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Apoptosis in murine tumors treated with chemotherapy agents

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There is increasing attention directed to the hypothesis that apoptosis plays a role in the response to cancer treatment including chemotherapy. However, the evidence to support this hypothesis has come almost entirely from experiments conducted in cultured cell systems. To extend this hypothesis to the therapeutic setting it is necessary to address this critical question in tumors treated *in vivo*. We have therefore evaluated the extent of apoptosis induced in murine tumors treated *in vivo* with cancer chemotherapy agents. Seven different murine tumors, comprising a mammary adenocarcinoma (MCa-4), an ovarian adenocarcinoma (OCa-I), a lymphoma (LY-TH), three sarcomas (FSA, NFSA and SA-NH) and a squamous cell carcinoma (SSC-7), were examined 8 and 24 h after treatment with cisplatin or cyclophosphamide (CY). Apoptosis was scored by morphometric analysis of histological sections of the tumors. The results showed that MCa-4, OCa-I and LY-TH had a significant apoptotic response to both cisplatin and CY, and the other tumors had essentially no apoptotic response. In addition, two of these tumors, MCa-4 and OCa-I, underwent apoptosis in response to adriamycin, 5-fluorouracil, Ara-C, etoposide, camptothecin and fludarabine. These observations demonstrate that apoptosis may be a feature of tumor response to chemotherapy *in vivo*, and illustrate the heterogeneity of apoptotic response amongst different tumor types and to different cytotoxic agents.

Key words: Apoptosis, chemotherapy, tumor.

Introduction

The success of cancer chemotherapy is primarily limited by drug resistance. Tumor cells that are resistant to the drug emerge during the course of therapy and repopulate the tumor. These cells are either already present in low numbers before therapy and are then selected by the treatment or arise during therapy due to the genomic instability inherent in many types of tumors. Drug resistance has been the topic of intensive research for many years and several mechanisms have been uncovered to account for this phenotype. These mechanisms include, but are not limited to, increased expression of cell membrane components that regulate drug uptake and efflux,[1] e.g. the multidrug resistance (MDR) glycoprotein; enhanced enzymatic activity to detoxify chemotherapy agents,[2] e.g. glutathione transferase; or enhanced cellular repair of the lesions produced by DNA-reactive drugs.[3]

Recently, however, another mechanism has been put forth to explain resistance to drugs: the failure of cells to undergo a particular mode of cell deletion known as programmed cell death, or apoptosis, on exposure to the agent.[4,5] Apoptosis is distinguished from other modes of cell death on the basis of characteristic biochemical and morphological features and patterns of occurrence in tissues.[6] In addition to its critical involvement in tissue remodeling during normal organism development, apoptosis has been implicited in a number of human diseases including cancer.[7] In cancer, loss of apoptosis propensity may be a necessary and early step in the progression of many different types of tumors, allowing the propagation of cells that have sustained DNA damage.[8] It also appears to be an important pathway for cell death following the exposure of tumor cells to a variety of therapeutic modalities including radiotherapy, chemotherapy, hyperthermia, hormonal ablation and certain types of immunotherapy.[9] Thus, the overall effectiveness of these therapeutic modalities may be dictated by the presence of cells that are resistant to apoptosis induction. This relatively recent hypothesis has stimulated investigations that seek to understand the biochemical and molecular mechanisms by which apoptosis-resistant cells emerge in tumor cell populations.[10]

These mechanistic approaches notwithstanding, the question that remains to be answered is, does apoptosis propensity govern tumor response in the

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therapeutic setting? With respect to chemotherapy, cells displaying the features of apoptosis have been observed following treatment *in vitro* with many if not most of the chemotherapy agents in current use, including but not limited to dexamethasone, cisplatin, methotrexate, 5-fluorouracil (5-FU), etoposide (ETP), camptothecin (CAM), melphalan, Ara-C and BCNU.[4,5,11] In this report, we have analyzed whether apoptosis is a feature of the cytotoxic action of several anticancer drugs when administered *in vivo*. In addition, this study has compared the response in several different types of tumors in an attempt to ascertain the generality of apoptosis induction in chemotherapy.

