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J. Sundström · L.J. Pelliniemi · E. Salminen
P. Pöllänen · E. Abdelwahid · E. Veräjänkorva
K.-O. Söderström

Effect of etoposide on experimental testicular teratoma in 129/SvJ mice

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Abstract To study the effects of etoposide on experimental testicular teratoma in 129/SvJ mouse we analysed the tumour growth, differentiation, apoptosis and the localisation of mdr_1 P-glycoprotein (mdr_1-Pgp). In this model the implanted gonadal ridges developed into testicular teratomas in 17 out of 56 implanted testes (30%) and in 14 out of 28 mice (50%). The tumour-bearing mice were treated with etoposide on 4 successive days either 4 weeks or 6 weeks after implantation, and killed 7 days after the last dose. The mice in the control groups did not receive etoposide. The teratomas consisted mainly of neural tissue. The etoposide-treated 4-week teratomas, but not the 6-week teratomas, were significantly smaller than those in the corresponding control groups. The density of apoptotic cells and the distribution of the mdr_1-Pgp were not altered by etoposide. The decreased proportion of immature neuroectodermal tissue components was observed in all treated teratomas, converting the histology towards that of a mature teratoma. In addition, a low proportion of immature tissue components was frequently combined with a low density of apoptotic cells. In conclusion, etoposide decreased the immature tissue components of teratomas, while mature tissues remained unaffected. These results may have clinical relevance in man, since they confirm that postchemotherapy mature teratomas cannot be treated with chemotherapy. Despite benign histology, the human residual tumours have a significant malignant potential and require complete surgical excision and close surveillance.

Key words Testis · Neoplasms · Tissue culture · Differentiation · Etoposide

J. Sundström (✉) · P. Pöllänen · E. Veräjänkorva
Department of Anatomy, University of Turku,
Kiinamyllynkatu 10, 20520 Turku, Finland
e-mail: Jari.Sundstrom@utu.fi
Tel.: +358-2-3337315, Fax: +358-2-3337352

E. Salminen
Department of Oncology, University of Turku, 20520 Turku, Finland

J. Sundström · L.J. Pelliniemi
Laboratory of Electron Microscopy, University of Turku,
20520 Turku, Finland

K.-O. Söderström · J. Sundström
Department of Pathology, University of Turku,
20520 Turku, Finland

P. Pöllänen
Department of Obstetrics and Gynaecology,
University of Turku, 20520 Turku, Finland

E. Abdelwahid
Department of Paediatrics, University of Turku,
20520 Turku, Finland

Introduction

Testicular cancer is the most common malignant neoplasm among young men in the western world [15], and the incidence is increasing [3]. It has a favourable prognosis, because most patients, even with advanced disease, can be cured [1]. High sensitivity of testicular cancer to cytostatic drugs has a key role in the treatment of patients with advanced disease [9]. Etoposide is a standard component of therapy for testicular cancers [2]. It acts by inhibiting the topoisomerase II enzyme, which belongs to a group of enzymes essential for DNA replication, transcription, chromatin segregation and DNA recombination [16]. Inhibitors of topoisomerase II affect the vital control mechanisms of the cell cycle, which induces a series of events finally considered to lead to apoptosis, or programmed cell death [8, 28]. One of the main difficulties hampering successful cancer chemotherapy is drug resistance, which can be either intrinsic or acquired [14]. Several mechanisms of drug resistance have been described, but the P-glycoprotein (Pgp) encoded by the *mdr_1* gene is the most widely studied factor in the development of drug resistance [11, 19].

The in vivo model of experimental testicular teratoma in 129/SvJ mouse was established in our laboratory [32, 33]. The tumours induced are composed of a variety of tissues, but neural tissue components predominate in these teratomas [29, 33]. The tumour growth is rapid

during the first 6 weeks after induction, and the teratomas include immature neuroectodermal tissues until 6–8 weeks of culture time have elapsed [33]. After this period the histology changes to that of a mature teratoma [33].

In the present study the effect of etoposide on teratomas during the rapid and the slowing phases of growth was investigated. The diameter of the tumours and the volume density of immature tissue components were measured. The amount of cells with DNA fragmentation was studied using the ISEL method. In addition, to study drug resistance, *mdr*$_1$ P-glycoprotein (mdr$_1$-Pgp) was localised in the host testis and in the teratomas.

