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The Hepatic Endothelial Carcinogen Riddelliine Induces Endothelial Apoptosis, Mitosis, S Phase, and p53 and Hepatocytic Vascular Endothelial Growth Factor Expression after Short-Term Exposure[1]

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Riddelliine is a naturally occurring pyrrolizidine alkaloid found in certain poisonous rangeland plants of the western United States. In National Toxicology Program 2-year studies, riddelliine induced high incidences of hemangiosarcoma in the liver of F344/N rats (both sexes) and B6C3F1 mice (males). To understand this pathogenesis, we tested short-term effects of riddelliine. Three groups (control; 1.0 mg/kg/day, high dose used in the 2-year study; and 2.5 mg/kg/day) of seven male F344/N rats per group were terminated after 8 consecutive doses and 30 doses (6 weeks, excluding weekends). Serum vascular endothelial growth factor (VEGF), histological, immunohistochemical [factor VIII-related antigen/von Willebrand factor (fVIII-ra/vWf)], VEGF, VEGF receptor-2 (VEGFR2), glutathione *S*-transferase-π, *S*-phase (BrdU), p53, apoptosis, and ultrastructural evaluations were performed on the liver. Following 8 doses of 1.0 and 2.5 mg/kg/day, increased numbers of apoptotic and *S*-phase nuclei appeared in hepatocytes and endothelial cells. Following 30 doses of 1.0 and 2.5 mg/kg/day, hepatocytes exhibited reduced mitosis, fewer *S*-phase nuclei, increased hypertrophy, and fatty degeneration, while endothelial cells showed karyomegaly, cytomegaly, decreased apoptosis, more *S*-phase nuclei, and p53 positivity. Hepatocytes of treated animals expressed higher VEGF immunopositivity. That altered endothelial cells were fVIII-ra/vWf and VEGFR2 positive confirmed their identity. These changes may have promoted hemangiosarcoma development upon long-term exposure through endothelial adduct formation, apoptosis, proliferation of endothelial cells having undamaged and/or damaged DNA, and mutation. Endothelial proliferation may also have been promoted through endothelial arrest at S phase, which was associated with endothelial karyo- and cytomegaly, resulting in hepatocytic hypoxia, triggering VEGF induction.

Key Words: **pyrrolizidine alkaloid; endothelium; hemangiosarcoma; cytotoxicity; vascular endothelial growth factor; VEGFR2; BrdU; factor VIII-related antigen; von Willebrand factor; p53.**

Riddelliine is a naturally occurring pyrrolizidine alkaloid in a class of compounds existing in rangeland plants of the genera *Crotalaria*, *Amsinckia*, and *Sencio* that grow in the western United States. Riddelliine, nominated by the Food and Drug Administration for toxicity and carcinogenicity testing, was mutagenic in *Salmonella typhimurium* strain TA 100 with S9 activation (Chan *et al.*, 1994; NTP, 1993) and induced sister chromatid exchanges in Chinese hamster ovary (CHO) cells with and without addition of the microsomal fraction S9. Riddelliine is metabolically activated by microsomal p450 systems, and the activated metabolite has been identified as dehydroretronecine (DHR) (Yang *et al.*, 2001). Chromosomal aberrations were induced in CHO cells only in the presence of S9 (NTP, 1993). A weak increase in micronucleated erythrocytes was noted in peripheral blood and bone marrow of male mice administered a single high dose of the compound by gavage. Unscheduled DNA synthesis was detected in cultured hepatocytes from rats and mice exposed to riddelliine by gavage.

Riddelliine has been shown to interact with DNA in the presence of S9, forming DNA adducts (Yang *et al.*, 2001). This

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[3] Abbreviations used: AB, automation buffer; bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2′-deoxyuridine; CHO, Chinese hamster ovary; DHR, dehydroretronecine; fVIII-ra/vWf, factor VIII-related antigen/von Willebrand factor; GST, glutathione *S*-transferase; GSTπ, glutathione-*S*-transferase-π; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

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TABLE 1
Summary of the Antibodies and Their Respective Protocols Used in the Evaluation of Riddelliine

