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Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with taxol

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Abstract The kinetics of taxol-induced mitotic arrest and apoptosis in murine mammary carcinoma MCA-4 and ovarian carcinoma OCA-I tumors were determined to establish a possible causative relationship between mitotic arrest and apoptosis and to see whether these cellular effects of taxol would correlate with the extent of its antitumor efficacy. Mice bearing 8-mm tumors in a hind leg were given taxol i.v. at a dose of 10–80 mg/kg. Both tumors responded to taxol by significant growth delay or transient regression; in general, the response was greater as the dose of taxol was increased. For kinetics studies the mice were treated with 60 mg/kg taxol given once when tumors were 8 mm in size or twice, with the second dose being given 3 days after the first. At various times ranging from 1 to 96 h after treatment with taxol, tumors were histologically analyzed to quantify mitotic and apoptotic activity. After a single dose of taxol, mitotic arrest was visible at 1 h, and the mitotic index increased with time to reach peak values of 36% in MCA-4 tumors and 22% in OCA-I tumors at 9 h. The index then declined to a baseline of 1%–3% at 3 days for MCA-4 tumors and 1 day for OCA-I tumors. Apoptosis followed mitotic arrest, beginning at the time of peak mitotic arrest, increasing to the highest level of about 20% at 18–24 h after treatment and gradually declining to the normal level of 3%–6% after 3–4 days. Nuclear material progressively condensed in mitotically arrested cells, culminating in the frank appearance of multiple apoptotic bodies. The change in cell morphology plus the dynamics of apoptosis development imply that a large percentage of tumor cells arrested in mitosis by taxol die by apoptosis. Kinetic analysis undertaken after the second dose of taxol showed a considerably lower percentage of cells arrested in mitosis as compared with that seen after a single dose, and the induction of apoptosis by the second dose was minimal. However, the antitumor efficacy of the second dose of taxol was similar to or better than that of the first dose, implying that in addition to mitotic arrest and apoptosis, there exist other mechanisms by which taxol exerts its antitumor action.

Key words Taxol · Mitotic arrest · Apoptosis

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Introduction

Taxol, a natural product isolated from the bark of the yew tree *Taxus brevifolia*, exhibits significant antitumor activity against various tumors of experimental animals [7] and humans [9]. Most clinical observations have been made in therapy of breast and ovarian cancers [9]. Although the exact mechanism by which taxol kills cells is unknown, it is generally considered to be related to the binding of taxol to tubulin structures [8, 9]. The drug enhances microtubule polymerization and promotes microtubule assembly [10, 11], which blocks transit of cells through the G2 and M phases of the cell cycle [10, 14]. The cells accumulate in these cell-cycle phases, but whether the arrested cells, or what proportion of them, are doomed to die is not known. A recent report showing that taxol enhanced cell killing by ionizing radiation through accumulation of cells in the radiation-sensitive G2 and M phases [14] suggests that taxol-arrested cells are capable of further survival. It has also been observed that taxol induces nuclear fragmentation in cultured cells [1], a feature characteristic of apoptotic cell death [15].

As both cell-cycle arrest and apoptosis induced by taxol have been observed almost exclusively in in vitro studies [1, 10], little is known about the effect of taxol on these processes in vivo. In the experiments described herein, we

investigated whether taxol would induce mitotic arrest and apoptosis of tumor cells in murine tumors and whether these cellular effects of taxol would correlate with the extent of antitumor activity manifested as tumor-growth delay. The experiments also determined the kinetics of mitotic arrest and apoptosis to address a possible causal relationship between the mitotic arrest and apoptosis induced by taxol.

Materials and methods

Mice and tumors

Female C3Hf/Kam mice, bred and maintained in our own specific-pathogen-free mouse colony, were 3–4 months old at the beginning of the experiments and were housed 3–5/cage. The transplantable tumors used were spontaneous mammary carcinomas (MCA-4) and ovarian carcinomas (OCA-I) syngeneic to this strain of mice. Both tumors are nonimmunogenic. MCA-4 was used in its fourth and OCA-I, in its seventh isotransplant generation.

Taxol

Taxol (Paclitaxel – Bristol-Myers Squibb Co., Wallingford, Conn.; batch 80610692A, lot 008) was initially dissolved in absolute ethanol with an equal volume of cremophor (Sigma, St. Louis, Mo.; lot 32H0925) and stored at 4 °C for up to week. This stock solution (32 mg/kg) was further diluted 1:4 with sterile physiological saline within 15 min of injection. The taxol solution was then injected i.v. in a volume of 0.08–0.31 ml/mouse, depending on the body weight and desired dose. The doses of taxol were 10, 20, 40, 60, and 80 mg/kg body weight; in most experiments, however, a dose of 60 mg/kg was used.

