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Histopathological changes in rabbit uterus carcinoma after transcatheter arterial embolization using cisplatin

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Abstract The effects of chemoembolization with cisplatin on gynecological malignancy were investigated using rabbit uterine tumors. A group of 20 rabbits were subjected to inoculation of the uterus with 5×10^7 VX2 carcinoma cells and 4 weeks later were divided into four groups, each consisting of five rabbits: an untreated control group, a group given cisplatin intraarterially (IA), a group subjected to transcatheter arterial embolization (TAE) with Gelfoam particles and a group subjected to transcatheter chemoembolization (TACE) with Gelfoam particles plus 1 mg/kg cisplatin. All groups were examined histologically 2 days after treatment. The untreated control group was further investigated 4 weeks after inoculation. In the untreated control group, the tumor cell nuclei varied in size and were irregular in form, and multiple nuclei and nuclear division were also observed. No necrotic zones were found up to 4 weeks after inoculation. The IA group showed no necrosis, but a few apoptotic cells were scattered throughout the tumor. In the TAE group, necrosis was observed in the center of the tumors, but proliferating cells persisted at the periphery. In the TACE group, necrosis was observed in the central part with many apoptotic cells surrounding the necrotic region in layers. The proliferating cell nuclear antigen (PCNA) index was 95.88% in the untreated control group, 86.6% in the IA group, and 8.62% in the TACE group, indicating a significant reduction in cell proliferation in the TACE group. These findings suggest that TACE results in more effective cytotoxicity than the other two treatments in uterine cancer tumor transplants.

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Introduction

Interventional radiology involves the use of angiographic techniques to treat various diseases and chemoembolization is such a technique used clinically for hepatic and pelvic cancer [1, 2]. We have used internal iliac transcatheter chemoembolization (TACE) using Gelfoam particles and cisplatin for advanced and recurrent gynecological malignancies and have obtained a response rate (complete plus partial responses) of 52.9%, suggesting considerable efficacy of this technique [3]. We have also demonstrated that TACE results in a tissue platinum concentration 2.25-fold higher than intraarterial (IA) infusion of cisplatin [4].

However, the reason for tumor regression after TACE therapy and the mode of cell death associated with this therapy remain unclear. In the present study, the histological changes 2 days after IA cisplatin infusion, transcatheter arterial embolization (TAE) using Gelfoam particles without cisplatin and TACE with cisplatin were compared. The objective of this study was to determine the mechanism of action of these three different therapies.

Materials and methods

VX2 tumor inoculation into the rabbit uterus

VX2 carcinoma cells (Funabashi Farm, Chiba, Japan) [5] were maintained subcutaneously in intact rabbits. Tumor tissue specimens were obtained aseptically, and a single-cell suspension was prepared from entire tumor specimens by mincing the tissue and filtering the suspension through a 50-mm nylon mesh. Cells were diluted to a concentration of 1×10^8 in 2 ml saline for injection. A group of 20 female New Zealand white rabbits weighing 3.3–4 kg were anesthetized with pentobarbital sodium (25 mg/kg) via the auricular vein and anesthesia was maintained with ether inhalation. A midline lower abdominal incision was made under aseptic conditions and the uterus was exposed. The vaginal side of the uterus was ligated, and thread was

applied to a site about 3–4 cm distal from the ligature. VX2 carcinoma cells were inoculated into one side of the bicornate uterus with an 18 G needle, and ligation was performed immediately after inoculation. A 30-mg dose of lincomycin hydrochloride (Upjohn, Kalamazoo, Mich.) was injected intramuscularly and the same amount was sprayed over the peritoneal cavity.

Experimental methods

The 20 rabbits were divided into four equal groups 4 weeks after inoculation of VX2 cells: an untreated control group, and the IA, the TAE and the TACE groups. The untreated control group was kept in a laminar air-flow room and sacrificed 4 weeks after tumor inoculation. The experimental animals were treated 4 weeks after tumor inoculation. First, the rabbits were anesthetized with pentobarbital sodium (25 mg/kg) injected into the auricular vein. With the animal in the supine position, a 3 Fr polyethylene catheter (Cook Co., Bloomington, Ind.) was inserted into the right femoral artery. Contrast medium (Omnipaque 350, Daiichi Pharmaceutical Co., Tokyo, Japan) was infused through the catheter under fluoroscopic control, the position of the abdominal aortic bifurcation was confirmed, and the tip of the catheter was advanced to the bifurcation.