Materials and methods

Mice and tumors

Inbred female C_3Hf/Kam mice that were bred and maintained in our specific-pathogen-free colony were used. The mice were 12–16 weeks old at the beginning of the experiments. Seven different murine tumors were examined, comprising one mammary adenocarcinoma (MCa-4), one ovarian adenocarcinoma (OCa-I), a lymphoma (LY-TH), three sarcomas (FSA, NFSA and SA-NH) and one squamous cell carcinoma (SSC-7). Many characteristics of these tumors, including their apoptotic response to radiation, have been previously described by us.[12] These transplantable tumors were syngeneic to the C_3Hf/Kam mice. Solitary tumors were produced in the muscles of the right hind limb by the innoculation of 5×10^5 viable tumor cells obtained by enzymatic digestion of tumors from source mice as described previously.[12] When the tumors grew to 8 mm in diameter, the mice were treated with the drug in question, further growth was monitored and apoptosis was scored at set times. The experimental groups consisted of three mice each for apoptosis measurements and seven to 10 mice in each group for the growth delay experiments.

Drug treatments

The various drugs were dissolved in the appropriate solvent at concentrations that would allow small-volume injections. Injections were i.p. unless otherwise noted. Cisplatin (Bristol-Meyers) was dissolved in saline and doses of 10–12 mg/kg of body weight were used. CY (Mead Johnson) was dissolved in distilled water and used at 200 mg/kg. Adriamycin (ADR, Cetus) was dissolved in saline and injected i.v. at 16 mg/kg. 5-FU was dissolved in water and given at 100 mg/kg. Ara-C (Cetus) was dissolved in water and given at 600 mg/kg. ETP (Bristol Laboratories) was dissolved in saline and given at 40 mg/kg. CAM (Sigma) was dissolved in sesame oil and injected i.m. at 4 mg/kg. Fludarabine (FLD, Berlex Laboratories) was dissolved in saline and given at 800 mg/kg.

Quantitation of apoptosis

Mice were killed by cervical dislocation at various times after treatment, and the tumors were immediately excised and placed in neutral-buffered formalin. This tissue was processed for embedding in paraffin blocks, from which 2–4 μm sections were cut and stained with hematoxylin and eosin (H&E). The morphological features we used to histologically identify and score apoptotic cells have been described previously.[13,14] Apoptosis was scored in coded slides by microscopic examination of H&E-stained sections at ×400 magnification. To determine the apoptotic index (AI), five fields of non-necrotic areas were selected in each specimen and in each field the number of apoptotic nuclei were recorded as numbers per 100 nuclei. The values of AI presented are expressed as percentages and are based on scoring 1500 nuclei, obtained from the three mice in each group.

Tumor growth delay

Tumor response was assessed on the basis of tumor growth delay measurements. Mice were treated with the drug under investigation when tumors growing in the right hind limb were 8 mm in diameter. The growth of non-treated and drug-treated tumors was determined by measuring three mutually orthogonal tumor diameters with a vernier caliper. The measurements were performed at 2–3 day intervals until tumors reached at least 14 mm in diameter.

Results

The goal of the present studies was to answer two questions raised in our earlier investigations demonstrating apoptosis induction by CY in the MCa-4 and OCa-I murine tumors.[15] First, would the degree of apoptotic response seen with CY extend to other cancer chemotherapy drugs? Second, would the degree of apoptic response seen in these two tumors extend to other types of tumors?

To address the first question, we treated the MCa-4 and OCa-I tumors with a variety of chemotherapy agents and analyzed them for the induced AI at 8 and 24 h following treatment. These times were chosen based on the previously reported response of these two tumors to CY which produced a rather broad distribution of apoptotic cells between these times.[15] Photomicrographs illustrating the features of apoptosis in the MCa-4 tumor following treatment with CAM are presented in Figure 1. The quantitative results of this analysis for all of the drugs examined are presented in Figure 2. As can be seen, with the possible exception of ADR and 5-FU where the response was modest, all of the drugs produced a fairly dramatic apoptotic response. Moreover, for most of the drugs the AI was higher at 8 h.