Tumor response to taxol

Solitary tumors were produced in the muscles of the right thigh of mice by the inoculation of 5×10^5 viable tumor cells. Tumor-cell suspensions were prepared by enzymatic digestion of nonnecrotic tumor tissue [6]; the viability of cells was in the range of 80%–85% for MCA-4 and more than 90% for OCA-I as determined by trypan blue exclusion and phase microscopy. Tumor growth was determined at 2- to 3-day intervals, or more frequently as needed, by measurement of three orthogonal tumor diameters with vernier calipers. The mice were treated with taxol or vehicle when their tumors had grown to 8 mm in diameter. In additional experiments, animals received two injections of taxol, one being given when tumors were 8 mm in diameter and the other, 72 h later. To calculate tumor-growth delay, tumor growth was then measured until tumors reached about 16 mm in diameter

Histological analysis of mitotic arrest and apoptosis

At different times ranging from 1 to 96 h after treatment with taxol, the mice were killed by cervical dislocation and the tumors were immediately excised and placed in neutral-buffered formalin. The tissue was then imbedded in paraffin blocks from which 4-µm sections were cut and stained with hematoxylin and eosin (H&E). Both mitosis and apoptosis were scored in coded slides by microscopic examination at 400 × magnification. The morphological features used for histological identification of apoptotic bodies have been described and illustrated in earlier publications from our laboratory [3–5, 12, 13]. Five fields of nonnecrotic areas were randomly selected in each histological specimen, and in each field the number of apoptotic nuclei and cells in mitosis were recorded as numbers per 100 nuclei and were expressed as a percentage. The percentage was based on scoring 1500 nuclei obtained from 3 mice/group.

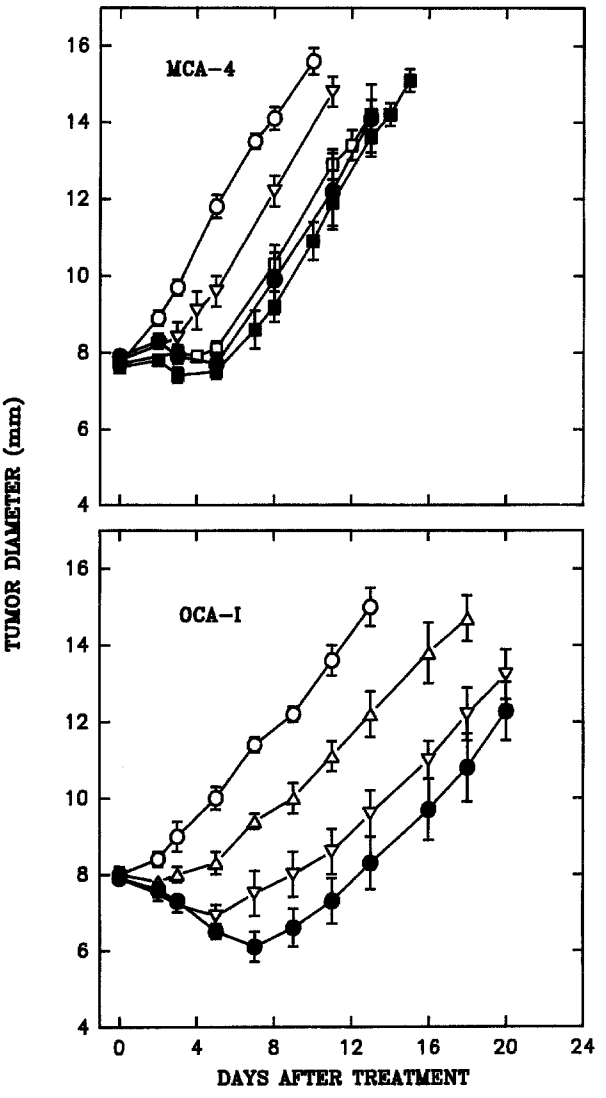

Fig. 1 Effect of taxol on the growth of MCA-4 or OCA-I tumors in C3Hf/Kam mice. Mice were treated i.v. with taxol when tumors were 8 mm in diameter. Control, ○; taxol at 10 (△), 20 (▽), 40 (□), 60 (●), and 80 (■) mg/kg. (*Vertical bars* SE of the mean) Groups consisted of five mice each