The IA group received a 5-s bolus injection of cisplatin (cis-diamminedichloroplatinum (II); Bristol-Myers-Squibb Co., New York, N.Y.) solution (1 mg/kg in 2 ml saline) via the catheter at the aortic bifurcation. The TAE group received a bolus injection of Gelfoam particles (about 1/4 sheet divided into 1-mm squares) mixed with 2 ml saline to the same site. The TACE group received a 5-s bolus injection of cisplatin solution (1 mg/kg in 2 ml saline) mixed with Gelfoam particles.

Tissue platinum concentration

Rabbits in the IA and the TACE groups were sacrificed 2 days after treatment by the rapid IA administration of an overdose of pentobarbital. The uterine tumors were immediately removed, washed with saline, and stored at –80 °C. The platinum concentration of tumor tissue samples was determined by atomic absorption spectrometry [6], and was expressed as micrograms of platinum per gram wet weight of tissue.

Histological examination

Specimens of the uterine tumors were fixed in 10% formalin for 48 h and embedded in paraffin according to routine procedures. Sections 3 μm thick were cut, and stained with hematoxylin and eosin.

Staining for proliferating cell nuclear antigen (PCNA) and PCNA index

Expression of PCNA was revealed by a mouse monoclonal IgG (PC 10) to rat PCNA (Dakopatts, Copenhagen, Denmark) using the ABC (avidin-biotin-complex; Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.) method. Sections from the untreated control group, and the IA and the TACE groups were examined at a magnification of ×250. Ten fields were assessed randomly for tumor cells and non-malignant uterine cells, and 1000 tumor nuclei per field were counted and the number showing positive nuclear staining was recorded. The percentage of positive cells was then calculated as the PCNA index [7].

Detection of apoptosis and apoptotic index

Apoptosis was identified by staining with an ApopTag kit (Oncor, Gaithersburg, Md.). The apoptotic cells were determined by microscopic examination of ApopTag-stained sections at a magnification of ×400. Apoptosis was quantitated by determining the percentage of tumor cells within a field of view at a magnification of ×400. A total of ten randomly chosen fields were counted per slide assayed, and the counts were averaged to obtain the apoptotic index.

Depending upon the intensity following H&E staining, PCNA staining, and ApopTag staining at a magnification of ×33, slides were graded as: –, ≤5%, no staining; 1+, 5–33%, weak staining; 2+, 33–67%, moderate staining; 3+, ≥67%, intense staining. The outer ten layers of tumor cells were identified as the tumor peripheral zone, and other inner ones as the tumor central area.

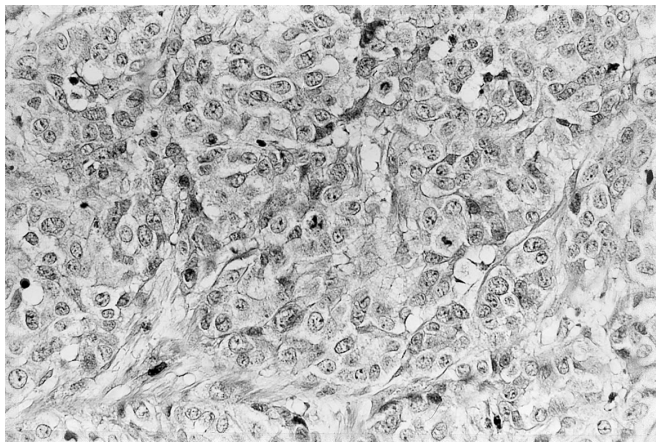

Fig. 1 Untreated VX2 carcinoma. The tumor cells are arranged in layers in a pavement pattern. The cells are markedly pleomorphic and show mitosis as tripolar mitotic figures (H&E, approx. ×130)

Statistical analysis

The significance of the differences in the cisplatin concentrations between the IA and the TACE groups was evaluated usign the two-tailed Student's *t*-test for analysis of variance. All *P*-values < 0.05 were considered significant. All data are given as mean values ± SD.