Materials and methods

Animals and induction of tumours

129/SvJ mice (stock no. 000691) from the Jackson Laboratory (Bar Harbor, Maine, USA) were used as experimental animals. One male was housed with one female per cage for breeding for 24 h, after which the females were examined for a vaginal plug. On the day on which the plug was found the age of the embryos was considered to be 0 days. Embryos were obtained at the age of 12 days. The females were killed with CO_2. A medial ventral incision was made and the uterus was opened. The embryos were dissected out from the uterus and fetal membranes using a dissecting microscope and killed with immediate decapitation. The abdominal contents of the embryos were removed under a dissecting microscope, and gonadal ridges were dissected out from the posterior abdominal wall, separated from the mesonephros and placed in fresh Hanks' medium (+4°C) for a few hours. The sex of the indifferent gonads was identified by X chromatin staining of the amniotic membranes (Orcein, Fluka). Adult male mice from the same strain (*n*=28) were anaesthetised with Hypnorm (Janssen-Cilag, Saunderton, High Wycombe, Bucks., UK), Dormicum (Hoffmann-La Roche, Basel, Schweiz) and sterile water (Kabi Pharmacia, Sweden) at 1:1:2, respectively. The testes were pushed to the abdomen through the inguinal canal and exposed through medial ventral incision. Gonadal ridges from male embryos were implanted into both testes of each adult mouse with a glass capillary. The animals were killed with CO_2 7 days after the last etoposide injection. The mice without cytostatic treatments were killed after a corresponding time. The testes were removed through a ventral medial incision and the diameter of the testes was measured in the proximal–distal axis (mm). The testes were cut into pieces, which were immediately fresh frozen in liquid nitrogen or fixed for conventional light and electron microscopy. In this strain the frequency of spontaneous testicular teratomas is low, under 2%, and spontaneous testicular teratomas in strain 129 mice can be easily detected by visual examination of exposed testis in animals over 1 week of age [31]. Thus, spontaneous testicular teratomas can be excluded in host animals before gonadal ridge implantation.

Intraperitoneal injection of etoposide

Etoposide was obtained from Bristol-Myers Squibb (Etopofos, 100 mg ampoule) and diluted to 2 mg/ml in 0.9% NaCl. Mice were given etoposide i.p. (19.5 mg/kg) on each of 4 consecutive days either 4 or 6 weeks after intratesticular implantation of male fetal gonadal ridges. The mice in the control groups did not receive etoposide. The animals were weighed every other day, and their condition was checked daily to check for possible adverse effects of cytostatic drugs. The mice were killed 7 days after the last cytostatic injection.

Light and electron microscopy

For light microscopy the testes with teratomas were taken from mice freshly sacrificed with CO_2, cut into pieces and fixed for 1 day in Bouin's fixative. The tissues were kept in 70% ethanol for another day, dehydrated in graded alcohol, embedded in paraffin and cut into 5- to 10-µm sections. The sections were stained with haematoxylin-eosin.

For electron microscopy, pieces of the testicular teratomas were fixed with 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/l s-collidine-HCl buffer (pH 7.4) and post-fixed with potassium ferrocyanide-osmium fixative [12]. The tissues were embedded in epoxy resin (Glycidether 100, Merck) and sectioned for light and electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate (Reichert Ultrostainer, Leica) and examined in a Jeol JEM-100SX electron microscope. For light microscopy, 1-µm-thick sections were stained with 0.5% toluidine blue.

Antibodies

For immunocytochemistry the purified polyclonal goat anti-human mdr$_1$ immunoglobulin fraction of goat antiserum (Santa Cruz, Calif.; mouse-, rat- and human-reactive) and peroxidase-conjugated rabbit anti-goat immunoglobulins (Dako, Glostrup, Denmark) were used. For in situ end-labelling (ISELs) the purified polyclonal alkaline phosphatase-conjugated goat anti-digoxigenin immunoglobulins (Boehringer, Mannheim, Germany) were used.

Immunocytochemistry

The tumour-containing testes were removed from the animals, and pieces of the teratomas were frozen immediately in liquid nitrogen. Sections 6 µm thick were cut in a cryostat and dried on slides. To localise mdr$_1$-Pgp from tumours the sections were fixed in cold (–20°C) acetone for 7 min. The acetone was allowed to evaporate before the sections were stored at –20°C. For staining, sections were soaked in 0.05 M TRIS-HCl buffer, pH 7.6 (Tham-HCl) containing 0.9% NaCl (TBS) at room temperature for 10 min to allow stabilisation of the temperature before incubation. Nonspecific binding sites were blocked by incubating the tissues in rabbit serum (Vector Laboratories, Burlingame, Calif.) diluted 16:1000 in TBS for 30 min. The excess of rabbit serum in TBS was removed from the slides and the sections were incubated with polyclonal goat anti-human mdr$_1$ immunoglobulins (Santa Cruz, Calif.) at +4°C overnight (diluted 1:100 in 1% bovine serum albumin [BSA, Sigma, Steinheim, Germany] in TBS). After washing in TBS the sections were incubated with the peroxidase-conjugated rabbit-anti goat immunoglobulins (Dako, Glostrup, Denmark; diluted 1:300 in 1% BSA in TBS) at room temperature for 1 h. Diaminobenzidine tetrahydrochloride (Sigma, Steinheim, Germany) was used as a chromogen. In control slides the primary antibody was replaced by the goat immunoglobulin fraction (Vector Laboratories). The slides were then dehydrated and embedded (Depex, Gurr). The sections were examined and photographed in bright field using a Leitz Diaplan light microscope (Ernst Leitz, Wetzlar, Germany).