Results

Antitumor effectiveness

To determine the therapeutic efficacy of taxol we gave mice bearing 8-mm MCA-4 or OCA-I tumors i.v. injections of either the drug at a dose of 10, 20, 40, 60, or 80 mg/kg or the vehicle. Figure 1 shows that taxol was effective against both tumors. The growth of MCA-4 was slowed or temporarily arrested. The OCA-I tumor slowed its growth or underwent transient incomplete regression before it

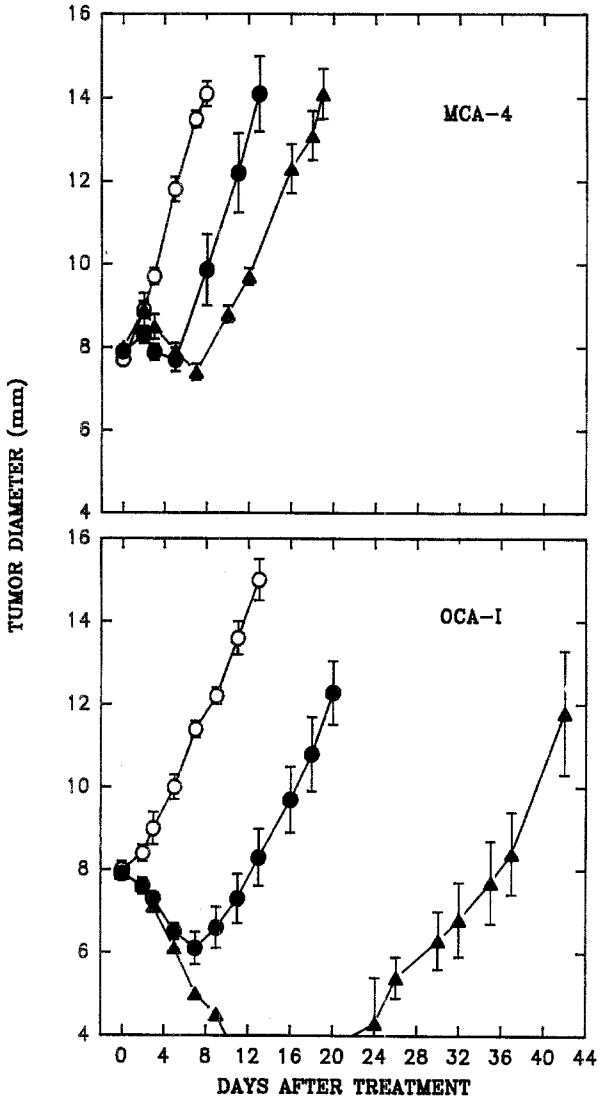

Fig. 2 Effect of taxol given as one (●) or two (▲) doses of 60 mg/kg each on tumor growth. Taxol was given i.v. to mice bearing 8-mm MCA-4 or OCA-I tumors. The second dose was given 3 days later (*Open symbols* Tumor growth in corresponding control mice, *vertical bars* SE of the mean)