Antibody dilution/incubation time[a]	IHC-positive control[b]	HIER[c]	IHC kit	Stain localization
Rabbit anti-factor VIII-ra/vWf 1:300/1 h (Biocare Medical, Walnut Creek, CA)	Normal rat tissue (liver, lung, kidney, intestine, pancreas)	Pepsin digestion[d]	Vector Rabbit Elite Kit (Vector Laboratories)	Cytoplasmic
Rabbit anti-GSTPi 1:500/1 h (Novocastra Laboratories, Newcastle, UK)	Dioxin-treated rat liver containing altered hepatocellular foci	Decloaker[e]	Vector Rabbit Elite Kit (Vector Laboratories)	Cytoplasmic and nuclear
Mouse anti-BRDU 1:50/30 min (BD Biosciences, San Jose, CA)	Intestine from a rat administered BRDU in drinking water	Trypsin digestion[f]	Vector Mouse Standard Kit (Vector Laboratories)	Nuclear
Rabbit anti-p53 1:500/1 h (Novocastra)	Mouse skin papilloma	Decloaker	Goat anti-rabbit for 30 min (Vector Laboratories) Vector Elite Label for 30 min	Nuclear
Goat anti-VEGF 1:1000/1 h (Santa Cruz Biotechnology, Santa Cruz, CA)	Normal rat pancreas and islets of Langerhans[g]	Microwave oven[h]	Goat Elite Kit	Cytoplasmic
Rabbit anti-Flk-1 1:200/30min (Santa Cruz Biotechnology, Santa Cruz, CA)	Adult rat lung	Trilogy[i]	Vector Rabbit Elite Kit (Vector Laboratories)	Cytoplasmic

[a] Source in parentheses.
[b] IHC, immunohistochemistry.
[c] HIER, heat-induced epitope retrieval.
[d] Slides incubated in Carezyme-II Pepsin (Biocare Medical) at 37°C for 5 min.
[e] Slides incubated in 1× citrate buffer, pH 6.0 (Biocare Medical), in a Decloaker (Biocare) for 5 min.
[f] Slides incubated in 0.01% trypsin at 37°C for 3 min, followed by a 30-min incubation in 2 N HCl.
[g] Watanabe *et al.* (2000).
[h] Slides placed in 1× citrate buffer, pH 6.0, and microwaved at 50% power for 5 min repeated for a total of three cycles. Between cycles, 50 ml of fresh citrate buffer added to prevent evaporation of the retrieval solution.
[i] Slides placed in 1× Trilogy (Cell Marque) in pressure cooker for 20 min.

work suggested that riddelliine induces liver tumors in rats through a genotoxic mechanism, and the eight DHR-derived DNA adducts are likely to contribute to liver tumor development. In purified liver parenchymal and endothelial cells from female rats treated with 1 mg/kg/day and male mice treated with 3 mg/kg/day for 2 weeks, the adduct levels at all time points were significantly greater in nonparenchymal cells than hepatocytes (NTP, 2001).

In National Toxicology Program 2-year studies with riddelliine administered by gavage, high incidences of hemangiosarcoma were induced in the liver of male and female rats and male mice (NTP, 2001). In rats, the incidences at 1.0 mg/kg/day were 43 of 50 in males and 38 of 50 in females vs 0 of 50 in the respective vehicle controls. In mice, the incidences at 3.0 mg/kg were 31 of 50 in males, 0 of 50 in females, and 2 of 50 in the control males. Although no hemangiosarcoma occurred, other treatment-related vascular lesions were noted in female mice administered the same dose, including chronic arteritis in ovaries, uterus, kidneys, mesentery, and small and large intestines. In the same studies, the incidences of hepatocellular neoplasms occurred with negative trends in male mice and were significantly decreased in females. The incidences of hepatocellular adenoma in male and female rats were significantly increased (0/50 and 4/50 in males and 1/50 and 7/50 in females, in the control and high-dose groups, respectively). Other pyrrolizidine alkaloids, such as lasiocarpine and clivorine, are also associated with the induction of hemangiosarcoma in rat liver (Kuhara *et al.*, 1980; Rao and Reddy, 1978).

That pyrrolizidine alkaloids are cytotoxic specifically to endothelial cells has been determined (Jones and Rabinovitch, 1996; Meyrick and Reid, 1982; Taylor *et al.*, 1997; Thomas *et al.*, 1998). Thrombi may be formed at the site of endothelial cell damage induced by these compounds (Reindel *et al.*, 1991). Thrombin and platelet activation causes the release of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), both of which exert mitogenic activity upon endothelial cells, inducing angiogenesis (Maragoudakis *et al.*, 2000). Considerable evidence exists that VEGF is a major tumor angiogenic factor, and VEGF mRNA is upregulated in numerous tumor types (Ferrara, 2001). Although VEGF is produced by several cell types, one of its receptors, VEGFR2 (VEGF receptor 2), or Flk-1 [fetal liver kinase/KDR (kinase insert domain-containing receptor)], is localized to endothelial cells and thus provides an additional marker for endothelial cell-specific staining (Millauer *et al.*, 1993).