Results

Untreated control uterine tumors

In a preliminary experiment, diagnostic laparotomy was performed up to 5 weeks after inoculation at weekly intervals to determine the appearance of the tumors and the extent of tumor growth. No tumor growth was observed at 1 and 2 weeks postinoculation. However, 3 weeks after inoculation the tumors were 15–20 mm in diameter but pelvic lymph node metastases were absent. By 4 weeks after inoculation, the tumors were about 30–50 mm in diameter and definite pelvic lymph node metastases were observed. The lymph node metastases reached a size of 5–10 mm and were apparent in 40% of the inoculated rabbits. Subsequent metastases to the upper abdomen and lungs resulted in death in more than 80% of the animals. Histological examination of tumor tissue specimens harvested 4 weeks after inoculation revealed papillary proliferation of tumor cells directed towards the uterine cavity. The interstitium consisted of fine vascularized connective tissue surrounded by proliferating tumor cells arranged in pavement pattern layers. The tumor cells had nuclei that varied in size and were irregular in form, and multinucleate cells

Table 1 Histopathological features of carcinomas transplanted into rabbit uterus (– ≦5% no staining, + 5–33% weak staining, ++ 33–67% moderate staining, +++ ≧67% intense staining)

	H & E stain				ApopTag stain		PCNA stain		
	Vacuolar degeneration		Necrosis		Apoptosis		PCNA-positive cells		PCNA index
	Center	Periphery	Center	Periphery	Center	Periphery	Center	Periphery	
Untreated	–	–	–	–	–	–	+++	+++	95.88 ± 0.79%
IA	++	++	–	–	+	+	++	++	86.6 ± 4.23%
TAE	–	–	+++	–	–	–	–	+	–[a]
TACE	++	+	+++	–	+	+++	–	–	8.62 ± 2.19%

[a] PCNA index not calculated because of the local presence of PCNA-positive cells in the tumor periphery

and mitotic figures were also observed (Fig. 1). No apoptosis, necrosis, or vacuolar degeneration were observed in the tumor periphery or the tumor center. The PCNA index was 95.88 ± 0.79%. On the basis of these findings, tumors 4 weeks after inoculation were considered appropriate specimens for the current investigation.

Platinum concentrations

In the tumor specimens removed 80 min after treatment, the tissue platinum concentration was 1.21 ± 0.35 μg/g in the TACE group and 0.48 ± 0.09 μg/g in the IA group, that is 2.25 times higher in the TACE group. In tumor specimens removed 2 days after treatment, the tissue platinum concentration was 0.71 ± 0.06 μg/g in the TACE group and 0.36 ± 0.20 μg/g in the IA group, that is 1.97 times higher in the TACE group.

Histological findings

The histological results in the IA group, the TAE group, and the TACE group are given in Table 1 and Figs. 2–4.

IA group

In the IA group, H&E staining revealed weakly stained apoptotic cells scattered throughout the tumor center and the periphery, while no necrosis but moderate vacuolar degeneration were apparent. Intensively stained proliferating cells were apparent in the tumor periphery and the tumor center following PCNA staining, and the PCNA index was 86.6 ± 4.2%. There were few ApopTag-positive cells, and the apoptotic index was 1.0 ± 0.3%.

TAE group

PCNA staining revealed intensely necrotic cells in the tumor center and weakly proliferating cells remaining in the tumor periphery. In the TAE group, the PCNA index was not calculated because of the local presence of PCNA-positive cells in the tumor periphery. No apoptosis or vacuolar degeneration were apparent after H&E staining in the TAE group. Thus, there were no positive cells following ApopTag staining.

