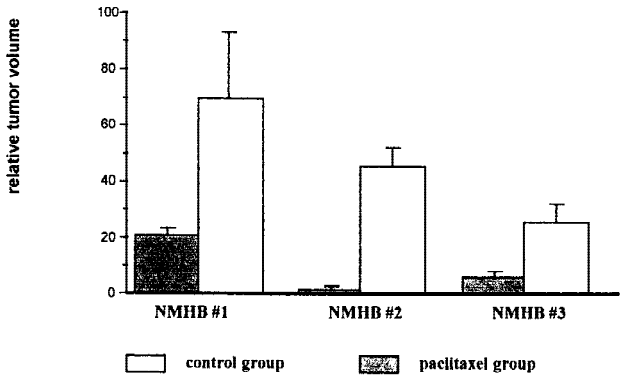

Fig. 1. Antitumor activity of paclitaxel against NMHBs 1–3 after 30 days (n = 10; mean, SD).

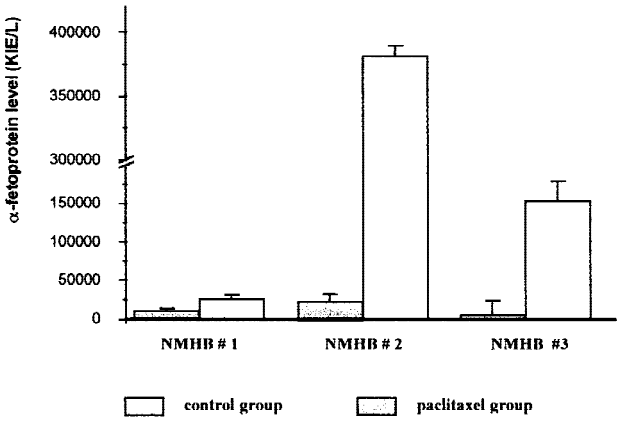

Fig. 2. α-fetoprotein levels with and without chemotherapy in NMHBs 1–3 after 30 days (n = 10; mean, SD).

after treatment of xenotransplanted HB with TAXOL. Immunostaining was performed on 5-μm cryostat sections using the APAAP technique essentially as described by Cordell et al. [21]. Antibodies against vimentin (DAKO A/S, Hamburg, Germany) were used for better differentiation between tumor components and mouse stroma. Briefly, the slides were fixed in ice-cold acetone for 10 min and air-dried. After preincubation, the primary antibody was added for 1 hr at room temperature. Thereafter, incubation followed with the APAAP-complex for 1 hr. Color development was performed with new fuchsin (Sigma, Deisenhofen, Germany). After washing with deionized water and counterstaining with hemalaun (Merck, Darmstadt, Germany), the slides were mounted with glycerol-gelatin (Merck). Positive reaction resulted in bright red staining.

Transmission Electron Microscopy

For ultrastructural analysis, small pieces of about one cubic millimeter were excised from nude mice HB, immediately fixed by immersion in a solution of 3% glutaraldehyde and 0.1-M cacodylate buffer (pH 7.4) for 8 hr and washed in the same buffer overnight.

Dehydration was performed in graded alcohol solutions and embedding in epoxy resin (Epon). After investigation of the gross morphology in light microscopic sections stained with toluidine blue, thin sections (70 nm) were stained with lead citrate and uranyl acetate and observed in a transmission electron microscope (Zeiss EM 10). All experiments were approved by the regional government's ethical committee for animal experiments, Hannover, Germany.

RESULTS

Reduction of Relative Tumor Volume

The difference in the relative tumor volume reduction as measured on day 30 between the treatment and control groups are reported in Figure 1. Compared to the untreated control groups, TAXOL produced a significant reduction of the relative tumor volume in all HBs after 30 days (NMHB 1, $P < 0.001$; NMHB 3, $P < 0.008$). NMHB 2 was very sensitive to paclitaxel ($P < 0.0001$).

