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Advances in Brief

Arsenic Trioxide Causes Selective Necrosis in Solid Murine Tumors by Vascular Shutdown[1]

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Abstract

To investigate the antitumor action of arsenic trioxide in solid tumors, we carried out quantitative tumor perfusion studies, using locally advanced methylcholanthrene-induced fibrosarcoma grown in BALB/c mice. The tumor perfusion studies were assessed by two separate methods: ^{99m}Tc clearance and ^{86}Rb uptake. A single administration of arsenic trioxide (10 mg/kg i.p.) produced a preferential vascular shutdown in the tumor tissue at 2 and 6 h, leading to massive necrosis in the central part of the tumor. The phenomenon was repeatable at intervals of weekly administration of the drug in the same tumor. Normal skin, muscle, and kidney were relatively unaffected by arsenic trioxide. These results suggest that the drug may be investigated as an adjunct to the standard cancer therapeutic modalities.

Introduction

Arsenic compounds have been used in traditional medicine in many countries for >1000 years. Approximately 100 years ago, Western medicine adopted some of these compounds, used them widely (1), and subsequently abandoned them because of their toxic and oncogenic effects after chronic exposure. Arsenic compounds have received renewed attention in recent years following clinical observations of their effect on APL.[3] In 1996, a group at Shanghai Institute of Hematology in China reported that As_2O_3 was an effective agent against APL even in patients who relapsed after retinoic acid-induced remission (2). The rate of clinically complete remission with As_2O_3 treatment was >80% without obvious acute toxicity, including bone marrow suppression. These spectacular clinical observations, confirmed by others (3–5), initiated investigations into the molecular mechanisms of As_2O_3-induced cell death.

It has been documented that arsenic can induce apoptosis in leukemic cells by activating apoptotic pathways (5, 6). However, the primary target of arsenic-induced cytotoxicity remains unknown. Trivalent arsenicals are regarded primarily as sulfhydryl reagents at the biochemical level. As_2O_3 inhibits many enzymes by reacting with biological ligands containing available sulfhydryl groups. Among them, arsenic targets vicinal thiol groups in tubulin with a high specificity (7). We postulated that As_2O_3 may have an antivascular effect on solid tumors because tubulin has been shown to be a primary target for other antitumor and antivascular agents, such as colchicine (8), flavone acetic acid (9), and combretastatin-A4 (10). We report the first *in vivo* observation demonstrating the antivascular effect of As_2O_3 in well-established murine solid tumors.

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[3] The abbreviations used are: APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; Meth-A, methylcholanthrene-induced fibrosarcoma.

Materials and Methods

Mice, Tumors, and Compounds

The murine tumor line used was Meth-A in BALB/c mice (11). Male mice, 6–8 weeks of age weighing 20–25 g were obtained from the Charles River Laboratories (Portage, MI). Meth-A cells were maintained by serial i.p. passage in syngeneic mice. For tumor implantation, Meth-A cells from ascitic fluid were collected between days 7 and 10 after cell implantation and washed twice with MEM medium. Approximately 1×10^5 viable Meth-A cells were inoculated into the skin of the right upper abdominal quadrant of the mouse. All experiments were carried out with intradermal tumors except for rubidium-uptake studies, which used s.c. tumors grown in the right rear leg of the mice.

As_2O_3 purchased from Sigma Chemical (St. Louis, MO) was dissolved in H_2O by continuous stirring. As_2O_3 was kept at 4°C as a stock solution. Dilution was made with 0.9% NaCl to a final concentration of 0.5 mg/ml As_2O_3. Dextrose was added to a final concentration of 5% to minimize acute arsenic toxicity (12).

Gross Morphology and Histopathology

Gross morphological changes were followed up to 48 h after a single injection of As_2O_3 (10 mg/kg i.p.). At selected time points, tumors were excised and histopathological examinations were performed. Tumor tissues from different groups were fixed in 10% neutral formalin and processed in paraffin wax. Sections (4 μm thick) were stained with H&E and assessed microscopically to investigate the temporal effect of As_2O_3.