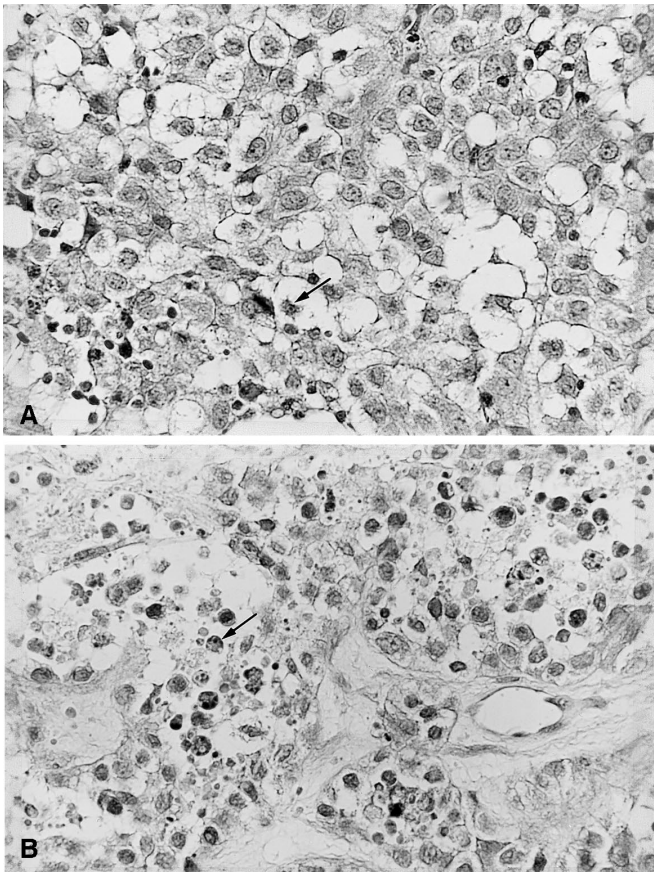

Fig. 2 A, B VX2 carcinoma 2 days after treatment in the IA (**A**) and the TACE (**B**) groups. **A** Morphologically the tumor does not appear to have been influenced markedly by the treatment. Among the vacuolar degenerated cells the apoptosis-like changes of the chromatin are few (*arrow*). **B** The center of the tumor is necrotic. At the periphery many cells with apoptotic changes (*arrow*) can be seen (H&E, approx. ×130)

TACE group

In the TACE group, H&E staining revealed necrotic cells in the tumor center with many intensely stained apoptotic cells

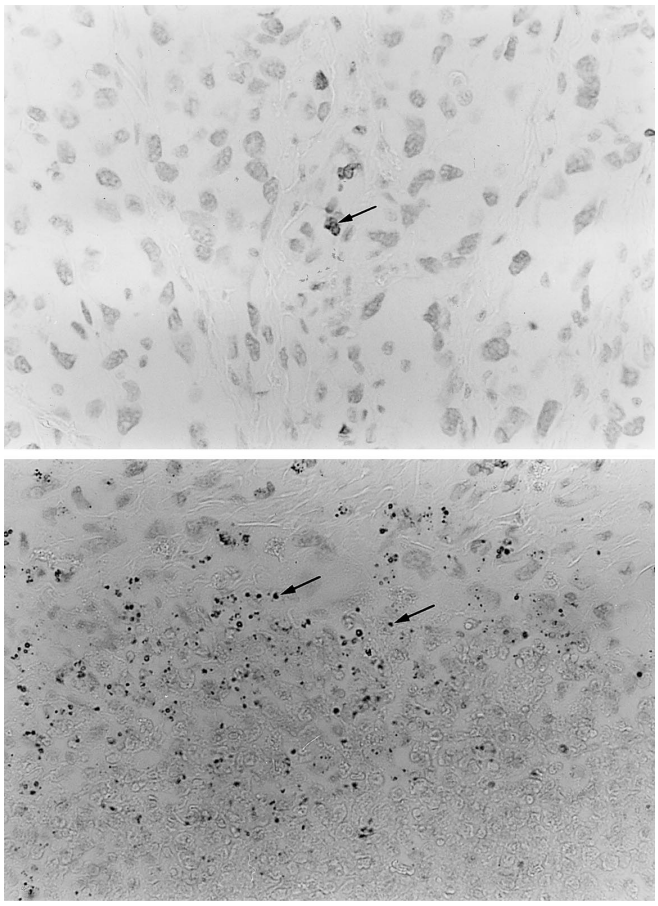

Fig. 3A, B Changes in ApopTag staining (approx. ×130). **A** In the IA group, apoptotic morphological changes, mainly vacuolar degeneration, can be seen in a few cells in both the center and the periphery of the tumor (*arrow*). **B** In the TACE group, many cells with morphological features of apoptosis expressed as apoptotic bodies are present (*arrows*)

in the tumor periphery. The PCNA index was $8.62 \pm 2.19\%$. Many intensely ApopTag-stained cells were also found. The apoptotic index was $5.9 \pm 0.5\%$. The difference between the PCNA and apoptotic index in the TACE and the IA groups was statistically significant for all criteria at $P < 0.001$.