Our working hypothesis was that the active metabolite of riddelliine, dehydroretronecine, interacts with endothelial DNA, causing damage that, at a certain threshold, leads to

TABLE 2
Effects of Riddelliine Administered by Gavage on Body Weight and Mean Absolute Liver Weight of Male Rats

Days of dosing	8			30		
Concentration of riddelliine mg/kg/day	0	1	2.5	0	1	2.5
Mean final body weight (g)	188.63 ± 6.70	178.79 ± 7.60	177.54 ± 7.74	279.19 ± 12.37	279.85 ± 11.63	270.59 ± 9.86
Absolute liver weight (g)	8.26 ± 0.42	7.96 ± 0.63	7.67 ± 0.36	12.79 ± 0.79	14.18 ± 0.72*	13.91 ± 0.90*

Note. Values are means ± SD with $n = 7$ animals per group.
* $p < 0.001$.

apoptosis, which is followed by endothelial cell proliferation of DNA-damaged cells, "fixation" of the adducts into mutations, and eventual culmination in hemangiosarcoma at 2 years. In an effort to understand the pathogenesis of hemangiosarcoma, we tested the capacity of riddelliine to damage the endothelial cells and to induce cellular proliferation and p53 accumulation in male rats. The same and a slightly higher dose than that used in the 2-year bioassay were incorporated into a short-term investigation, up to 6 weeks of administration.

Immunohistochemical stainings were performed for factor VIII-related antigen/von Willebrand factor (fVIII-ra/vWf) and VEGFR2 endothelial cell markers (Hieken *et al.*, 2001; Torres *et al.* 1997; Yamane *et al.*, 1994).

MATERIALS AND METHODS

Chemical. Riddelliine (CAS No. 23246–96-0), supplied by Dr. Russell J. Molyneux (Western Regional Research Center, Agriculture Research Service, U.S. Department of Agriculture), was extracted and purified from Riddell's groundsel (*Senecio riddellii*) collected from rangeland (Molyneux *et al.*, 1979). Cumulative analytical data based on magnetic resonance spectroscopy, thin-layer chromatography, and high-performance liquid chromatography using solvent systems indicated a purity of 92% riddelliine, 5% retrorsine, and 1.4% seneciphylline.

Animals. Male F334/N rats were obtained from Taconic (Germantown, NY) at 4–5 weeks of age. After quarantine for 11–12 days, the animals were randomly assigned to riddelliine-exposure or control groups and housed three or four per cage in polycarbonate cages. Pelleted food (NTP-2000, Zeigler Bros., Gardners, PA) and water were available *ad libitum*. The animals were observed twice daily and weighed weekly. Animal husbandry and handling were conducted in accordance with the NIH Guidelines (Grossblatt, 1996).

Doses and study design. Dose formulations were prepared by dissolving the appropriate quantity of riddelliine in corn oil. Dose levels were 0 (vehicle control), 1.0, or 2.5 mg/kg/day administered by gavage to six groups of seven male Fischer rats. Animals from each dosage group were euthanized after 8 consecutive daily doses or 30 doses given for 6 weeks, 5 doses/week, excluding weekends.

Necropsy and tissue handling. At euthanasia rats were anesthetized using 70% CO_2. Blood was then collected without anticoagulant by cardiac puncture for VEGF determination. The blood was allowed to clot at room temperature and was centrifuged. The serum was separated, frozen in liquid nitrogen, and stored at −70°C until analysis. Necropsy was performed, and the liver was weighed. Samples of liver tissue for transmission electron microscopy were removed as quickly as possible from the animals, placed immediately in McDowell–Trump fixative (McDowell and Trump, 1976) on dental wax, and cut into pieces approximately 1 mm^3. The tissues were then placed in vials containing the same fixative for storage. The remainder of the liver and the duodenum were fixed in formalin for 24 h and then transferred to 70% alcohol. For light microscopic examination, tissues were processed, embedded in paraffin, sectioned at 5–6 μm, and stained with hematoxylin and eosin.

To better address the objectives of our investigation, we distinguished between parenchymal (hepatocytes, composing about 80% of the total cellular

TABLE 3
Effects of Riddelliine Administered by Gavage on Incidence and Severity of Histopathology in the Liver of Male Rats

Days of dosing	8			30		
Concentration of riddelliine mg/kg/day	0	1	2.5	0	1	2.5
Microscopic diagnosis						
Mitoses, hepatocytes						
Not seen	0	6	7	2	7	7
Minimal	1	1	0	4	0	0
Mild	4	0	0	1	0	0
Moderate	2	0	0	0	0	0
Vacuolation, hepatocytes						
Not seen	7	7	7	7	5	3
Minimal	0	0	0	0	2	1
Mild	0	0	0	0	0	3
Hypertrophy, hepatocytes						
Not seen	7	7	7	7	2	0
Minimal	0	0	0	0	5	4
Mild	0	0	0	0	0	3
Apoptosis, nonparenchymal cells						
Not seen	1	2	0	2	3	6
Minimal	6	4	1	5	3	1
Mild	0	1	2	0	1	0
Moderate	0	0	3	0	0	0
Marked	0	0	1	0	0	0
Cytomegaly and karyomegaly, nonparenchymal cells						
Not seen	7	7	1	7	7	0
Mild	0	0	6	0	0	6
Moderate	0	0	0	0	0	1