Inhibition of Tumor Growth

Mice, typically five per group, with mean tumor diameters of 12.0 ± 1.0 mm were used in treatment or control studies. As_2O_3 was administered i.p. in doses of 10 mg/kg once a week for the duration of the experiment, starting 13 days after tumor inoculation. The same volume of normal saline with 5% dextrose was given to the control group. Tumor volume was assessed three times a week. Tumors were measured with a caliper, and tumor volumes were calculated assuming cylindrical growth using the formula $a/2 \times b/2 \times h \times \pi$, where a, b, and h are the minor and major dimensions and height, respectively. No attempt was made to correct the tumor volume measurement for the area of central necrosis.

Blood Perfusion Measurement

Two methods, technetium clearance and rubidium uptake, were used to assess the antivascular effect of a single injection of As_2O_3 (10 mg/kg i.p.). Both methods have been described in detail elsewhere (13, 14).

^{99m}Tc Clearance. Clearance measurements were performed as described previously (13). Briefly, mice were anesthetized with a 60 mg/kg i.p. injection of sodium pentobarbital and were immobilized using a jig that did not restrict blood flow to the tissue of interest. Technetium bound as sodium pertechnetate, $Na^{99m}TcO_4$ (typical activity, 6 μCi/ml), was injected directly into the central area of the intradermal tumor 10 min after the pentobarbital injection. A total volume of 10 μl was deposited, using a 29-gauge needle. Radioactivity was measured and recorded every 10 s, 200 times, using a multichannel scintillation counter (AccuSpec; Canberra Industries, Meriden, CT) interfaced with a personal computer. Raw data were corrected for the physical decay of tech-