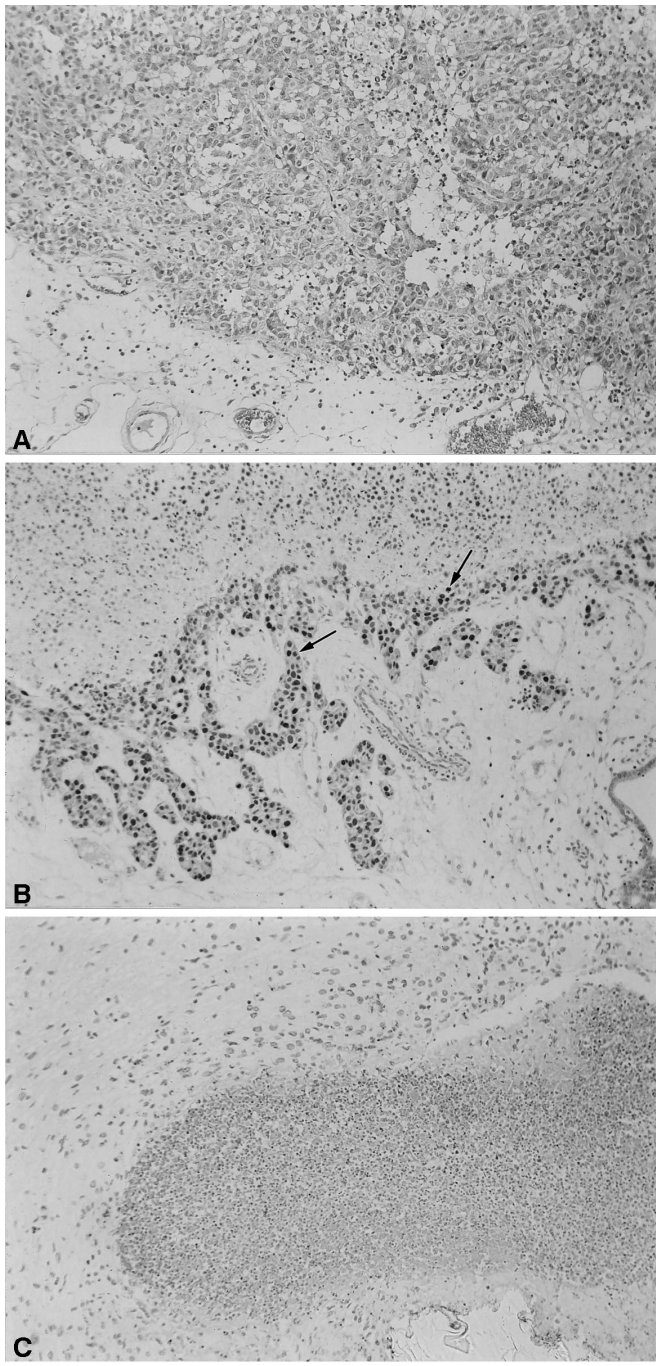

Fig. 4A–C Morphological changes in VX2 carcinomas 2 days after treatment in the IA (**A**), the TAE (**B**), and the TACE (**C**) groups (approx. ×30). **A** A few apoptotic changes can be seen in vacuolar degenerative cells (H&E). **B** PCNA-positive cells are distributed mostly in the periphery of the tumor nest and surround capillaries in the necrotic area (*arrows*) (PCNA staining with hematoxylin counterstaining). **C** ApopTag-positive apoptotic features are located around the central necrotic area (ApopTag staining with methyl green counterstaining)

Discussion

There are many reports suggesting that cisplatin is most useful as an anticancer agent for the treatment of uterine cancer [8]. Our clinical experience of the treatment of uterine cancer has shown a favorable therapeutic efficacy of TACE coupled with cisplatin administration [3]. The objective of the present study was to determine the clinical efficacy of TACE using cisplatin as the anticancer agent.

Since the 1970s cell death has been classified morphologically as necrosis or apoptosis [9]. Apoptosis differs from necrosis both morphologically and biochemically. Apoptosis is characterized by cell shrinkage and chromatin condensation. Biochemically one of the main events in the early stages of the process is the digestion of DNA. In contrast to lysosomal digestion during necrosis, lysosomes remain intact, and endonuclease is involved [9]. Recently, it

has been suggested that apoptosis may play an important role in the cytotoxic effects of anticancer drugs [10] and radiation therapy [11]. Furthermore, the gene associated with apoptotic induction has now been identified [12] and the mechanism of apoptosis has gradually been clarified. Apoptosis has conventionally been detected by electrophoresis using agarose gel to identify fragmentation of the nucleus by extracting the DNA at the time of condensation and destruction of the nucleus. ApopTag staining used in this study is a useful staining method because it interacts with the terminal 3'-OH group of the DNA which undergoes fission through the action of an endonuclease during apoptosis [13], so the method is able to stain less frequent apoptoses which fail to be detected by conventional methods.