ISEL of apoptotic cells

Bouin-fixed paraffin-embedded sections were cut and mounted on Superfrost Plus (Menzel-Gläser) slides. Frozen acetone-fixed sections, formalin- and Bouin-fixed paraffin-embedded sections were tested for ISELs with both microwaves and proteinase K. The Bouin-fixed, paraffin-embedded sections with proteinase K pretreatment gave the best reactions, and this fixative is also optimal for studying the routine light microscopic morphology of tissues [21]. In situ end-labelling of DNA was performed as described elsewhere [4]. After deparaffinization, the glasses were soaked in Tris buffer (100 mM Tris, 150 mM NaCl, pH 7.5) at room temper-

ature for 10 min. Thereafter the sections were incubated in 2× SSC at 80°C for 30 min and rinsed briefly in water for 5 min. They were incubated in proteinase K (6 µg/ml in 20 mM Tris, 2 mM $CaCl_2$, pH 7.4, Boehringer Mannheim) at 37°C for 20 min, rinsed three times in water and covered by TdT buffer (0.2 M $C_2H_6AsO_2K$, 25 mmol/l Tris-HCl, 0.25 mg/ml bovine serum albumin, 5 mM $CoCl_2$, Boehringer Mannheim) for 10 min. Digoxigenin-dideoxy-uridine-phosphate (dig-ddUTP) was linked to free DNA 3'-endings by incubation with terminal transferase (1 U/µl terminal transferase, 5 µM dig-ddUTP in TdT buffer, Boehringer Mannheim) for 60 min at 37°C. Terminal transferase was omitted from the control slides. After washing in Tris buffer for 10 min the sections were incubated with blocking buffer (100 mmol/l Tris, 150 mmol/l NaCl, pH 7.5, 0.5% [w/v] blocking reagent, Boehringer, Mannheim, Germany) for 30 min at room temperature, followed by incubation with alkaline phosphatase-conjugated digoxigenin antibody (Boehringer Mannheim, diluted 1:4000 in blocking buffer) for 2 h at room temperature. The sections were rinsed in Tris buffer and equilibrated in alkaline phosphatase buffer (100 mmol/l Tris, 100 mmol/l NaCl, 50 mmol/l $MgCl_2$, pH 9.5). The colour was developed by adding the substrate (nitroblue tetrazolium/5-bromo-4-chloro3-indodyl-phosphate-stock [Boehringer Mannheim] diluted 1:50 in alkaline phosphatase buffer). The colour reaction was stopped by the addition of 10 mM Tris and 1 mM EDTA (pH 8) after a developing time of 30 min to 2 h. The slides were then embedded in Aquamount (Gurr).

Testis extracts and Western blot analysis

Testes from a 129/SvJ mouse with bilateral testicular teratomas cultured for 7 weeks were freshly removed, pooled, weighed and homogenised in a glass homogeniser (1 g tissue/3 ml distilled water) supplemented with 1 µg/ml aprotinin (Sigma, St. Louis, Mo.) and soybean trypsin inhibitor (Sigma, St. Louis, Mo.) to avoid proteolysis. The homogenates were centrifuged at 250 *g* for 15 min. The supernatant was collected and centrifuged at 10 000 *g* for 30 min to separate soluble material from membranes and debris. Salts were removed from the second supernatant in a Sephadex G-25 PD-10 column (1.5×5 cm, Pharmacia, Uppsala, Sweden) chromatography, and freeze-dried. Protein in the samples was measured and diluted to 1 µg/µl in 2× Laemmli solution (1% SDS, 10% glycerol, 0.01% bromophenol blue and 2% β-mercaptoethanol in 50 mM Tris buffer, pH 6.8). The samples were boiled for 5 min.

Denaturating 7.5% SDS-polyacrylamide mini-gels were prepared and 10 µl of the extracted sample were loaded to the wells. Low-molecular-weight markers (Pharmacia, Uppsala, Sweden) were run parallel to the samples. Gels were run at a 150-mA current and, after electrophoresis, proteins were transferred in to nitrocellulose filter for 60 min as described [37]. The nitrocellulose filter was stained with Ponceau S, and each separate line was cut off. Strips were incubated with blocking buffer (1% BSA [Sigma, Steinheim, Germany], 2% nonfat dry milk in Tris-buffered saline) for 30 min and then incubated for 1 h at room temperature with polyclonal goat anti-human mdr_1 immunoglobulins (Santa Cruz, Calif.) diluted 1:50 in the blocking buffer. For the control strip the primary antibody was replaced by the goat immunoglobulin fraction (Vector Laboratories). After incubation, strips were washed three times with TBS and then incubated for 1 h at room temperature with peroxidase-conjugated rabbit-anti-goat immunoglobulins (Dako) diluted 1:100 in the blocking buffer. Strips were washed again with TBS and then allowed to react with 0.6 mg/ml diaminobenzidine tetrahydrochloride (Sigma, Steinheim, Germany) and 0.003% H_2O_2 in 0.005 M Tris (pH 7.6) for 10 min. Reactions were stopped with TBS, and strips were blotted dry before photography.