α-Fetoprotein Levels

The initial serum levels of α-fetoprotein were elevated in all tumor-bearing mice before chemotherapy. The pure embryonal NMHB had lower levels of α-fetoprotein than the differentiated NMHBs 2 and 3. There was a relatively wide distribution of the α-fetoprotein values for each HB. The serum levels of α-fetoprotein in nude mice after chemotherapy were related to the growth columns in all NMHBs comparing the treatment and control groups (Fig. 2). The differences between the control groups and treatment groups were significant in all three HBs ($P < 0.0001$).

Proliferation Activity

The semiquantitative determination of the proliferation activity of the tumor cells in NMHBs after application of TAXOL showed a reduction of positively labeled cells in the treatment groups compared to the control group (Table I). Nevertheless, in all NMHBs, approximately 30 to 50 cells/300 counted cells showed a proliferation activity after chemotherapy. This phenomenon related to the increase of relative tumor volume after chemotherapy in the treatment groups (Fig. 1).

Histological Findings

NMHB 1 was an embryonal HB with anaplastic components in treatment and control group of over 50%. The mean tumor regression in the treatment group was 49% (25%–95%). The main components of regression, with an equal distribution, were tumor bleeding and necrosis. A fibrotic tumor transformation of 5% was found only in 2 of 10 tumor specimens. In the control group, the tumor regression was approximately 40%.

TABLE I. Proliferation Activity After Chemotherapy Proliferating Cells/300 Counted Cells (Mean, n = 50)

	NMHB 1	NMHB 2	NMHB 3
Paclitaxel	50	35	33
Control group	85	68	65

NMHBs 2 and 3 were similar in their biological behavior under chemotherapy. Both NMHBs were fetal/embryonal HB with a predominance of embryonal differentiation. No areas of anaplasia were found. The mean tumor regression in NMHB 2 was 45% (range, 15%–80%) and in NMHB 3 only 35% (range, 15%–65%). The main components of tumor regression in both NMHBs were also tumor bleeding and necrosis, but, especially in NMHB 3, 9 from 10 tumors had a fibrotic tumor transformation (mean, 7%; range, 5%–30%). Interestingly, no fetal components were found in the tumor specimens of NMHBs 2 and 3 after chemotherapy. In the control groups of these NMHBs, some fetal areas were found and tumor regression was 40%–50%, with large areas of bleeding and central necrosis.

Immunhistochemistry With Antitubulin Antibody

Immunhistochemistry with the antitubulin antibody demonstrated the promotion of microtubules by shifting the dynamic equilibrium toward microtubule assembly in all NMHBs. The distribution of microtubules was different. The pure embryonal NMHB 1 showed only a moderate reactivity of hepatoblastoma tissue to the antibody, whereas the reaction in NMHB 2–3 was strong (Table II, Fig. 3). In the control group, the reaction of the tumor cells with the antitubulin antibody was negative (Fig. 4). In control staining, these were negative for vimentin, in contrast to the strong reaction of the stroma. The host connective tissue of the mouse surrounding the tumor areas reacted with antibody. The murine monoclonal antibody against vimentin was detectable only in connective tissue, because the embryonal and fetal HBs showed negative for vimentin staining [23].

Transmission Electron Microscopy

HB tumors not treated with TAXOL showed dense tissue arranged in nodules separated by thin layers of connective tissue. Blood vessels developed in the connective tissue and inside the nodules, where the cells appeared relatively homogeneous; occasionally, there were foci of hematopoiesis. The cells within the HB nodules, in the main, resembled hepatocytes, but were arranged rather densely instead of forming trabeculi. Interspersed were some nonparenchymal liver cells (Fig. 6).

Cells showing different amounts of intracytoplasmatic microtubules were also found between the normal ones in TAXOL-treated HB. Microtubules were primarily found near the nuclei. Cells with lower microtubule accumulations showed several normal cell organelles, i.e., mitochondria without swelling and rough-surfaced endoplasmic reticulum, indicating a still viable metabolism (Fig. 5A). Other cells were totally occupied with dense accumulations of microtubules and showed clear signs of cell death and disintegration (Fig. 6B). This was seen

TABLE II. Reactivity of Hepatoblastoma Cells for Antitubulin Antibody*

	Tubulin
NMHB 1	
Paclitaxel group	+
Control group	–
NMHB 2	
Paclitaxel group	+++
Control group	–
NMHB 3	
Paclitaxel group	++
Control group	–

*–, negative; +, moderate; ++, strong; +++, very strong.