With regard to the second question, we compared the apoptosis induced by CY and cisplatin in seven different murine tumors. A variety of different tumors histologies was examined: in addition to the original two adenocarcinomas, we determined the apoptotic response in a squamous cell carcinoma, three sarcomas and a lymphoma. The results of these studies are presented in Figure 3. Limited results from a very similar, previously published[12] study of apoptosis following radiation are replotted in Figure 3 for the sake of comparison. The patterns of apoptotic response for the various tumors displayed in this manner suggest that apoptosis propensity is a feature of tumor type and may be independent of the inducing agent because four of the tumors, SCC-7, FSA, NFSA and SA-NH, showed very little or no apoptosis after any of the three treatments, whereas the MCa-4, OCa-I and LY-TH tumors displayed substantial apoptotic response to all three treatments. Photomicrographs depicting the lack of an apoptotic response in the SCC-7 tumor following treatment with CY are presented in Figure 1.

The four tumors that displayed resistance to apoptosis induction by these agents were examined for the influence of CY and cisplatin on their tumor growth kinetics (Figure 4): growth kinetics after CY and cisplatin had been examined in two of the sensitive tumors, MCa-4 and OCa-I, in previous reports[15,16] and data depicting their responses have been replotted in Figure 4 for the sake of comparison. Whereas two of the tumors, SSC-7 and NFSA, appeared to have very little growth delay (Figure 4), consistent with their resistance to apoptosis induction (Figure 3), two other tumors, SA-NH and FSA, displayed a substantial regression and growth delay at least for CY in spite of the fact that they had little or no apoptotic response.

In light of the regression seen in the SA-NH and FSA tumors (Figure 4) in the absence of any apoptotic response determined at 8 or 24 h (Figure 3), these two tumors were re-examined for an apoptotic response at those times after CY injection when regression occurred. Thus, SA-NH tumors were examined at 2, 4 and 6 days following CY treatment, and FSA tumors were examined at 2, 4, 6, 8 and 12 days after CY injections. Whereas no apoptosis was evident in either of these tumors at any of these additional time points, both tumors displayed hemorrhagic coagulative necrosis that progressed with time, reaching a maximum 6–8 days after treatment and greatly reducing the cellularity of these tumors by times near the nadir of the growth delay. Photomicrographs illustrating hemorrhagic necrosis in the SA-NH tumor following treatment with CY are presented in Figure 1.

Discussion

Knowledge about programmed cell death or apoptosis has significantly advanced over the last 5 years, and we now are beginning to appreciate some of the mechanisms that regulate this process, including the signal transduction pathways[17] and certain genes that control the propensity for apoptosis at the molecular level,[18] including the proto-oncogene *bcl-2*[19] and the tumor suppressor gene p53.[8] In parallel with these very important mechanistic studies has come the realization that apoptosis is also a major mode of cell death in the cytotoxic response of cells to certain cancer treatment modalities, especially radiotherapy and chemotherapy.[9] These observations have given impetus to the idea that apoptosis might be modulated to enhance the therapeutic effectiveness of radiation and anticancer drugs. However, strategies designed to enhance cancer therapy by enhancing apoptosis inherently rely on the assumption that the propensity for tumor cells to undergo apoptosis *in vivo* dictates tumor response to therapy in the clinical setting, and the question becomes, can the degree of apoptosis that does occur in tumors treated *in vivo* account for the subsequent response of the tumor to the therapy?