Statistical analysis

The differences in the diameters of the tumours between the etoposide-treated and non-etoposide-treated groups were analysed using Student's *t*-test. To measure the volume density of immature neuroectodermal tissue component in teratomas, random photographs were taken from tumours. The area of immature tissue and that of the whole tumour were measured in each section with weighing of the cut-out pieces of respective regions in the photographic prints. The statistical significance of the differences in the volume densities of immature tissue components in tumours with and without etoposide treatment were calculated with Student's *t*-test.

Results

Number, size and histology of teratomas with and without etoposide treatment

Testicular teratomas developed in 17 out of 56 implanted testes (30%) and in 14 out of 28 mice (50%). Three mice had bilateral testicular teratomas. There were at least three mice in each group with testicular teratomas (Table 1). The histology varied between individual tu-

Table 1 Number and size of teratomas with and without etoposide treatment, volume fractions of their immature tissue components, and the density of their apoptotic cells studied with the ISEL method (only three teratomas studied for apoptosis in each group). The tumours were induced by implanting 12-day-old male fetal gonadal ridges to adult mouse testis from the same strain

	Number of teratomas/ implanted testes[a]	Macroscopic size of tumours[b] (SEM)[c]	Volume fraction of immature tissue components (%)	Teratomas positive for apoptosis[e]
Teratomas treated with etoposide 4 weeks after implantation	4/18	10 mm[d] (0.9 mm)	0.42%*	1/3[f]
Controls for the group treated with etoposide at 4 weeks	3/12	14 mm (1.0 mm)	13.3%	3/3
Teratomas treated with etoposide 6 weeks after implantation	5/16	16 mm (1.4 mm)	0.42%*	2/3
Controls for the group treated with etoposide at 6 weeks	5/10	16 mm (1.5 mm)	4.6%	1/3

* P=0.016 between all the treated and nontreated tumours, Student's *t*-test

[a] The mice were killed 7 days after the last cytostatic injection

[b] Proximal-distal diameter (mm)

[c] Standard error of mean

[d] The tumours of the treated four-week-group were smaller than the 4-week-controls without etoposide (P=0.044), Student's *t*-test

[e] The apoptotic tumours had a higher volume density of immature tissue components than the non-apoptotic tumours (P=0.047), Student's *t*-test (see Results for details)

[f] Only three teratomas from separate tumour-bearing mice were studied for apoptosis in each group