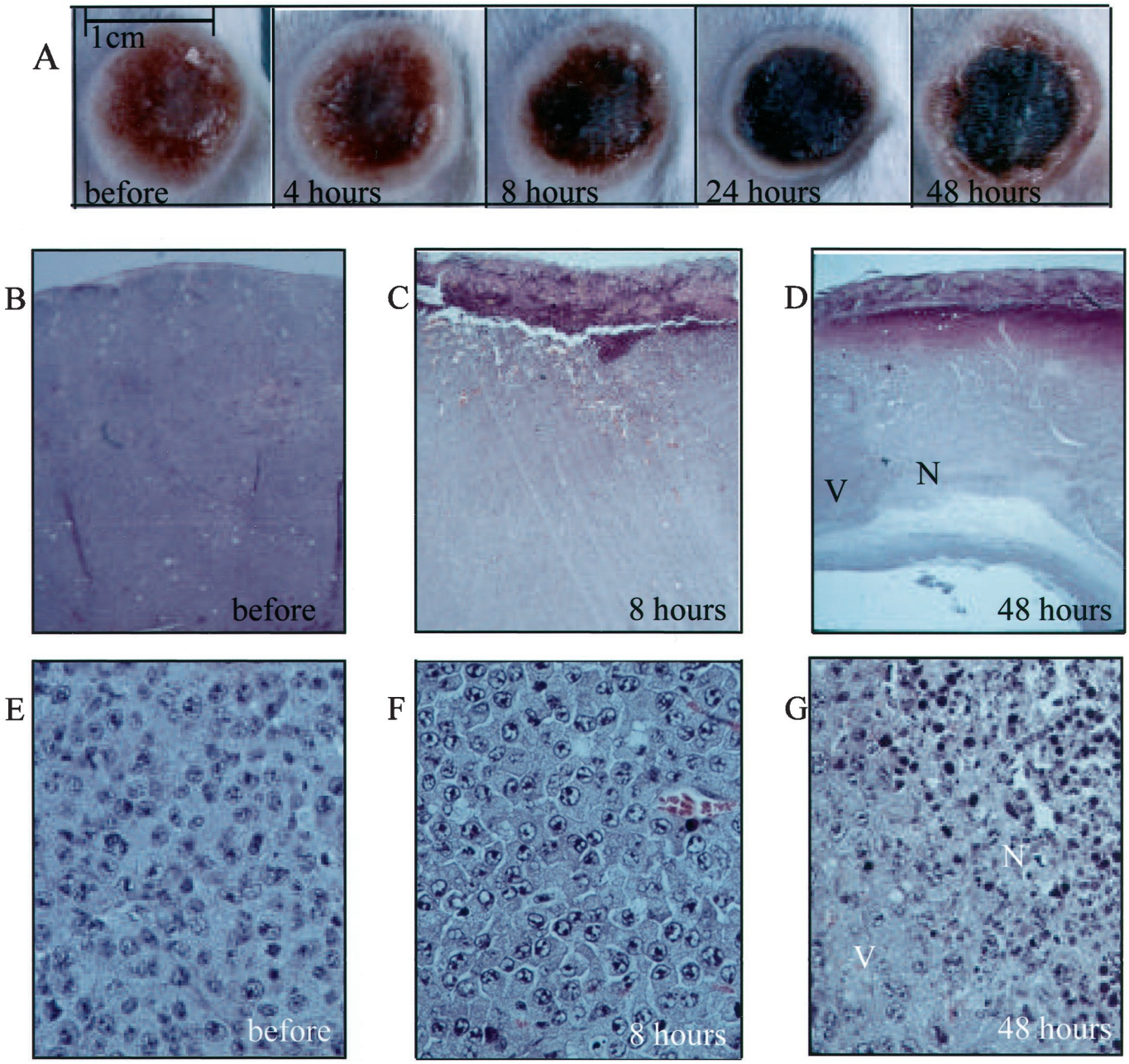

Fig. 1. Morphological features of Meth-A tumors in BALB/c mice. *A*, sequential gross morphological changes before and after the administration of As_2O_3 (10 mg/kg i.p.). *B–G*, histological features of the tumors stained with H&E. *B–D*, ×25 magnification; *E–G*, ×400 magnification. *V*, nonnecrotic zone; *N*, necrotic zone.

netium (6 h) and fit to a single exponential decay constant. No attempt to measure the partition coefficient was made, and the results are reported as clearance rate, not blood perfusion. The clearance rate was measured in each mouse before and 2, 6, and 24 h after arsenic treatment. Six tumor bearing mice with 12.0 ± 1.0 mm mean diameter tumors were used to study the effect of As_2O_3 on tumor clearance rate at each time point. Control experiments included clearance rate measurements in normal skin after As_2O_3 and also in untreated control tumors.

^{86}Rb Uptake. Changes in blood perfusion were measured in mouse tissue, using the rubidium uptake method (14). The rubidium studies were performed using s.c. Meth-A tumors. The tumor site was not critical to the results because we obtained similar results with rubidium-uptake studies using intradermal tumors at selected time point. In all experiments, 5 μCi of ^{86}RbCl in a 0.1-ml volume were injected through a tail vein after anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine, and mice were sacrificed by cervical dislocation 60 s after injection. The tumors, kidney, skin, and muscle were excised and counted in a well-counter (1282 Comugamma Cs; Pharmacia LKB Nuclear Inc., Gaithersburg, MD). The ratio of radioactive counts from the tissue of interest to the counts in the standard ^{86}Rb solution equivalent to the total ^{86}Rb activity injected multiplied by 100 gives the percentage of cardiac output to the tissue of interest. In this study, we did not attempt to measure absolute blood flow; relative blood perfusion values are presented. ^{86}Rb uptake was measured before and 2, 6, and 24 h after arsenic treatment. At least 10 tumor-bearing mice were used for each time point. The results are reported as the percentage of ^{86}Rb uptake per gram of tissue and plotted as a percentage of the control tissue.

Results

Tolerance and Acute Effect of As_2O_3. The toxicity of As_2O_3 was studied using the BALB/c mouse strain prior to initiating the tumor response studies. The approximate LD_{50} dose of an i.p. administration of As_2O_3 was 17 mg/kg. We used the maximally tolerated dose in this strain, which was 10 mg/kg. This dose induced reliable antitumor

effects on locally advanced tumors and was well tolerated even in repeated administrations. Within 30 min after injection, mice showed signs and symptoms of acute toxicity, consisting of slurred motion, lethargy, and piloerection, but recovered within 6 h. No weight loss or other subacute toxicity was observed during the course of experiment.

Gross Morphological and Histopathological Changes. Fig. 1*A* illustrates gross tumor morphology before and after a single injection of the drug. As_2O_3 induced a pronounced central necrosis in the tumor, which was observed within 4 h and steadily progressed until at least 48 h. More than 70% of the tumor tissue underwent central necrosis, and the necrotic area formed a crust that became stony hard by 48 h. The tumor tissue under the crust was malleable. The peripheral zone of tumor tissue appeared unaffected. Tumor regrowth appeared to be initiated from the peripheral zone.