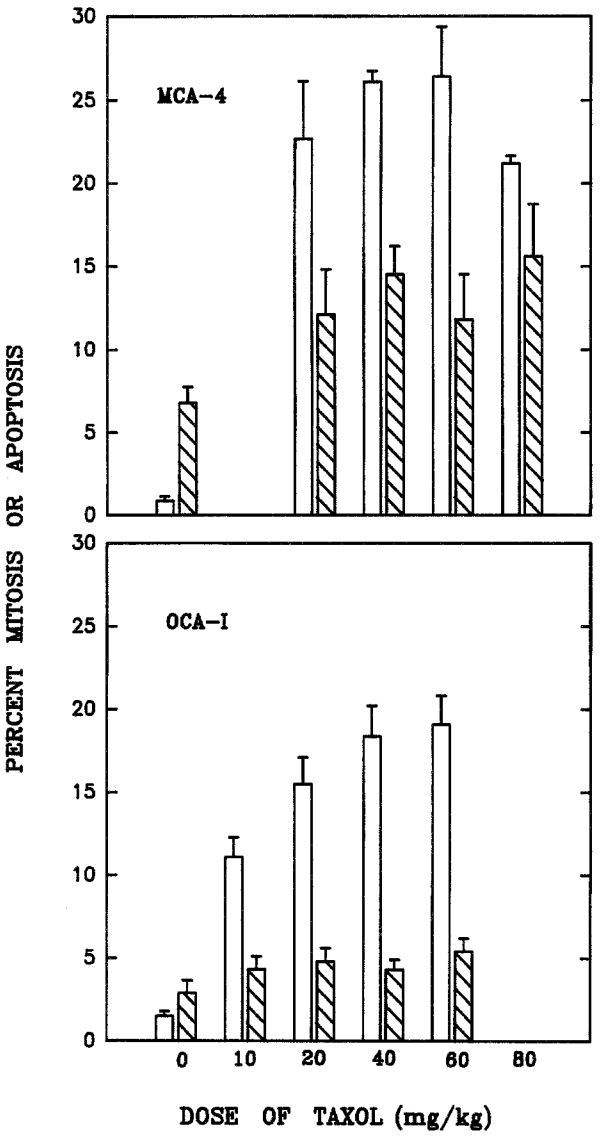

Fig. 3 Percentage of mitosis (*open bars*) or apoptosis (*hatched bars*) induced in tumors at 8 h following treatment with taxol at different doses. Mice bearing 8-mm MCA-4 or OCA-I tumors were injected i.v. with taxol at doses of 10–80 mg/kg. The percentage of mitosis or apoptosis was scored from histological sections made from control or treated tumors. (*Vertical bars* SE of the mean) Groups consisted of three mice each

regrew. None of the doses used killed any mice with the exception of the highest dose used, 80 mg/kg, which caused the death of 1 of 9 mice treated (11%).

The following experiment tested the antitumor effect of two i.v. injections of 60 mg/kg taxol given 3 days apart, with the first injection being given when tumors were 8 mm in diameter. The curves plotted in Fig. 2 show that two injections of taxol were much more effective than a single dose. In the case of the MCA-4 tumor, the effect was nearly additive; one injection of taxol delayed the time required for tumors to grow from 8 to 12 mm by 5.4 days, whereas two injections of taxol delayed it for 10.2 days. The effect of two injections of taxol on the OCA-I tumor was more than additive. The time required for this tumor to enlarge from 8 to 12 mm was 11.0 days after one injection and 31 days after two injections. It should be noted that two injections of taxol caused transient complete regression (tumor size, <4 mm) of OCA-I tumors (Fig. 2).

Mitotic arrest and apoptosis

Tumors from each group of mice treated with different single doses of taxol (see above) were histologically analyzed for mitotic arrest and apoptosis at 8 h after treatment. The 8-h point was selected to approximate the time of the peak of apoptosis development as based on earlier observations of apoptosis induction by cisplatin and cyclophosphamide in these tumors [4, 5].

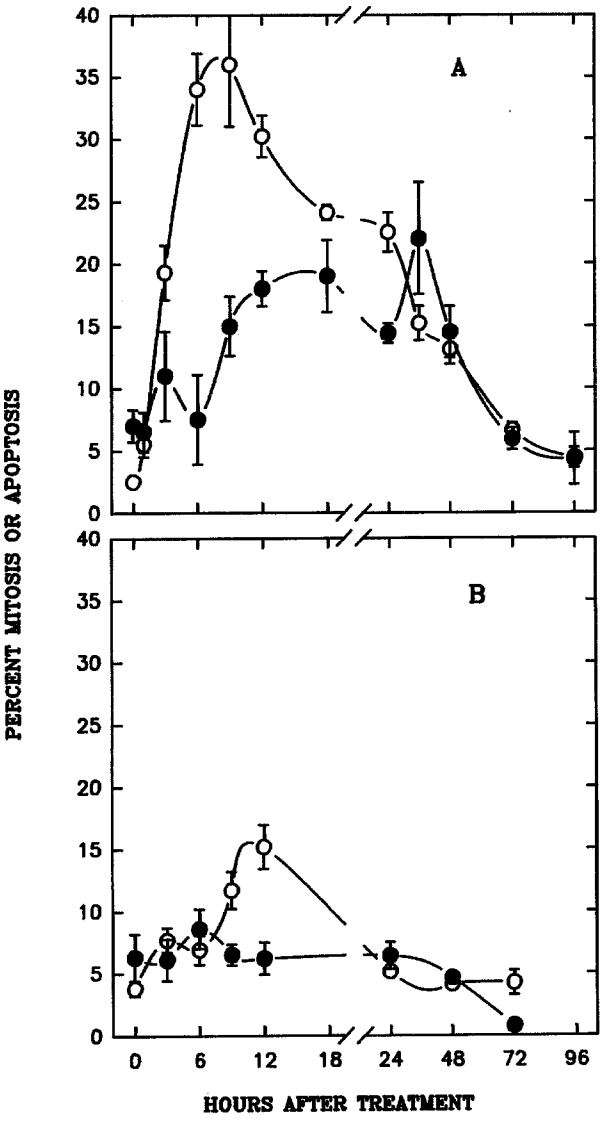

Fig. 4 A, B Percentage of mitosis (○) or apoptosis (●) induced in MCA-4 tumors as a function of time **A** after a single dose of 60 mg/kg taxol or **B** after a second dose of 60 mg/kg given 3 days after the first dose. Mice bearing 8-mm MCA-4 tumors were injected i.v. with taxol; the percentage of mitosis or apoptosis was scored from histological sections made from tumors at 0–96 h after treatment (*Vertical bars* SE of the mean)