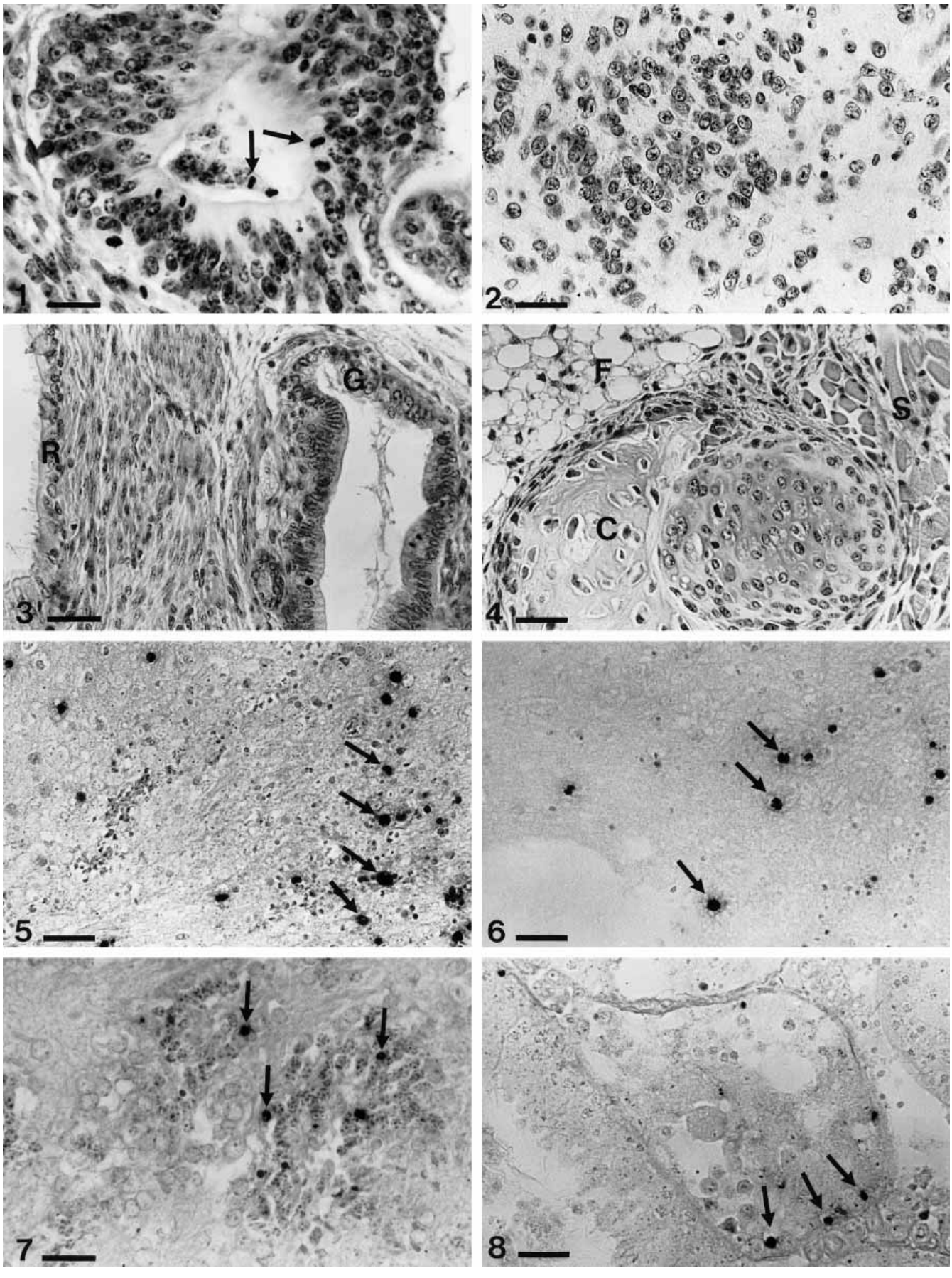

Fig. 1–8 (Legend see page 612)

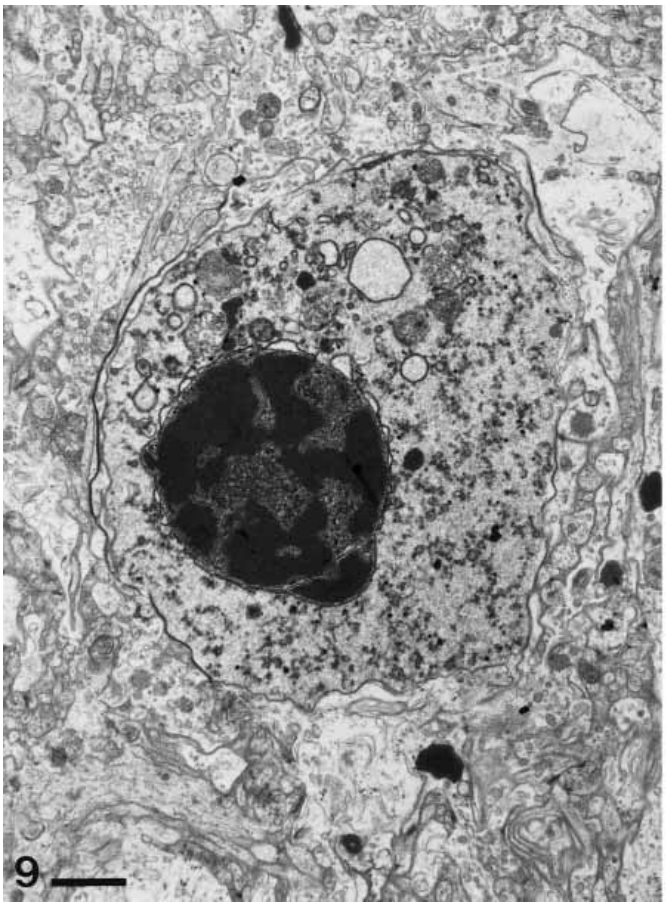

Fig. 9 Electron micrograph of a cell at an early stage of apoptosis in testicular teratoma of 129/SvJ mouse. The condensation of the chromatin in the nucleus is evident. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to adult mouse testis from the same strain for 8 weeks. *Bar* 1.5 µm