Note. $n = 7$ animals per group. Apoptosis grading scale/slice of liver (20×): not seen = 0; minimal = 1–2; mild = 3–6; moderate = 7–10; and marked > 10. Mitosis grading scale/10 fields (40×): none seen; minimal = 1–3; mild = 4–6; moderate = >7.

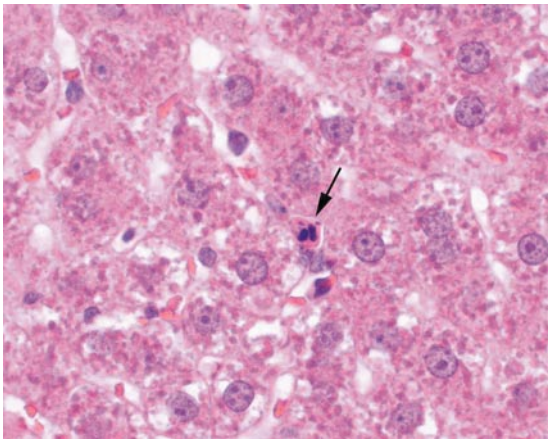

FIG. 1. Endothelial cell apoptosis (arrow) in the liver following eight consecutive daily doses of 2.5 mg/kg/da riddelliine. Magnification 400× (H & E).

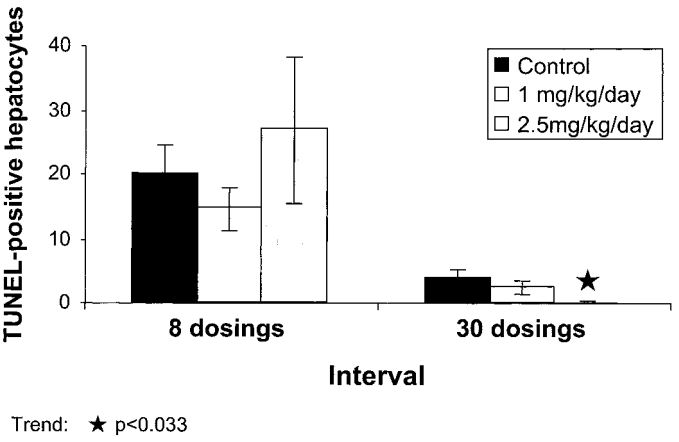

FIG. 3. Apoptosis evaluation in the hepatocytes following 8 consecutive daily and 30 (excluding weekends) doses. The data presented are means ± SE.

volume in the liver) and nonparenchymal (bile duct, endothelial, Kupffer, stellate, and a majority of endothelial sinusoidal) cells (Evans and Brian, 1997). Both cell types were identified using morphologic and localization criteria. Parenchymal cells are arranged in plates and have round nuclei; nonparenchymal cells, frequently associated with sinusoids, display elongated, oval, or irregular nuclei. Due to the immunopositivity of the affected nonparenchymal cells for the endothelial-specific markers fVIII-ra/vWf and VEGFR2, we considered these cells to be endothelial. For the immunohistochemical evaluation, serial sections from paraffin-embedded liver were used. All slides in this study were initially evaluated by a "blind" method (i.e., evaluators did not know identities of animal number and treatment group).

Determination of serum VEGF. Concentrations were determined using a quantitative polyclonal sandwich immunoassay (Quantikine M mouse VEGF immunoassay, R & D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol. The reliability of this kit has been verified previously in the measurement of rat VEGF in culture media (Seko *et al.*, 1999; Sugishita *et al.*, 2000).