The sequential histopathogical changes after drug treatment are shown in Fig. 1, *B–G*. Untreated control tumors consist of undifferentiated round cells with evidence of focal necrosis and a thin layer of amorphous debris on the surface. Beginning at 8 h after the treatment, there were early signs of cell death. The majority of cells had separated from one another and rounded. Amorphous debris on the surface of the tumor became thicker. As early as 4 h, there were foci of vascular congestion and a minimal amount of interstitial hemorrhage continuing up to 8 h, but there was no evidence of intravascular coagulation or thrombosis. By 24 h, the central portion of the tumor became uniformly necrotic. Most of necrotic cells showed faint cytoplasmic staining and condensed, pyknotic nuclei. The peripheral area of the tumor was unaffected, with a well-demarcated interface between the viable and necrotic tissues. This phenomenon became more pronounced by 48 h.

Tumor Growth Delay. Fig. 2 shows changes in the tumor volume as a function of time after a weekly administration of the drug. Immediately after the drug was administered, the tumor ceased to grow for ~2 days and then resumed growing. The regrowth was always seen from the periphery of the tumor. After administration of a second dose of the drug, tumor growth stopped for ~2 days, after which the tumor continued growing. A similar growth inhibitory pattern was observed after a third dose of the drug.

Effect of As_2O_3 on Regional Blood Perfusion in Tumor Tissue and Various Organs. Fig. 3 shows the ^{99m}Tc clearance rate and ^{86}Rb uptake as a function of time after a single administration of As_2O_3.

Clearance. In the absence of As_2O_3, technetium cleared from the tumor site rapidly. The initial half-time of clearance was ~8–9 min,

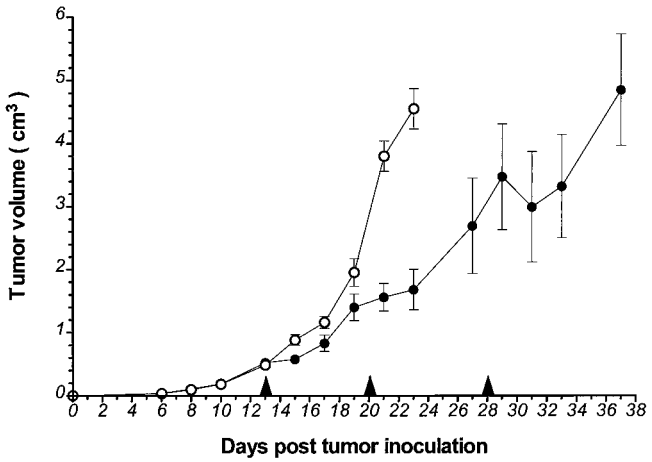

Fig. 2. Effect of As_2O_3 on the growth of Meth-A tumors in BALB/c mice. ○, untreated control group (vehicle only); ●, As_2O_3 group, 10 mg/kg i.p. The *arrowheads* indicate the times of As_2O_3 administration. Data are the means of five animals per group. *Bars*, SE.

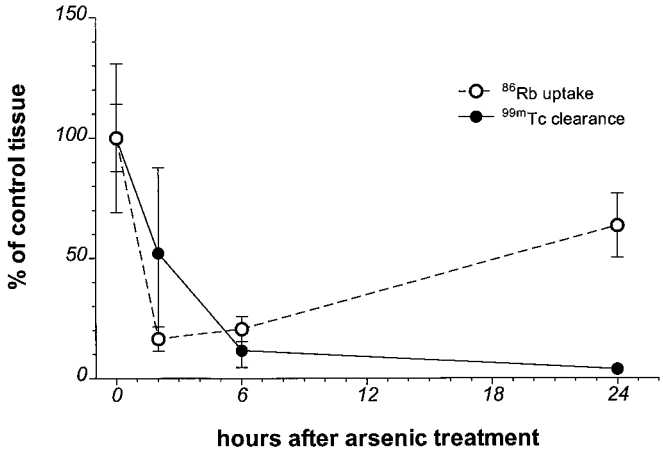

Fig. 3. Effect of As_2O_3 (10 mg/kg) on the blood perfusion in Meth-A tumors in the BALB/c mice measured by ^{86}Rb uptake and ^{99m}Tc clearance. Each *point* represents the mean of >10 mice for the rubidium uptake and 6 mice for ^{99m}Tc clearance. *Bars*, SE.

and this clearance rate did not change with subsequent injections even with seven repeat injections in the same area (data not shown). In contrast, the rate of technetium clearance was markedly reduced by the administration of As_2O_3 and remained low at 24 h, consistent with morphological observations.