As shown in Fig. 3, taxol was very effective in arresting tumor cells in mitosis in both MCA-4 and OCA-I tumors. The percentage of mitotic cells in MCA-4 tumors rose from the control value of $<1\%$ to more than 20%. Overall, no strong dependence of the degree of mitotic arrest on the delivered dose of taxol was observed within the range of doses used. Even the dose of 20 mg/kg, which was much less effective than higher doses in slowing the growth of MCA-4 tumors, was highly effective in inducing mitotic arrest. The mitotic arrest seen in the OCA-I tumor was similar to that observed in MCA-4; the lowest dose of taxol used (10 mg/kg) was significantly less effective than other doses, and in general, the percentage of mitosis increased as the dose of taxol was increased. This corresponded well with the dose dependence of tumor growth delay (see Fig. 1, lower panel).

Figure 3 also shows the effect of different doses of taxol on induction of apoptosis. All doses of taxol significantly increased the proportion of apoptotic cells in the MCA-4 tumor from the control value of $6.8\% \pm 1.0\%$ to mean values ranging from 11.8% to 15.6%, depending on the dose of taxol used. However, as was the case for the induction of mitotic arrest in this tumor, there was no clear dependence of the magnitude of apoptosis induction on the delivered dose of taxol. In contrast, no significant induction of apoptosis in the OCA-I tumor was observed at 8 h after treatment with any of the doses of taxol used.

To obtain more complete insight into taxol-induced mitotic arrest and apoptosis we determined in separate experiments the kinetics of development of the two parameters after a single dose or two injections of 60 mg/kg taxol. Mitotic arrest and apoptosis were scored at 1, 3, 6, 9, 12, 18, 24, 36, 48, 72, and 96 h after a single injection of taxol and at 3, 6, 9, 12, 24, 48, and 72 h after the second dose of taxol (given 3 days after the first dose). The treatments were started when tumors were 8 mm in diameter. The results are presented in Fig. 4 for MCA-4 tumors and in Fig. 5 for OCA-I tumors.

At 1 h after a single dose of taxol there was an increase in arrested mitoses in MCA-4 tumors (Fig. 4 A). The percentage of arrested cells increased rapidly with time, achieving its peak at 9 h after taxol administration, at which time more than 30% of tumor cells were arrested in mitosis. Thereafter, there was a gradual decline in the percentage of arrested mitoses, with values returning to normal by 72–96 h after treatment with taxol. The dynamics of apoptosis induction by a single dose of taxol was different from that of mitotic arrest. The increase in apoptosis began at about 6 h after taxol treatment and gradually increased until it plateaued at a level of about 20% at 12 h, where it remained relatively constant, with some minor fluctuations, until 48 h. The percentage of apoptosis then declined to the pretreatment background level by 72 h after taxol treatment.

The kinetics of induction of mitotic arrest seen in the OCA-I tumor (Fig. 5 A) was similar to that observed in MCA-4. The peak in the arrest also occurred at 9 h after taxol administration, but unlike that in MCA-4 tumors, the percentage of mitotic cells in OCA-I tumors returned to the normal value more rapidly (by 24 h). The overall magnitude of mitotic arrest was smaller than that for MCA-4, the peak value being $22.6\% \pm 1.1\%$. The dynamics of apoptosis induction in OCA-I was also somewhat different from that in MCA-4. There was no significant increase in the percentage of apoptotic cells until 12 h after taxol administration, at a time when the percentage of cells arrested in mitosis started to decline. Thereafter the percentage of apoptotic cells increased to reach a peak at 24 h after taxol treatment and then rapidly declined to 8% at 36 h, at which level it remained until the end of the observation period of 96 h. Thus, even at 4 days after treatment with taxol, the percentage of apoptotic cells was above the pretreatment background level.