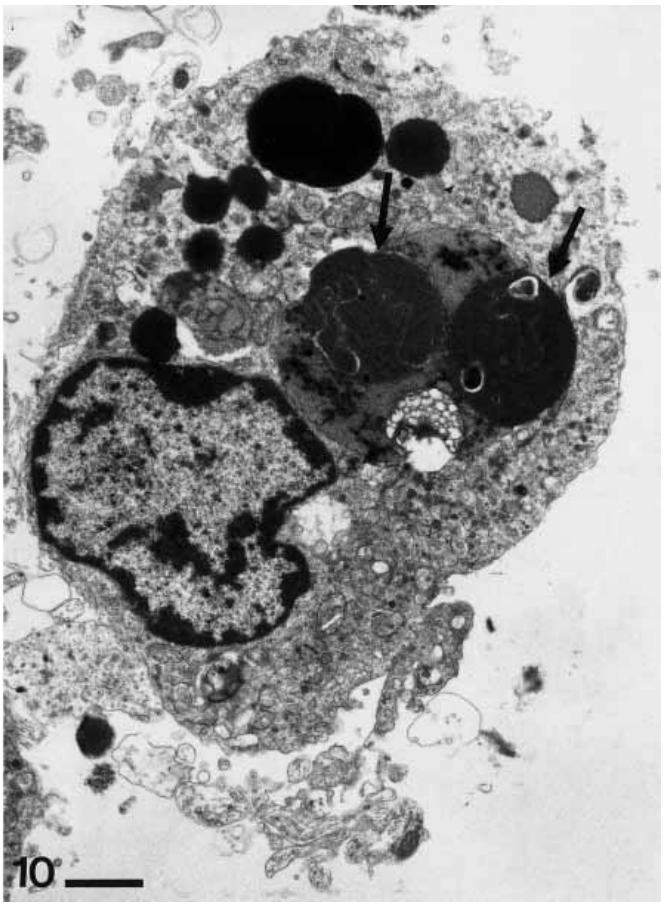

Fig. 10 Electron micrograph of a cell in testicular teratoma of 129/SvJ mouse. The cell has phagocytosed apoptotic bodies (*arrows*). The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to adult mouse testis from the same strain for 8 weeks. *Bar* 1.5 µm

◀ **Fig. 1** Light micrograph of neural epithelium with mitoses (*arrows*) in testicular teratoma. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. *Bar* 20 µm

Fig. 2 Light micrograph of glial cells in testicular teratoma. They form the major tissue component in experimental testicular teratomas of 129/SvJ mice. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 8 weeks. *Bar* 30 µm

Fig. 3 Light micrograph of respiratory-like epithelium (*R*) and glandular epithelium (*G*) in testicular teratoma treated with etoposide after culturing for 6 weeks. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 8 weeks. *Bar* 30 µm

Fig. 4 Light micrograph of striated muscle (*S*), fat tissue (*F*) and cartilage (*C*) in testicular teratoma treated with etoposide after culturing for 6 weeks. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 8 weeks. *Bar* 30 µm

Fig. 5 Apoptosis in glial cells (*arrows*) of teratoma as shown by ISEL. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. *Bar* 30 µm

Fig. 6 Apoptosis in glial cells (*arrows*) as shown by ISEL in teratoma treated with etoposide after culturing for 4 weeks. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. *Bar* 30 µm

Fig. 7 Apoptosis in neural epithelium (*arrows*) in teratoma as shown by ISEL. It has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. *Bar* 20 µm

Fig. 8 Apoptosis in a seminiferous tubule (*arrows*) of the testis with teratoma as shown by ISEL. The teratoma in the same testis has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 8 weeks. *Bar* 30 µm