Immunohistochemical staining. Immunohistochemistry was performed using the avidin–biotin peroxidase method (Hsu and Raine, 1981). Two 6-μm serial sections were used for each stain: fVIII-ra/vWF, VEGF, VEGFR2, glutathione *S*-transferase-π (GSTπ), 5-bromo-2′-deoxyuridine (BrdU), and p53 (Table 1). Section 1 was incubated with primary antibody. The serial section was stained with the respective negative control serum in which the primary antibody was made to account for any nonspecific staining from the primary. All negative controls (normal rabbit, mouse, and goat) of sera were commercially produced (Jackson Immunoresearch Labs, West Grove, PA). For all stains, slides were deparaffinized, hydrated through a series of graded alcohols to 1× Automation Buffer (AB) (Biomeda, Foster City, CA), and then blocked for endogenous peroxidase acitivity with 3% H_2O_2. Heat-induced epitope retrieval was required for all stains. Incubations were conducted in a humidified chamber at room temperature. A block for endogenous biotin (Avidin–Biotin blocking kit, Vector Laboratories, Burlingame, CA) was completed for all stains. Vector Elite kits were used according to the manufacturer's recommendations for the blocking serum, the biotinylated secondary, and the avidin–biotin enzyme complex, with the exception of p53, for which the concentration of the blocking antibody was increased (10%) and the secondary antibody was titrated to minimize nonspecific background staining. The antibody complex was made visible using Dako Liquid DAB (Dako Corporation, Carpinterina, CA) in a process that required 6 min development in the dark. Slides were counterstained with Harris Hematoxylin (Harelco, Gibbstwon,

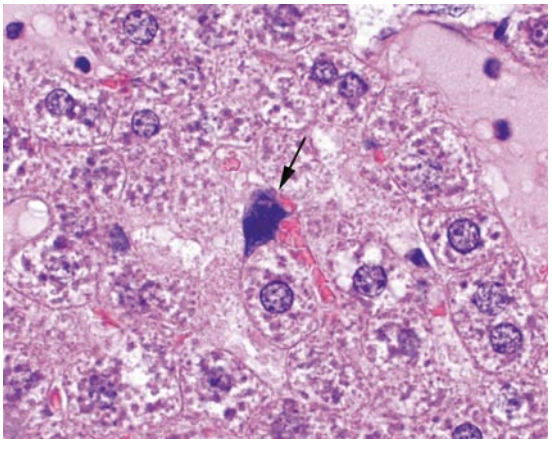

FIG. 2. Endothelial-cell karyomegaly and cytomegaly (arrow) in the liver following 30 daily doses (excluding weekends) of 2.5 mg/kg/day riddelliine. Magnification 400× (H & E).

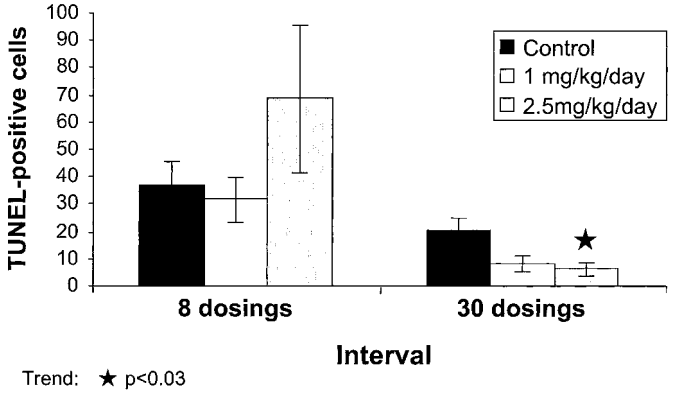

FIG. 4. Apoptosis evaluation in the endothelial cells following 8 consecutive daily and 30 (excluding weekends) doses. The data presented are means ± SE.

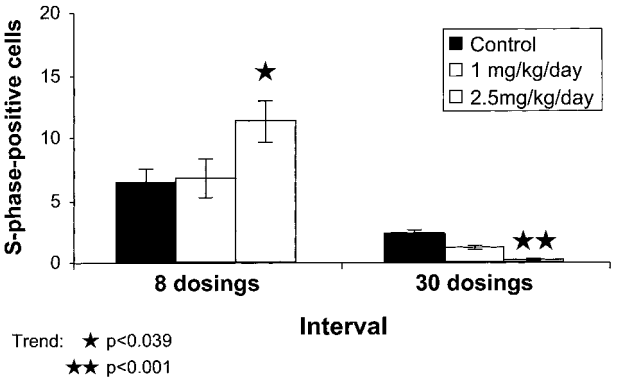

FIG. 5. *S*-phase evaluation in the hepatocytes following 8 consecutive daily and 30 (excluding weekends) doses.

NJ), rinsed in 1× AB, and coverslipped with Permount (Surgipath, Richmond, IL).

Immunoreactivity for fVIII-ra/vWF, VEGF, VEGFR2, and p53 was scored by semiquantitative assessment of the relative area of tissue or the relative number of relevant cells in the section expressing immunopositivity, as follows: no immunoreactivity detected = 0, equal to or less than 10% nuclear or cytoplasmic reactivity = 1 (minimal), greater than 10% up to 25% = 2 (mild), greater than 25% up to 50% = 3 (moderate), and greater than 50% = 4 (marked).