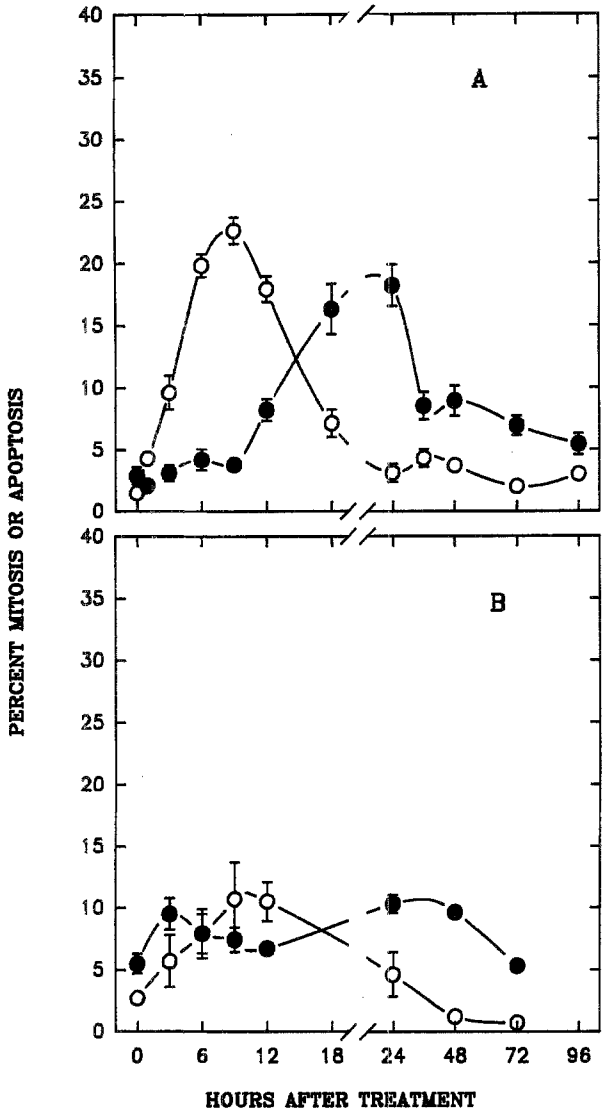

Fig. 5 A, B Percentage of mitosis (○) or apoptosis (●) induced in OCA-I tumors as a function of time **A** after a single dose of 60 mg/kg taxol or **B** after a second dose of 60 mg/kg given 3 days after the first dose. Mice bearing 8-mm OCA-I tumors were injected i.v. with taxol; the percentage of mitosis or apoptosis was scored from histological sections made from tumors at 0–96 h after treatment (*Vertical bars* SE of the mean)

The magnitude of mitotic arrest and apoptosis induction seen in both tumors after the second dose of taxol differed considerably from that observed after the first injection of taxol (Figs. 4 B, 5 B). Both tumors responded to the treatment by mitotic arrest, and the kinetics of mitotic response was in general similar to that seen after the first injection of taxol. However, the percentage of cells arrested in mitosis after the second dose was much smaller than that after the first dose. The apoptotic response to the second injection of taxol was minimal, if at all present, in MCA-4 tumors (Fig. 4 B). In the OCA-I tumor the percentage of apoptotic cells increased somewhat and fluctuated within the level of 6%–10% throughout the observation period of 3 days (Fig. 5 B).

Histology features

Both MCA-4 and OCA-I tumors were largely composed of well-defined lobules surrounded by various amounts of stroma containing blood vessels. The central portions of many lobules contained some necrosis. After treatment with taxol, mitotically arrested cells were seen throughout the lobules, although they were predominant at the periphery of lobules, closer to blood vessels. Apoptotic cells were more evenly distributed throughout the lobules, although they also showed some tendency to be more frequent at the periphery.

The morphological appearance of mitotic, necrotic, and apoptotic cells is shown in Fig. 6. Within the first 12 h after treatment with taxol, mitotic cells contained condensed chromosomes, their nuclear membrane was lost, and their plasma membrane was well preserved. Many mitotic cells had a characteristic "wagon wheel" appearance in which chromosomes were arranged in a concentric zone beneath the plasma membrane (Fig. 6 B). The central eosinophilic cytoplasm of taxol-treated mitotically arrested cells has been shown to consist of bundles of microtubules [10, 11]. After 12 h an increasing number of these unique mitotic cells with a coronal appearance underwent death that manifested as membrane disruption and spilling of the nuclear material into the extracellular space. The extracellular, non-membrane-bound nuclear material had two appearances: (1) dark pyknotic nuclear debris and (2) karyolytic condensed chromatin (Fig. 6 B).

In addition to the lytic, necrotic death of these cells described above, many taxol-treated cells formed multiple discrete apoptotic bodies that consisted of membrane-bound, small nuclear fragments surrounded by a narrow rim of cytoplasm (Fig. 6 B). These widely dispersed extracellular apoptotic bodies were similar to those induced by irradiation of these tumors [3].