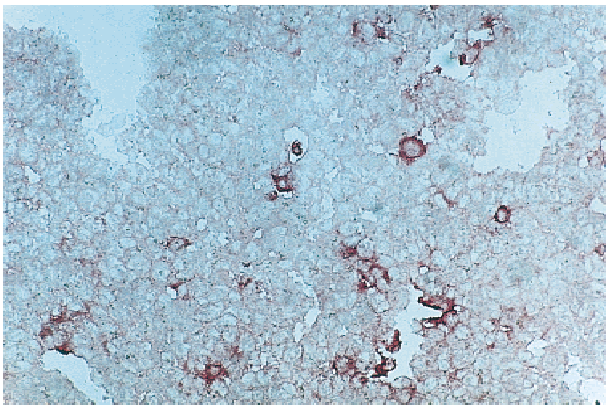

Fig. 3. NMHB 2 after chemotherapy with multiple hepatoblastoma cells with strong expression of perinuclear promotion of microtubules (5-μm cryostat section; APAAP, ×310).

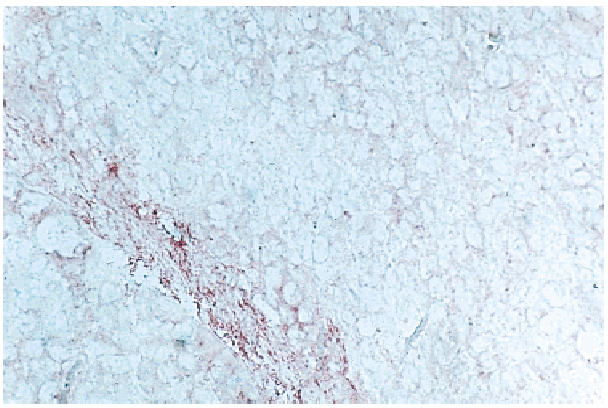

Fig. 4. NMHB 2 control group with antitubulin antibody staining. The reaction of tumor tissue against antitubulin was negative, but the mouse connective tissue cells surrounding the tumor areas reacted with antibody (5-μm cryostat section; APAAP, ×310).