S-*phase (BrdU) evaluation.* The *S*-phase nonparenchymal cell labeling was evaluated using the labeling agent BrdU administered in drinking water, as described by Ton *et al.* (1997). BrdU was given at a concentration of 80 mg/100 ml during the last 3 days before euthanasia. Cells that had incorporated BrdU were identified by a brown to black nuclear pigment. Every slide contained a section of small intestine and three sections of liver. Positive staining for BrdU was confirmed by examining the duodenum, a tissue with a normally high rate of cellular proliferation. The number of BrdU-positive hepatocytes was scored within five randomly selected fields at 200× by light microscopy. The number of unlabeled hepatocytes was also scored, and the BrdU-labeling index was calculated as the percentage of BrdU-labeled hepatocytes. A total of at least 1500 hepatocytes was scored per animal. Nonparenchymal cells were scored separately within the same fields evaluated for hepatocellular proliferation. Because unstained nonparenchymal cells were difficult to discern, only BrdU-labeled cells were counted. Thus, the labeling index was calculated as the total number of BrdU-labeled nonparenchymal cells counted within the five fields scored per animal.

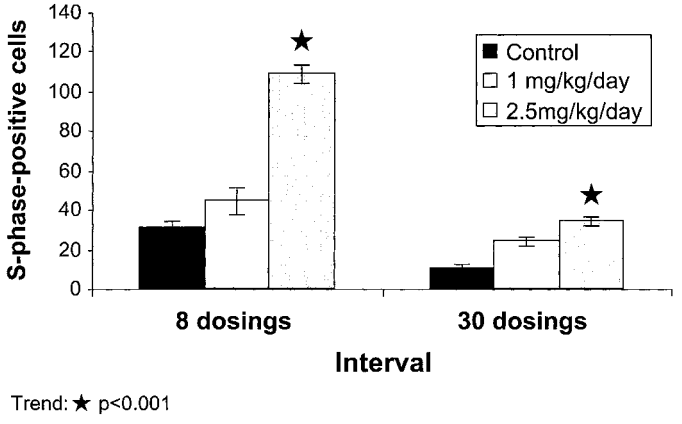

FIG. 6. *S*-phase evaluation in endothelial cells following 8 consecutive daily and 30 (excluding weekends) doses.

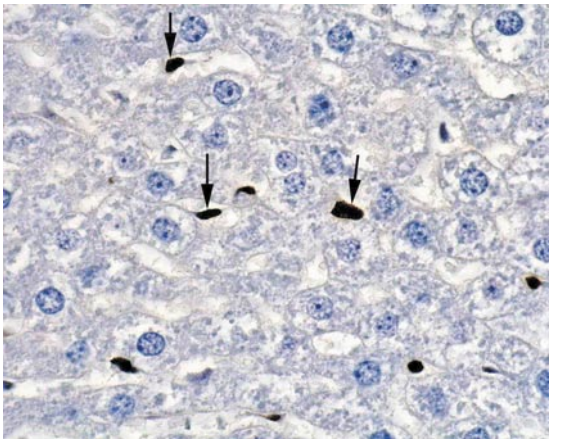

FIG. 7. *S*-phase nuclear immunohistochemical positivity of karyomegalic endothelial cell (arrows) in the liver following 30 daily doses (excluding weekends) of 2.5 mg/kg/day riddelliine. Magnification 400× (BrdU).

Apoptosis evaluation. ApopTag *In Situ* Apoptosis Detection Kit (Intergen Company, Purchase, NY) was used to detect apoptotic cells by the TUNEL assay (Gavrieli *et al.*, 1992). The reagents provided in the kit are designed to label the free 3′OH DNA termini *in situ* with chemically labeled and unlabeled nucleotides. Apoptotic cells were identified by a brown to black nuclear pigment. One slide from every animal was stained for apoptosis by the TUNEL assay. One negative control slide was prepared from every animal. Every slide contained a section of small intestine and three sections of liver. Positive staining for apoptosis was confirmed by examining the duodenum, a tissue with a normally high rate of epithelial cell apoptosis at the villus tips. The total number of TUNEL-positive cells located within the central section of the three on the slide was scored according to standard NTP practice.