Technetium clearance was 4.94 ± 1.53 h^{-1} before treatment and 2.57 ± 1.70, 0.58 ± 0.35, and 0.18 ± 0.09 h^{-1} at 2, 6, and 24 h after treatment, respectively. The calculated percentages relative to the control tumor were 52.1 ± 35.5, 11.6 ± 7.1, and 3.9 ± 1.8% of the control at 2, 6, and 24 h, respectively.

Uptake. Rubidium uptake in Meth-A tumors showed that a significant decrease in tumor blood perfusion occurred at 2 and 6 h after As_2O_3 injection and that tumor blood perfusion had recovered by 24 h. Rubidium uptake was 1.50 ± 0.21%/g tissue before treatment, and it decreased to 0.20 ± 0.07, 0.30 ± 0.08, and 1.0 ± 0.20%/g tissue at 2, 6, and 24 h after treatment, respectively. The calculated percentage relative to control tumor blood perfusion was 16.4 ± 5.0, 20.5 ± 5.2, and 64.3 ± 13.3% of the control at 2, 6, and 24 h respectively. The data of 2 and 6 h after the drug treatment were similar to the trends for the clearance rate. The uptake data at 24 h were inconsistent with both technetium clearance and morphological observation (see "Discussion"). Similar trends in uptake were observed in two other tumor lines studied (FSaII in C3H mice and SCK in A/J mice; data not shown).

Fig. 4 illustrates the effect of As_2O_3 (10 mg/kg) on rubidium uptake in the kidney, skin, and leg muscle measured at the same time as the measurement in the tumor. Blood perfusion in the skin and muscle was not changed significantly compared with control values. The maximum reduction of blood perfusion in normal tissues was that of the kidney at 2 h after As_2O_3 (60.0 ± 5.5% of control). The reduction in blood perfusion reduction to the tumor was much greater than that in the normal tissues studied.

Discussion

The data clearly demonstrate that As_2O_3 induces a prompt selective vascular shut down leading to massive central necrosis in Meth-A sarcoma grown in BALB/c mice. The profound tumor response occurred at a dosage of As_2O_3 that was well tolerated even in repeated administrations. The phenomenon was repeatable on subsequent administration on the same tumor.

Two different methods of blood perfusion measurements gave complementary information. Technetium clearance, which allows

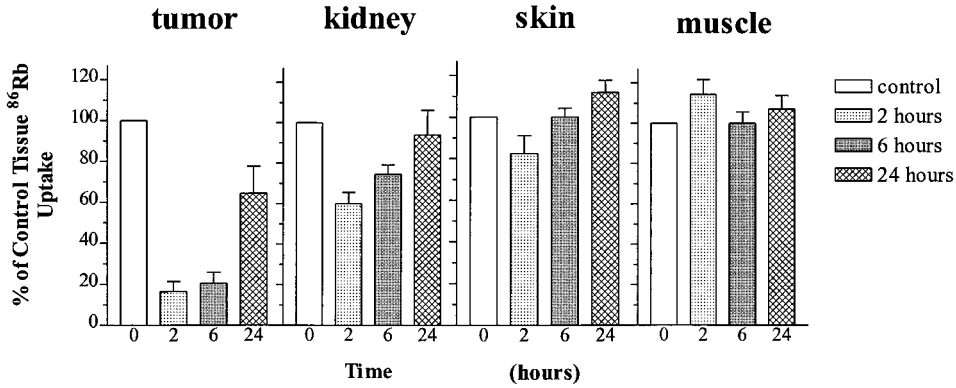

Fig. 4. Changes in blood perfusion as a function of time in normal tissues and Meth-A tumor measured by ^{86}Rb uptake. Values are the means of >10 mice at each time point.