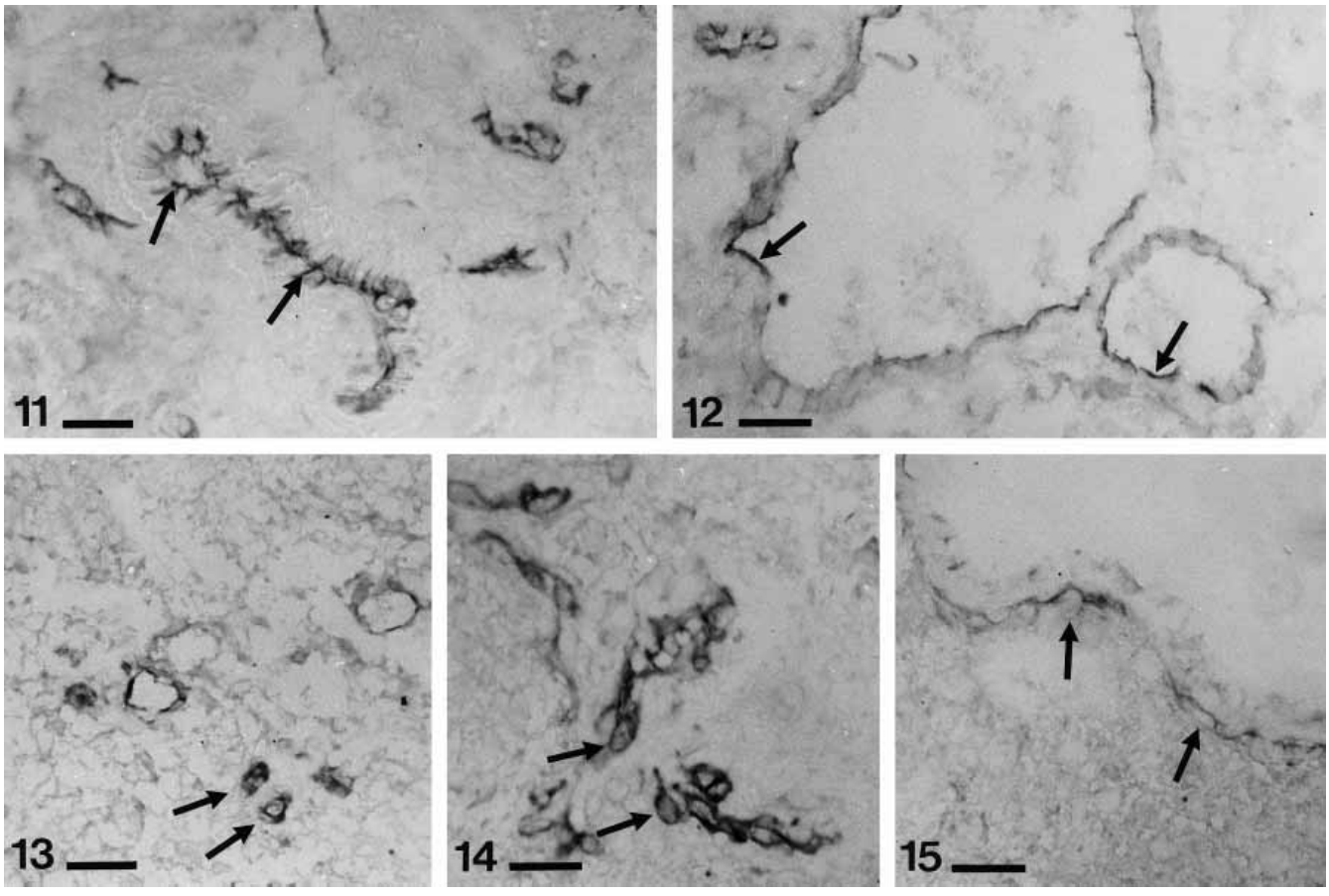

Fig. 11 Mdr_1 P-glycoprotein in epithelium (*arrows*) of testicular teratoma as shown by immunocytochemistry. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. No background staining, *bar* 30 µm

Fig. 12 Mdr_1 P-glycoprotein in epithelium (*arrows*) of testicular teratoma as shown by immunocytochemistry. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 8 weeks. No background staining, *bar* 30 µm

Fig. 13 Mdr_1 P-glycoprotein in capillary endothelium (*arrows*) of tumour stroma as shown by immunocytochemistry. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. It was treated with etoposide after culturing for 4 weeks. No background staining, *bar* 30 µm

Fig. 14 Mdr_1 P-glycoprotein in cells of tumour stroma (*arrows*), as shown by immunocytochemistry. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. It was treated with etoposide after culturing for 4 weeks. No background staining, *bar* 30 µm

Fig. 15 Mdr_1 P-glycoprotein in subepithelial parts of the tumour (*arrows*) as shown by immunocytochemistry. The teratoma has grown from the male gonadal ridge of a 12-day-old fetal 129/SvJ mouse implanted to an adult mouse testis from the same strain for 6 weeks. It was treated with etoposide after culturing for 4 weeks. No background staining, *bar* 30 µm

mours, but neural tissue formed the largest component in all teratomas (Figs. 1, 2). Some tumours consisted of several tissues (Figs. 3, 4), and the systematic description of the morphology of these teratomas has been presented before [33].

The tumours cultured for 4 weeks before etoposide treatment were smaller when they were collected for analyses (mean proximal–distal diameter, 10 mm) than were the tumours grown for the same time without cytostatic treatment (mean proximal-distal diameter, 14 mm, P=0.044). There was no difference in the size of tumours cultured for 6 weeks before etoposide treatment and the corresponding controls without cytostatic treatments. The volume density of immature neuroectodermal tissue components (Fig. 1) was significantly lower in all treated teratomas as a single group than in all teratomas cultured without etoposide (0.41% % versus 8.6%, P=0.016). The results have been summarised in Table 1.

Apoptotic cells in tumour tissue

Three teratomas from separate tumour-bearing mice were studied for apoptosis in each group. The tumours were divided into two classes according to the incidence of apoptosis. The apoptotic tumors contained >30 positive cells per section and the negative tumors ≤3 positive cells per section (Table 1). The tumours with a high volume density of immature tissue components were more

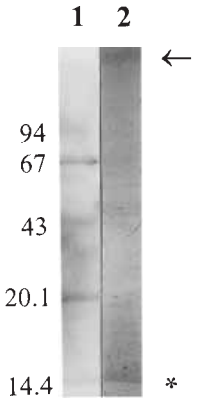

Fig. 16 Immunoblotting of mdr_1 P-glycoprotein from the testes with teratomas cultured for 7 weeks. The *lanes* are as follows: *lane 1* standard; *lane 2* mdr_1 P-glycoprotein, a band with M_r of 170 k corresponds to the right molecular weight (*arrow*), while another band with M_r of 15 k (*asterisk*) could not be explained

frequently apoptotic than those with low volume density of immature components (P=0.047).