In our analysis of 15 different tumors following irradiation, we observed a positive correlation between the degree of apoptotic response and the magnitude of tumor growth delay as well as the rate of tumor cure.[12] We have attempted to address the role of apoptosis in tumor response to chemotherapy in the present study. In two previous examinations, we demonstrated that CY[15] and

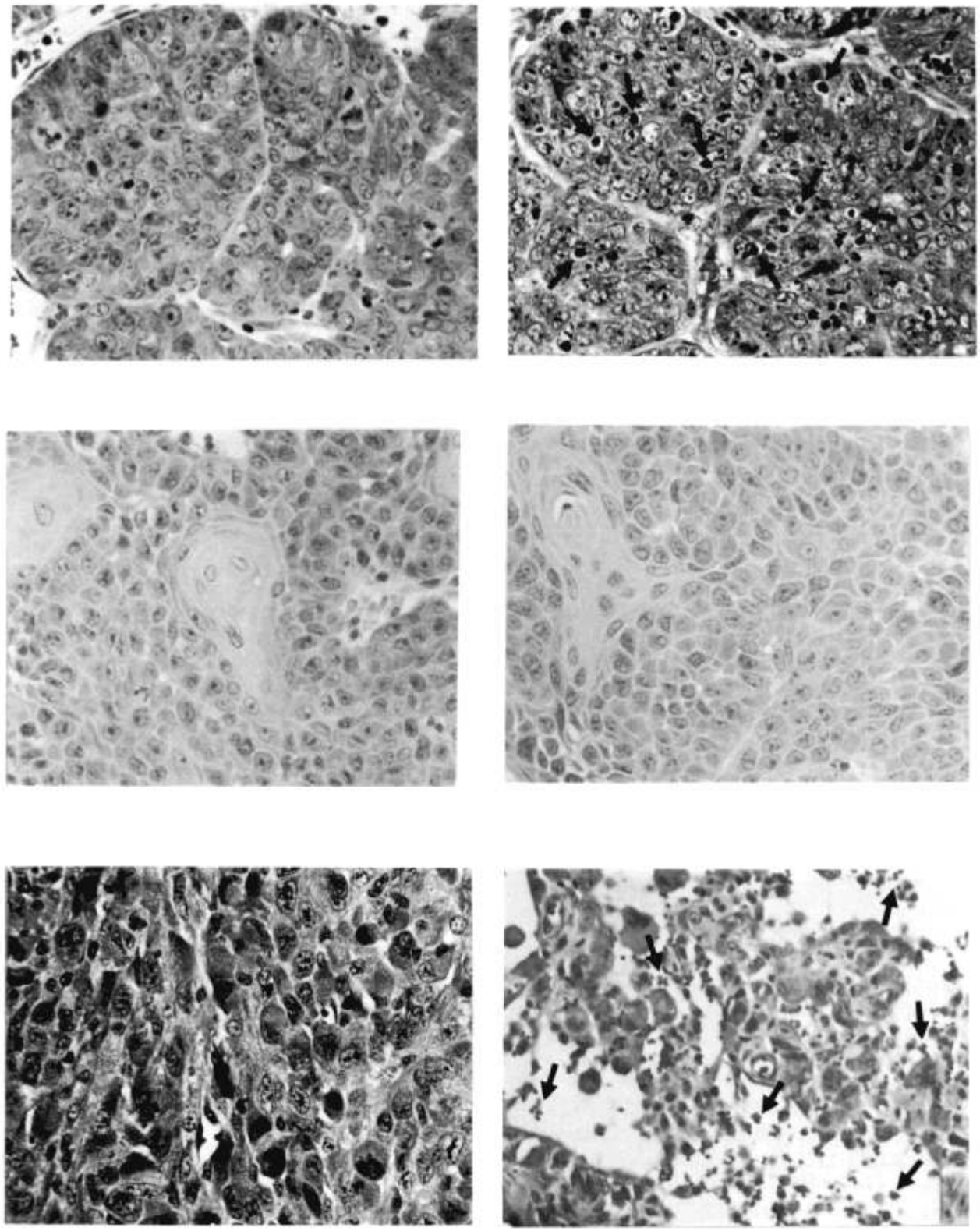

Figure 1. Photomicrographs (×284) of H&E-stained sections of murine tumors. Top panels represent MCa-4 tumors from untreated mice (left) or mice that received 4 mg/kg CAM, 8 h (right). Middle panels represent SCC-7 tumors from untreated mice (left) or mice that received 200 mg/kg of CY, 8 h (right). Bottom panels represent SA-NH tumors from untreated mice (left) or mice that received 200 mg/kg of CY, 6 days (right). Arrows in the top panels point to apoptotic cells and to red blood cells in the bottom panels.