multiple measurements in the same tumor, demonstrated a decreased clearance rate with increasing time after As_2O_3 treatment. This finding corresponded well with the morphological findings. Rubidium uptake, a single measurement in different animals, yielded decreased blood perfusion relative to controls up to 6 h after As_2O_3 treatment, with some recovery at 24 h. Further analysis of the perfusion data at the 24-h time point showed that perfusion in half of the group of tumors ($n = 6$ of 14) remained low (mean ± SE, 24.4 ± 10.9% of the control tumor) and that perfusion in the other tumors ($n = 8$) recovered to near control values (94.2 ± 46.1%). Separate uptake studies with intradermal Meth-A tumors showed results similar to the results for the s.c. tumors. When rubidium activity was assessed from the central and peripheral parts of the tumor separately, the central part of the tumors had a lower uptake than the tumor periphery (37.6 ± 25.1% *versus* 82.2 ± 53.3%). These data indicate that the discrepancy between technetium clearance and rubidium uptake could be explained by the rubidium activity in the peripheral zone of the tumor. Regardless of the 24-h data, As_2O_3 treatment causes a rapid, selective shut down of tumor blood perfusion and induces massive necrosis in the central part of the tumor.

Several other antitumor agents, including combretastatin A-4 (10), colchicine (8), flavone acetic acid (9) and hydralazine (15), have been shown to have their primary effects on tumor vasculature. Their effects are characterized by massive vascular shut down in the central region of the tumor. Flavone acetic acid, hydralazine, and colchicine are either ineffective or toxic in species other than mice. Combretastatin A-4 and new flavone acetic acid derivatives have shown promise in overcoming these past problems. Similarly, As_2O_3 has been used in humans for many years, and preliminary studies on rats in this laboratory indicate that its antitumor effects may not be species specific.[4]

The underlying mechanisms of vascular shut down and central necrosis are not easily explained by our current knowledge of the known biological effects of As_2O_3. However, several possible scenarios are being entertained to illustrate this phenomenon. One possible mechanism is that As_2O_3 can induce selective endothelial cell injury, resulting in vascular shut down to tumor tissue. This mechanism is based on the observation that As_2O_3 is a noncompetitive inhibitor of GTP binding to tubulin (7). Combretastatin A-4 affects normal microtuble function and has been shown to have a greater toxicity toward endothelial cells compared with tumor cells *in vitro* and induces selective vascular shut down in P22 rat carcinosarcoma *in vivo* (10). Another tubulin-binding agent, colchicine, produced hemorrhagic necrosis in experimental tumors (8).

Another possible scenario is tumor necrosis factor-mediated vascular shut down, because the gross observation after As_2O_3 treatment is similar to that seen with endotoxin-induced necrosis. The evidence is strengthened by the observation that cultured human keratinocytes overexpress tumor necrosis factor mRNA after arsenic exposure (16).

Apoptosis or/and necrosis represents a third possible scenario. As_2O_3 triggers apoptosis in APL cells by degrading promyelocytic leukemia/retinoic acid receptor-α fusion protein (6, 17), but it also induces apoptosis in other hematological malignancies and several solid tumor cells *in vitro* (7, 18, 19) at a lower concentration (0.5–5 μM). At higher concentrations (>5 μM), As_2O_3 causes acute necrosis in various cell lines (7). In this scenario, we can speculate massive apoptosis or/and necrosis in the central area of the tumor after As_2O_3 exposure.

The decrease in blood perfusion in the kidney and tumors observed with rubidium uptake suggests a systemic effect of As_2O_3. Changes in blood pressure measured using plethysmography on the tail revealed that the systolic pressure dropped to 50% of the original pressure 1 h after As_2O_3 injection (data not shown), remained low for ~1 h, and recovered gradually to near normal levels by 6 h. As_2O_3-induced systemic hypotension in the presence of high tumor interstitial fluid pressure, especially in the central area of the tumor (20), may be considered a contributing factor in inducing a vascular shut down.

We currently are investigating these possible scenarios, but most of our preliminary data do not unequivocally confirm any of the possible scenarios. Nonetheless, the present findings that As_2O_3 induces a rapid tumor mass reduction with a viable, presumably well-oxygenated peripheral zone of the tumor may provide an opportunity to combine this approach with standard cancer therapy that includes radiation and cytotoxic agents.

[4] Y. S. Lew *et al.*, unpublished results.

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