Electron microscopic examination. Liver samples (1 mm^3) were processed into Spurr's resin for ultrastructural examination. Thick (1 μm) sections were cut, stained with toluidine blue, and evaluated to select representative

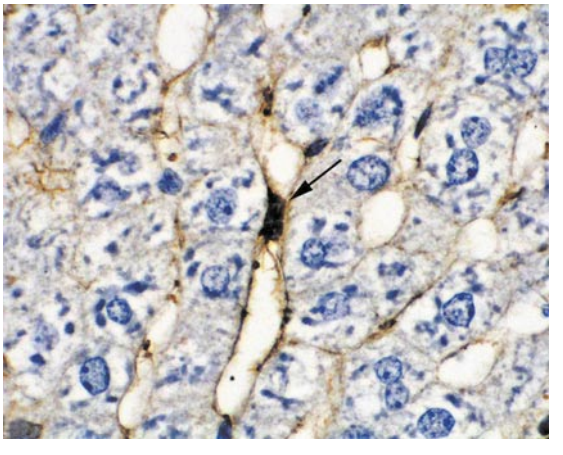

FIG. 8. Factor VIII-related antigen/von Willebrand factor (fVIII-ra/Vwf) cytoplasmic immunohistochemical positivity of karyomegalic, cytomegalic endothelial cell (arrow) in the liver following 30 daily doses (excluding weekends) of 2.5 mg/kg/day riddelliine. Magnification 400×.

areas for examination. Thin sections (approximately 90 nm) of selected blocks were cut, mounted on 150-mesh copper grids, stained with 5% methanolic uranyl acetate and Reynold's lead citrate, and examined in a Zeiss EM10C transmission electron microscope. Representative electron micrographs were taken of each sample, and significant features were described and recorded.

Statistical methods. Mean liver weights of the dosed animals were compared with those of the control animals after adjusting for body weights using the Dunnett–Hsu test procedure available within PROC GLM in the statistical software SAS (SAS, 2000). Dose-related trends in apoptosis, S-phase, and VEGF data were analyzed using a general methodology introduced by Peddada *et al.* (2001). This methodology evaluates the trend in mean response over dose using isotonic regression. The test statistic is defined to be the difference between the largest isotonized mean response and the smallest isotonized mean response. The *p* values for the test are derived using bootstrap methodology. A total of 5000 bootstrap samples were obtained under the null hypothesis of no difference between the dose groups. Corresponding to each of the 5000 bootstrap samples, the above-described test statistic was calculated, thus obtaining the null distribution of our test statistic, which was then used for obtaining the *p* value of the test procedure.

RESULTS

Serum VEGF. Analysis of the serum VEGF demonstrated no effect of treatment (mean values after 8 consecutive days of dosing 89.7, 78.5, and 97.7 pg/ml and after 30 doses 84.7, 90.1, and 96.1 pg/ml, respectively, for control, 1.0, and 2.5 mg/kg/day).

Body and liver weight. No treatment-related effect on body weight was noted. Following 8 dosings (Table 2), no statistically significant differences between 1.0 mg/kg and control ($p = 0.99$) and between 2.5 mg/kg and control ($p = 0.62$) were noted in liver weight. Data from animals receiving 30 doses of the compound indicated that in both dose groups–1.0 and 2.5 mg/kg–liver weights were significantly increased ($p < 0.0001$) compared to the controls.

Histopathological findings. These results are presented in Table 3. In the liver of rats euthanized after 8 high doses, changes consisted of increased apoptosis (Fig. 1) and mild nuclear enlargement (karyomegaly) in the nonparenchymal cells. The number of hepatocellular mitoses was reduced in both dosed groups. In the liver of rats euthanized after 30 doses, essentially no morphologic evidence of apoptosis, seen in the earlier-sacrificed animals, was found. Other dose-related changes included reduction of hepatocellular mitoses, minimal to mild hepatocellular hypertrophy, and centrilobular fatty vacuolization. Relatively frequent (i.e., more than half of the cells in sections), mild to moderate endothelial cell enlargement (cytomegaly), and irregularly shaped, karyomegalic nuclei (Fig. 2) were present in the high-dose animals only.

Apoptosis data. In the liver of rats euthanized after 8 high doses, a slight but not significant increase in apoptosis in parenchymal as well as nonparenchymal cells in the riddelliine-treated animals compared to controls was observed (Figs. 3 and 4). In the liver of rats euthanized after 30 high doses, a statistically significant decrease was noted in the parenchymal ($p < 0.033$) and nonparenchymal cells ($p < 0.03$) compared to controls. In general, apoptosis values in the control and dosed groups at 30 doses were lower than those at 8 doses. The decrease appeared to be age related.

S-*phase analysis.* As shown in Figs. 5 and 6, in the liver of rats euthanized after 8 doses, a statistically significant increase in *S*-phase parenchymal ($p = 0.039$) and nonparenchymal cells ($p < 0.001$) was noted in riddelliine-treated animals. In the liver of rats euthanized after 30 doses, a statistically significant decrease in S phase in the hepatocytes ($p < 0.001$) was noted, while an opposite statistically significant effect of increase was noted in the nonparenchymal cells ($p < 0.001$) (Fig. 7). The numbers of *S*-phase cells in the hepatocytes and nonparenchymal cells were lower in the older rats than in the younger rats.