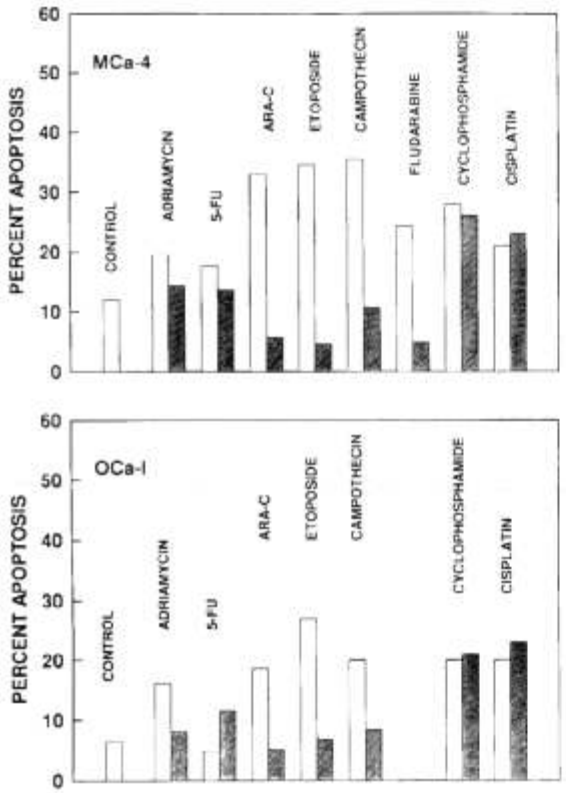

Figure 2. The induction of apoptosis in MCa-4 and OCa-I tumors in mice treated with different chemotherapy agents. Doses of the various drugs are given in Materials and methods. Mice were killed at either 8 (□) or 24 (■) h after treatment.

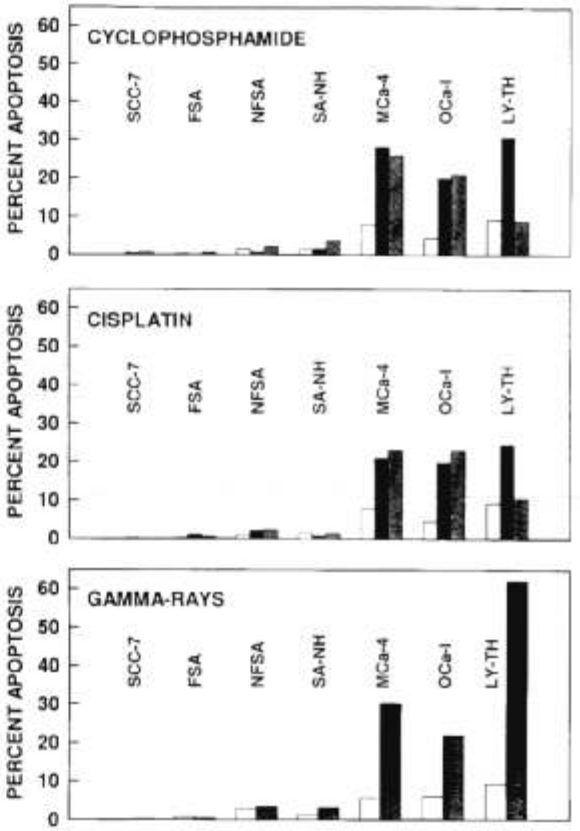

Figure 3. The induction of apoptosis in seven different murine tumors by 12 mg/kg of cisplatin, 200 mg/kg of CY or 25 Gy or radiation. Mice were killed as 8 (■) and 24 h (■) after CP or CY administration and 4 h after irradiation. Untreated controls are also shown (□). The data for radiation are redrawn from elsewhere[12] and are shown for the sake of comparison.