Discussion

The results presented herein show that taxol was highly effective in inducing mitotic arrest and apoptosis in two murine tumors and that these cellular effects manifested macroscopically as tumor-growth delay. In general, the growth delay of both MCA-4 and OCA-I tumors was more pronounced as the dose of taxol was increased. This was particularly evident in OCA-I tumors. When the dose of taxol (60 mg/kg) was repeated 3 days later the effect was either additive or supra-additive, suggesting that repeated administrations of taxol are likely to be more effective than single doses in tumor treatment.

In most reports on taxol the antitumor activity of this drug has been attributed to its effects on microtubules, resulting in the arrest of cells in the G2 and M phases of the cell cycle and in cell cytotoxicity. These observations on the cellular effects of taxol have been made primarily using cell-culture systems [10, 14]. Our present study clearly demonstrated that taxol causes a profound mitotic arrest

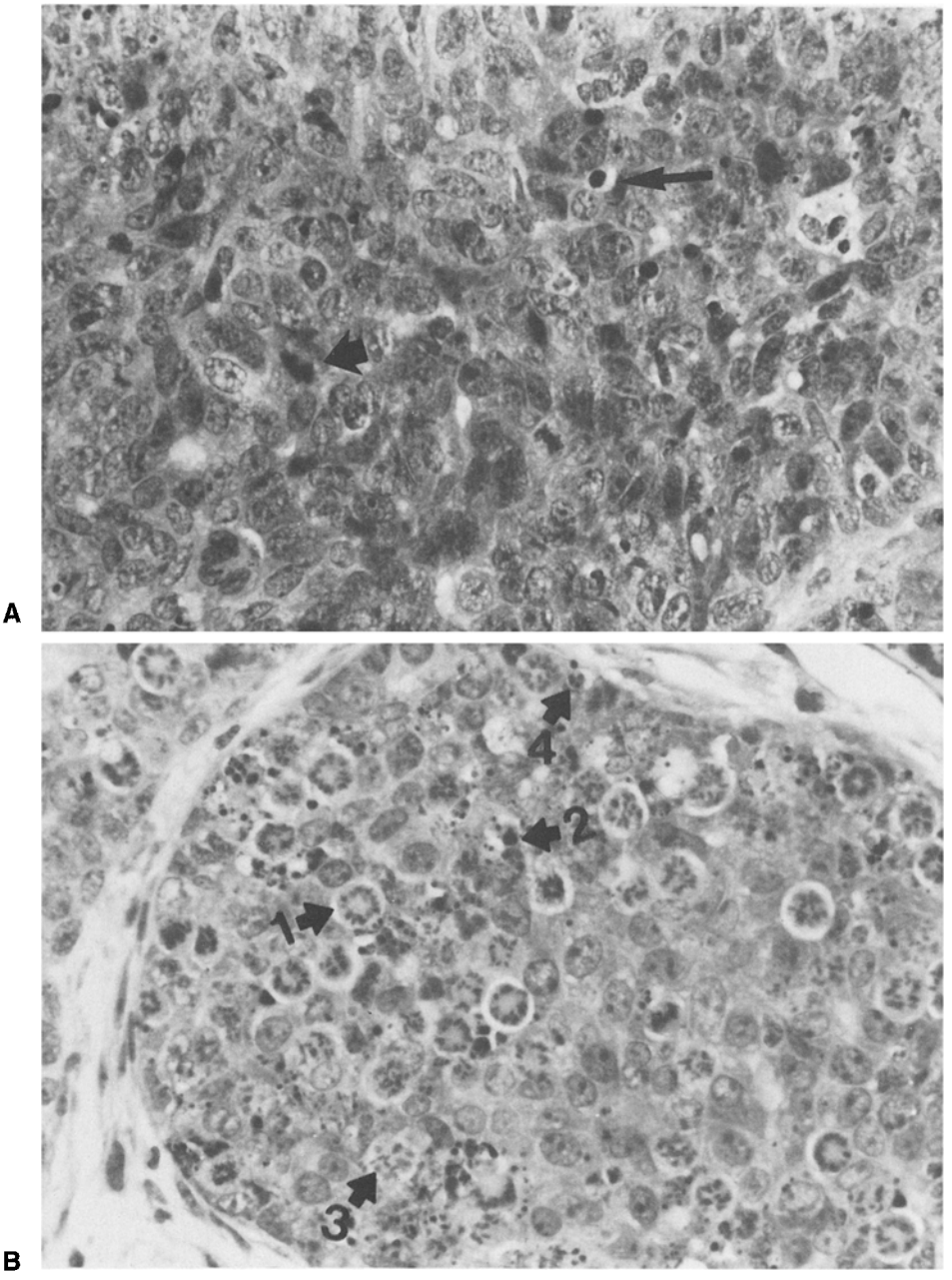

Fig. 6A,B Histological appearance of MCA-4 tumors **A** left untreated or **B** at 24 h after treatment with 60 mg/kg taxol. **A** untreated tumors show rare mitotic figures (*short arrow*) or apoptotic cells (*long arrow*). **B** Taxol-treated tumors show mitotically arrested cells with the characteristic coronal appearance of condensed chromatin (*arrow 1*). Necrotic cells exhibited pyknosis (*arrow 2*) and karyolysis (*arrow 3*). Apoptotic bodies consisted of small nuclear fragments surrounded by a narrow rim of cytoplasm (*arrow 4*)