ApopTag staining detected apoptosis after IA and TACE cisplatin administration, but positive cells were not detected after TAE. Positive cells appeared more frequently in the periphery than in the tumor center. Moore [14] reported that in tumor tissues apoptosis occurs near blood vessels but that apoptosis can occur in hypoxic lesions distant from blood vessels. In the present study, induction of more marked apoptosis by TACE than IA administration in the periphery than the tumor center might be attributable to the distribution of blood vessels, to the oxygen concentration in the tissues or to the duration of the interaction with the drug. Barry et al. [15] have previously reported that apoptosis is induced in vitro by cisplatin. They challenged Chinese hamster ovary cells with cisplatin at a low concentration (0.4 μM) for 2 h and detected DNA ladders 48 h later. Exposure to cisplatin at a high concentration (60 μM) for 2 h promptly induced digestion of DNA, leading to frequent appearance of DNA ladders. We also investigated the evolution with time of drug concentrations in tumor tissues following IA administration and TACE, and found that after 80 min TACE achieved concentrations 2.52-fold higher than IA administration ($P < 0.01$) and on the second day, TACE achieved concentrations 1.97-fold higher than IA administration ($P < 0.01$) [4]. This higher drug concentration may have been responsible for the higher incidence of apoptosis in the periphery of the tumors following TACE.

Necrosis was not found following IA administration but marked necrosis was observed following TAE or TACE in the tumor center. Necrosis is not caused by cisplatin but is induced in the tumor tissue by the embolus-induced interruption of the supply of oxygen and nutrients from the tumor blood vessels and interstitial fluid. However, it has been reported that necrosis occurs in tumor cells located 70–150 mm away from tumor blood vessels [16]. In necrotic lesions induced by the blockade of blood vessels and hypoxia, energy metabolism dysfunction has been shown in the membranes of certain cells leading to the elimination of physiological membrane permeability resulting in the swelling of mitochondria and cells, followed by the destruction of the membrane, the release of enzymes and their subsequent activation [17]. This process is considered to be the cause of the marked TAE- and TACE-induced necrosis in the tumor center. In liver cancer, Yu et al. [18] found necrosis in the center of the tumor tissues an average of 2.4 months after TACE, in accordance with our findings.

Vacuolar degeneration as shown by H&E staining was found in the IA and the TACE groups but not in the TAE group. The presence of vacuolar degeneration in the tumor center and the periphery suggests almost equivalent penetration of cisplatin into tumor tissue. In this connection, Kuo [19] reported vacuolar degeneration 4–14 days after IA administration of cisplatin at a dose of 2.5 mg/kg to VX2 tumors transplanted into rabbit hind paws. We also found vacuolar degeneration in patients with advanced cervical cancer who had undergone TAE combined with cisplatin administration. These clinical findings are consistent with the present study results.

The number of PCNA-positive cells in both the tumor center and the tumor periphery was decreased in the IA, the TAE and the TACE groups in this decreasing order. The PCNA index [20], as a marker of proliferation ability, in the IA group was not significantly different from the control group. Thus, the control, single bolus injection of cisplatin resulted in a markedly weaker cytotoxicity of the drug. Possible mechanisms by which chemoembolization produces its superior effect, as has been shown previously by us [4] and other researchers [21], include the substantial increase in drug retention, and the prolonged dwell-time of the neoplastic agent within the tumor, which leads to an increased first-pass fraction of the drug extracted. Our previous study [4] showed that the half-life of platinum in arterial blood following IA administration is 6 min but that following TACE is 11 min ($P < 0.001$).

In summary, the histological changes induced by the three procedures in the present study suggest the following. First, the distribution and interaction of cisplatin was necessary for the induction of vacuolar degeneration and apoptosis in the IA and the TACE groups and the degree of these effects depended on the local concentration of cisplatin and the period of its interaction with the tissue. It has also been found that the cytotoxicity of cisplatin is dose dependently stimulated by hypoxic conditions rather than by aerobic environments [22]. This is consistent with the findings that intense apoptosis was found in the TACE group. Second, significant necrosis observed in the tumor center in the TAE and the TACE groups was attributed to environmental changes in the tumor tissue. The augmentation by TACE of the antitumor effects of cisplatin was indicated by the lack of proliferation of the tumor cells both in the center and the periphery as shown by PCNA staining. Daniels et al. [21] reported that the efficacy of TAE combined with chemotherapy results from direct ischemia due to embolization of blood vessels and the confinement of the drug within the tumor bed. The outcome of the present study suggests that TACE induced necrosis in the tumor center and apoptosis in the tumor periphery early on the second day after treatment, and these two different types of cell death contributed to the enhancement of the antitumor effects.

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