cisplatin[16] induce a very dramatic apoptotic response in the MCa-4 and OCa-I tumors, and here we have extended these observations to six additional chemotherapy agents. All of the agents induced an apoptotic response in the MCa-4 and OCa-I tumors. Thus, it appears that an apoptotic response to chemotherapy is a feature of certain tumors and that many different agents, including many of those important clinically, are capable of apoptosis induction in such tumors. The levels of apoptosis induced by the different agents cannot be directly compared because we did not do dose escalation studies; in any case, the difference in limiting toxicities make such a comparison difficult. Even so, the single doses of CAM and ETP we chose produced very high levels of apoptosis in the MCa-4 tumor, reaching AI values of 35% within 8 h of injection of the drug, a value higher than those achieved by high doses of radiation in the same tumor.[12] Although we did not perform detailed time courses for AI, the data shown in Figure 2 are consistent with what we observed for radiation, demonstrating that, in tumors that have a propensity for this mode of cell death, the apoptosis appears within just a few hours following treatment.

Analysis of the apoptotic response of the seven different tumors following exposure to CY or cisplatin when compared to our previous results for the same tumors exposed to radiation[12] yielded a striking pattern (Figure 3): that is, tumors that responded to radiation by apoptosis (MCa-4, OCa-I and LY-TH) also had an apoptotic response to both CY and cisplatin and tumors that were resistant to radiation-induced apoptosis (SSC-7, FSA, NFSA and SA-NH) were cross-resistant to CY- and cisplatin-induced apoptosis. We earlier speculated that the heterogeneity in apoptotic response to radiation among various tumors could be due to intrinsic tu-