in in vivo settings as well. A time-course microscopic analysis of mitotic cells in tumors treated with taxol revealed that at 1 h after treatment the arrest in mitosis was visible and that it reached its peak at about 9 h after treatment, at which time more than one-third of MCA-4 cells and about one-fourth of OCA-I tumor cells were arrested in mitosis. At 24 h after treatment of OCA-I tumors and 3–4 days after treatment of MCA-4 tumors, mitosis returned to its pretreatment level.

Histological analysis of these tumors revealed unique morphological features associated with taxol treatment. The chromatin of many neoplastic cells assumed a peripheral distribution as shown in Fig. 6 B. Most cells exhibiting the characteristic appearance of taxol-induced mitotic arrest were doomed to die either by necrotic lysis or by apoptosis. That taxol kills cells by two modes of death is not unique; this death resembles that induced by other cytotoxic agents, including other chemotherapy agents and radiation [1–5, 12, 13]. What may be unique about taxol is the association of both modes of cell killing (necrosis and apoptosis) with aberrant mitosis. Necrosis was characterized by lysis of the cell membrane and loss of the altered nuclear material into the extracellular space. Apoptotic death occurred as a transition of peripherally oriented coarse chromatin into classic membrane-bound apoptotic bodies.

Apoptosis has recently attracted significant interest from cancer biologists and clinical investigators [16]. There is increasing evidence that various anticancer agents, including radiation [12] and chemotherapeutic drugs [2], induce

apoptosis in tumors and that the degree of induced apoptosis correlates with the antitumor effectiveness of the cytotoxic agents [3, 5]. The data presented herein show that taxol is capable of inducing apoptosis in both MCA-4 and OCA-I tumors.

Development of apoptosis lagged several hours behind mitotic arrest, plateauing in MCA-4 tumors at between 12 and 48 h and reaching its peak in OCA-I tumors at 24 h after treatment with taxol. The most plausible explanation for this is that most apoptotic cells developed from the mitotic cells doomed to die, a process that can be histologically followed as described in Results. At the time of the peak of apoptosis the increase in the percentage of apoptotic cells was 3–4 times the background values. However, this increase was much lower than the increase in the percentage of mitotically arrested cells. As discussed above, mitotically arrested cells may die by other additional modes or they may successfully complete mitosis and continue living.

The mitotic arrest and apoptosis induction observed after the second injection of taxol were quite different from those seen after the first treatment. In both MCA-4 and OCA-I tumors the number of mitotic cells increased within a few hours after treatment. The increase peaked at 9–12 h after treatment and normalized by 24 h. However, the percentage of arrested cells noted after the second injection was much lower than that observed after the first administration of taxol. A likely explanation for this is that at 3 days after treatment with the first dose of taxol, only a small proportion of tumor cells were in the proliferative pool and, thus, amenable to arrest in mitosis by the second dose of taxol. In contrast to its effect on mitosis, the second dose of taxol was virtually ineffective in inducing apoptosis in MCA-4 tumors and was only weakly effective in inducing apoptosis in OCA-I tumors. This suggests that the first dose of taxol eliminated almost totally a subpopulation of tumor cells susceptible to induction of apoptosis by this agent.

Clearly, both mitotic arrest and apoptosis are mechanisms by which taxol can induce growth delay and temporary regression of both MCA-4 and OCA-I tumors. However, the tumor-growth delay induced by taxol was more clearly dose-dependent than was the effect of taxol on mitosis and apoptosis. Furthermore, after the second dose of taxol the tumor-growth delay was equal to (MCA-4) or bigger than (OCA-I) that resulting from the first dose, but this did not correspond to the effect of taxol on mitosis and apoptosis. These observations indicate that in addition to mitotic arrest and apoptosis, taxol must exert other as yet unknown antitumor effects. Nonetheless, knowledge about the kinetic profiles of mitotic arrest has important therapeutic implications, especially in therapy where taxol is combined with other agents such as cell-cycle-specific drugs and radiation.

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