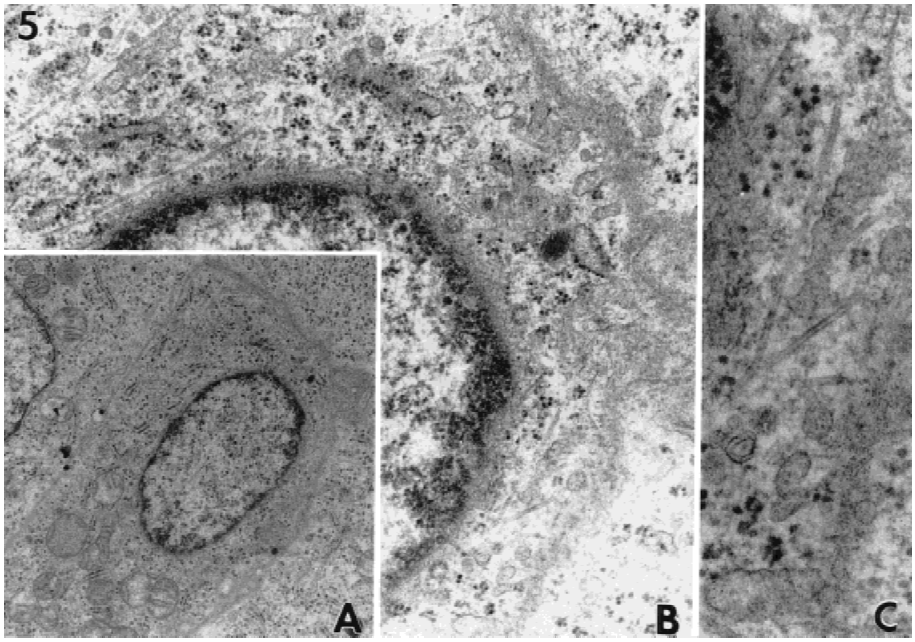

Fig. 5. A hepatoblastoma cell with a moderate accumulation of microtubules as seen in the transmission electron microscope (**A**). Further enlargement shows multiple microtubules in the perinuclear area. These are randomly arranged and only those meeting longitudinally are well identified (**B**). High power magnification (**C**) clearly identifies these microtubules with a double dense outer lining and a brighter central line. TEM: A ×10,000, B ×40,000, C ×80,000.

as cell death with nuclear condensation and fragmentation. Formation of apoptotic bodies containing parts of the nucleus and cytoplasm was also seen in these cells.

DISCUSSION

The taxanes are a new group of anticancer agents with a novel mechanism of action. The drug promotes microtubule assembly and stabilizes the microtubules. This is distinctly different from the action of other agents, such as colchicine and vinca alkaloids [24]. TAXOL, an effective agent in the treatment of non–small-cell lung cancer, ovarian cancer, and breast cancer [2], is currently in phase I pediatric oncology trials [3–5].

The treatment of hepatoblastoma has improved over the years, largely through cooperative multicentric trials. Surgical strategies and chemotherapy regimes have been defined [16,17,25]. However, the prognosis of children with advanced HB is still dismal, due in part to the development of drug resistance in nonresectable tumors [6,26–28]. Hence, an in vivo model is an important requirement for evaluating the effectiveness of new single cytotoxic agents that are needed in order to improve results.

The treatment of xenotransplanted tumors with cytostatic agents in pediatric tumors is a well-known model [29–31]. We have demonstrated that it is possible to transplant fresh HB cells into nude mice and achieve stable growth over multiple generations without major histological and functional alterations [14]. The evaluation of the response of TAXOL in xenotransplanted HB is described in this study for the first time. The results demonstrate that TAXOL is an active agent in the treatment of xenotransplanted HB. After chemotherapy, all three HBs showed a significant reduction in tumor volume, a decrease of serum α-fetoprotein, a decrease of proliferation activity, and histologic evidence of tumor response.

There was tumor regression with hemorrhage, necrosis, and partially fibrotic transformation of the HB. The last was not observed in the untreated control groups. Comparison of tumor regression in percent between the treatment and control groups is impossible, because the relative tumor volume of the control groups increased 30 to 70 times in 30 days and the nutrition of the isolated subcutaneous mouse tumor is therefore very limited. The result of this phenomenon is a high rate of spontaneous intratumoral bleeding and necrosis. The disappearance of fetal tumor areas under chemotherapy can possibly be explained by different dynamics of cell proliferation in the animal model vs. human hepatoblastoma, or by the hypothesis that HB cells arise from pluripotent cells and differentiate under chemotherapy [27,32].

Recent investigation of NMHB 1 with the five drugs CDDP, IFO, DOXO, carboplatin (CARBO), and etoposide (VP-16) demonstrated that only CDDP was an active agent against this HB [33]. In contrast to DOXO, IFO, VP-16, and CARBO, TAXOL showed an effective