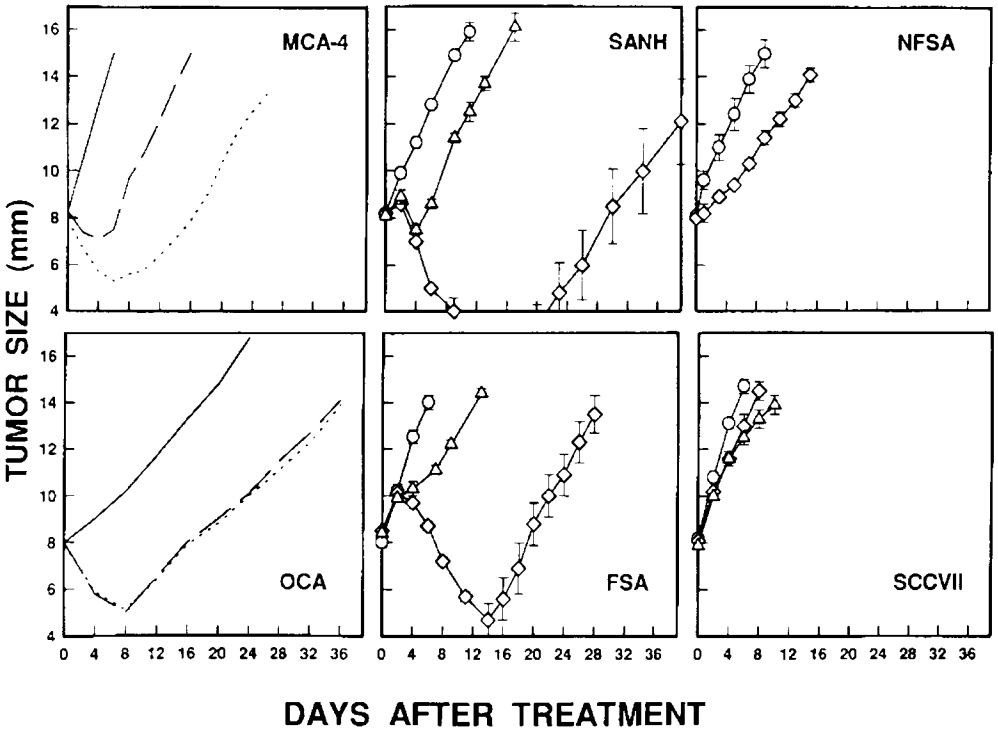

Figure 4. The kinetics of tumor growth delay for the MCa-4, OCa-I, SA-NH, FSA, NFSA and SSC-7 tumors treated with 200 mg/kg CY or 12 mg/kg cisplatin. The different symbols represent: no treatment (○), cisplatin (△) and CY (◇). The data for MCa-4 and OCa-1 tumors are redrawn from elsewhere[15,16] and are shown for the sake of comparison: no treatment (——), cisplatin (— — —) and CY (-----).

mor factors.[12] The results presented in Figure 3 lend additional support to this idea. Among the various tumor properties known to regulate apoptosis are growth factors and cytokines and oncogenes and tumor suppressor genes.[20] Of the latter the oncogene *bcl*-2[10,21] and the tumor suppressor gene p53[22,23] profoundly influence tumor cell propensity for radiation- and chemotherapy-induced apoptosis. We are currently studying expression and mutation status of these genes in the seven tumors we used.

There are additional factors that may explain the apparent heterogeneity in apoptotic response observed amongst these tumors (Figure 3). In a previous paper we pointed out that the AIs reported in these studies may underestimate the true proportion of apoptotic cells because they are rapidly phagocytosed *in vivo* and the window of time for visualizing an apoptotic cell may be as short as 3 h.[24] Thus, the heterogeneity could represent a difference in the rate of phagocytosis in different tumors. This might especially be true if the tumors had different macrophage contents. While we cannot rule out a contribution of different rates of phagocytosis to the heterogeneity, it seems unlikely in light of the fact that we have shown that the tumor that has a substantial apoptotic response, OCa-I, has a very rapid phagocytosis mechanism.[25] Moreover, we have examined the macrophage content of these tumors previously and there is no correlation with their apoptotic response, e.g. MCa-4 has 31% macrophage content whereas FSA has 27%.[26]

We compared the apoptotic response in six of the tumors used in our studies to their respective tumor growth delay following treatment with CY or cisplatin. That two of these tumors, MCa-4 and OCa-I, respond by growth delay and apoptosis had been shown in our previous investigations.[15,16] Of the other four, none of which responded by apoptosis, two showed substantial growth delay, FSA and SA-NH, and two did not, NFSA and SCC-7.

This lack of correlation between apoptotic response and tumor growth delay in response to chemotherapy may be explained on the basis of multiple pathways of cell death. It seems reasonable that in some tumors, tumor regression results from reproductive cell death or necrosis. We confirmed this for the SA-NH and FSA tumors, which appar-

ently regressed after CY treatment through massive hemorrhagic necrosis. Even in the apoptosis-sensitive MCa-4 and OCa-I tumors, not all of the tumor growth delay following CY or CP was due to apoptosis. The influence of these other pathways of cell death may also highly depend on the particular drug being investigated since the regression in the FSA and SA-NH tumors was most dramatic for CY treatment (Figure 4).

In conclusion, the results presented here suggest that apoptosis may play an important role in the response of certain types of tumors following treatment with chemotherapy agents and this appears to be largely independent of the agent in question. The important question remains, why do some tumors undergo apoptosis following treatment with anticancer drugs and others are resistant to apoptosis? Clues to the answer may lie in a particular tumor's expression of certain oncogenes and tumor suppressor genes such as *bcl*-2 and p53. Additional studies of the role of these genes in regulation of anticancer therapy-induced apoptosis are critical in this regard. Thus, the possibility of ultimately enhancing apoptosis or instilling apoptosis propensity in apoptosis-resistant tumor cells remains an important approach to enhance tumor response to therapy.

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