Clusters of cells with ISEL-positive nuclei were frequently found among the glial cells of the tumour stroma (Figs. 5, 6) and in the neural epithelium (Fig. 7). Very few ISEL-positive cells were present in glandular epithelium. The remnants of seminiferous tubules of the host testis under the tunica invariably contained apoptotic cells (Fig. 8). Apoptosis in the ISEL-positive tissues was confirmed by recording the typical nuclear and cytoplasmic changes with electron microscopy (Figs. 9, 10).

Localization of mdr_1 P-glycoprotein in tumour tissue

All tumours, with and without cytostatic treatment, were clearly positive for mdr_1-Pgp. The immunocytochemical reactions were localised in the cytoplasm of cells in epithelial structures (Figs. 11, 12), in endothelia of capillaries in the tumour stroma (Fig. 13) and in capillaries of the host testis. Clusters of positive cells were also found in the tumour stroma (Fig. 14) and in subepithelial areas (Fig. 15). The presence of mdr_1-Pgp was confirmed by Western blotting (Fig. 16).

Discussion

Etoposide disturbs the cell cycle in a phase-specific manner in the beginning of mitosis and in the G2 phase [6]. The suggested mechanism is that etoposide up-regulates certain caspases, important regulators of the cell cycle, and this leads to induction of apoptosis in affected cells [7]. The harmful effect of etoposide on the growth of our teratomas cultured for 4 weeks is apparently explained by the abundance of proliferating tissue in these tumours. The teratomas cultured for 6 weeks have almost achieved their final size [33] and therefore the proportion of proliferating cells is much lower, which explains the smaller effect of etoposide.

The decreasing volume density of immature neuroectodermal tissue components in treated teratomas supports the effect of etoposide on mitotic cells. The diagnosis of mature teratoma in man in the residual mass after chemotherapy of disseminated nonseminoma is not rare [27]. It has been suggested that chemotherapy of nonseminomatous germ cell tumours causes selective destruction of more undifferentiated components than differentiated teratoma [24]. Despite the benign histology of human postchemotherapy residual teratomas they preserve malignant potential, as indicated by flow cytometry and immunocytochemistry [26]. This is indicated by the risk of non-germ-cell malignancies developing within mature teratomas [38], or the growing teratoma syndrome [17]. Consequently, complete surgical excision of residual tumour and close surveillance is important for patients after chemotherapy of disseminated nonseminomatous germ cell tumour [27].

It was surprising that the amount of ISEL-positive cells did not correlate with cytostatic treatment. However, apoptosis is a rapid process, and it is possible that the resulting apoptotic bodies are so rapidly phagocytosed that they cannot be observed in later analyses. The 7-day interval between the completion of cytostatic treatment and the collection of samples was long enough for the disappearance of apoptotic bodies through phagocytosis by neighbouring cells. It is notable that some teratomas not treated with etoposide had plenty of ISEL-positive cells. This may be explained by the observation that spontaneous apoptosis is a normal phenomenon in neoplastic and developing neural tissue [18], since neural tissue components constitute the main part of these induced teratomas [32, 33].

The presence of mdr_1-Pgp in epithelial structures has been described before [35]. The blood vessels are generally devoid of mdr_1-Pgp in normal and neoplastic tissues except the capillaries in the blood–brain and other blood–tissue barrier sites [5]. In addition, mdr_1 is expressed by endothelial cells of newly formed capillaries in human gliomas, but it is not expressed in the neovasculature of other primary tumours [34, 36]. This is in accordance with our results, since mdr_1-Pgp was present in capillaries of both the host testis and the teratoma, which consists mainly of glial cells and other tissues of neural origin [33]. The intercellular junctions of capillary endothelial cells play a central role in the formation of the blood–tissue barrier [13, 25]. In addition to these intercellular junctions, mdr_1-Pgp in capillary endothelium of the testis and the brain may reduce the concentration of drugs in these sites during systemic chemotherapy, which can impair the results of cancer treatment [5]. It is well known that acute lymphocytic leukaemia may relapse in the testis [22] or in the meninges [10] following remission achieved by systemic chemotherapy. However, the blood–tissue barrier in the tumour area is obviously disrupted along with the progressive tumour growth [39], as in the present teratomas. This is in accordance with our results, since the disappearance of proliferative tissue components suggest the entrance of etoposide to intratesticular teratoma.

The behaviour of teratoma in man is determined by its location [30]. Ovarian teratomas are usually benign, while teratomas in the testis are often combined with an

embryonal carcinoma [20]. In our animal model the testicular teratomas did not metastasise [33]. Rodents develop teratomas directly, without the carcinoma in situ (CIS) stage, which is regarded as the origin of seminomas and nonseminomatous germ cell tumours in man [40]. However, the present experimental testicular teratoma in 129/SvJ mouse may be a good model for the teratoma in the infantile human testis, which obviously also lacks the CIS stage [23]. The present results may have clinical relevance in man, since they confirm that post-chemotherapy mature teratomas cannot be treated with chemotherapy. Despite benign histology the human residual tumours have a significant malignant potential and require complete surgical excision and close surveillance.

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