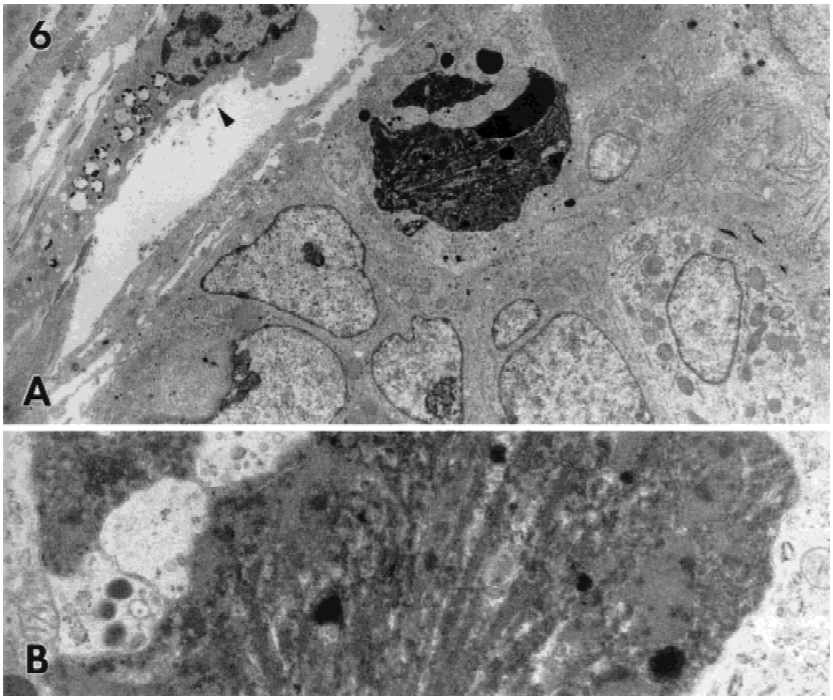

Fig. 6. Transmission electron micrograph of the periphery of a xenotransplanted hepatoblastoma nodule encircled by layers of host connective tissue with a fibrocyte (arrowhead in **A**). A dead hepatoblastoma cell at the nodular border shows a dense transformation of the cytoplasmic content. This cell is encircled by vital neighbor cells. These have intact cell organelles but show frequent and large nucleoli, representing a sign of cellular activation. The cytoplasm of the dead cell is almost completely filled by parallel arrays of densely accumulated microtubules (**B**). TEM: A ×4,000, B ×10,000.

activity against NMHB 1. In the parameter of tumor volume reduction, modification of α-fetoprotein level under chemotherapy, and proliferation activity, the efficiency of TAXOL was comparable with the cytostatic drug CDDP, although there was only a moderate promotion of microtubule assembly in the antitubulin staining. A possible explanation is the histologic subtype of the tumor. NMHB 1 is an embryonal HB with multiple areas of anaplasia [33,34].

NMHBs 2 and 3, as fetal/embryonal tumors, were similar in their biological behavior under chemotherapy, i.e., there was a good correlation between tumor reduction, α-fetoprotein levels, and proliferation activity. The reactivity and size of foci from assembled microtubules around the nucleus was greater in the fetal/embryonal HB than in the purely embryonal HB.

The electron microscopy findings confirmed the results of immunohistochemistry with the antitubulin antibody by finding different grades of a diffuse accumulation of microtubules in several cells inside the HB nodules, but still containing vital cell organelles. HB cells without vital cell organelles and undergoing cell death were always completely occupied by microtubules; also, they were only focally detected by transmission electron microscopy. Besides the topographic location of alterations, these results also verify the mechanism of action of TAXOL in the specimens investigated in the present study.

In contrast to other in vivo models with xenotransplanted pediatric tumors, such as rhabdomyosarcoma, TAXOL, CDDP, IFO, DOXO, CARBO, and VP-16 alone cannot totally eliminate the subcutaneous tumors in xenotransplanted HB [35,36]. The partial survival of tumor cells is also morphologically indicated here by the high number of cells that still appear viable. They show only moderate antitubulin staining in immunohistochemistry and relatively small accumulations of microtubules on transmission electron microscopy. This point also demonstrates the malignant behavior of this tumor in xenotransplanted HB.

In conclusion, TAXOL is an active anticancer agent in the treatment of xenotransplanted HBs. The efficiency of TAXOL is similar to CDDP representing, as yet, the most efficient cytostatic agent in the treatment of the three NMHBs [33]. Further investigations are necessary to check the effectiveness of TAXOL alone or the combination CDDP plus TAXOL as possible regimens in the treatment of HB. Immunohistochemistry and electron microscopy established the effect and mechanism of TAXOL in HB cells. Preliminary results in the phase I trials in children with refractory solid tumors documented an antitumor effect [3–5]. The activity in heavily

pretreated patients strongly suggests that TAXOL may, in the future, play an important role in treatment of children with cancers.

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