Immunohistological evaluation. The immunohistochemical markers revealed distinct patterns of localization. Staining for fVIII-ra/vWF, which is widely used as a marker of endothelial cells *in vitro* and on histological sections, revealed a moderate cytoplasmic expression in the nonparenchymal cells (Fig. 8), suggesting that the altered nonparenchymal cells are endothelial. Endothelial cell identity was further confirmed by moderate positive VEGFR2 staining in the cytoplasm of the cytomegalic sinusoidal cells (Fig. 9). Staining for p53 protein indicated minimal (equal to or less than 10% of the cells in section showing reactivity) nuclear positivity in the nonparenchymal cells of liver from all animals following 8 and 30 doses of riddelliine (Fig. 10). GSTπ is a physiological metabolic barrier and an inducible phase II detoxifying enzyme suggested to decrease the responsiveness of reactive oxygen species and organic electrophilic compounds (Nyska *et al.*, 2001); no GSTπ immunolabeling was seen in the nonparenchymal cells or hepatocytes of control and treated animals. Staining for VEGF protein indicated minimal cytoplasmic positivity in hepatocytes of control animals. Increased expression (mild to moderate) of immunopositivity was noted in groups of hepatocytes in animals following 8 and 30 doses of riddelliine (Figs. 11A and 11B). In some of the animals, the grade of staining was greater in the animals dosed for 8 days than in those administered 30 doses. No apparent lobular predilection for staining was noted.

Transmission electron microscopic observations. Findings (Table 4 and Figs. 12A–D) included endothelial cell swelling, hypertrophy, and hyperplasia. These changes in endothelial cells were observed in both the 8-day and 30-dosage animals given 2.5 mg/kg. Karyomegaly and apoptosis were less frequently observed in treated rats.

DISCUSSION

Our study demonstrates that, following 8 doses of 1.0 or 2.5 mg/kg/day, an increase in apoptosis and numbers of *S*-phase

TABLE 4
Effects of Riddelliine Administered by Gavage on Ultrastructure in the Liver of Male Rats

Days of dosing	8			30		
Concentration of riddelliine mg/kg/day	0	1	2.5	0	1	2.5
Ultrastructural findings						
Endothelial apoptosis	0	1	0	0	1	0
Endothelial swelling	0	1	0	1	3	3
Endothelial hypertrophy	0	1	1	0	0	2
Endothelial degeneration	0	1	0	0	1	2
Kupffer cell degeneration	0	1	0	0	2	0
Lipid in space of Disse	0	0	3	0	1	2
Endothelial cell karyomegaly	0	0	1	0	2	2
Endothelial hyperplasia	0	0	2	0	0	0

Note. $n = 3$ animals per group examined by electron microscopy.

nuclei occurred in nonparenchymal (i.e., endothelial) cells. Following 30 doses at 1.0 and 2.5 mg/kg/day, nonparenchymal cells exhibited karyomegaly, cytomegaly, decreased apoptosis, more *S*-phase nuclei, and, sporadically, p 53 nuclear positivity. That morphologically altered nonparenchymal cells were fVIII-ra/vWF and VEGFR2 positive indicated that they were endothelial cells. Accordingly, we are suggesting a possible role for endothelial apoptosis, proliferation, and mutation (Chan *et al.*, 1994; NTP, 1993) in the process of endothelial carcinogenesis (Fig. 13).

Interestingly, the administration of riddelliine for 2 years in the NTP studies was associated with arteritis development only in females, involving multiple organs, but not the liver. Why the female mice were the only animals that did not develop hemangiosarcoma in these experiments is not clear. Further investigations may clarify whether the mechanism of riddelliine-induced arteritis observed in the female mice may be different (i.e., autoimmune) from that involved in vascular tumor formation in rats and male mice.

The p53 tumor-suppressor protein plays a central role in maintaining genomic stability by modulating cellular responses to cytotoxic stresses and contributing to both cell-cycle arrest and programmed cell death (Hussain and Harris, 1998; Kirsch and Kastan, 1998; Schwartz and Rotter, 1998). Mutation in the p53 tumor-suppressor gene is common in many human and experimental cancers, including hemangiosarcoma, as well as preneoplastic lesions (Batheja *et al.*, 2000; Hong *et al.*, 2000; Hussain and Harris, 1998; Nylander *et al.*, 2000; Smith *et al.*, 1998; Soini *et al.*, 1995). Detection of p53 mutation at an early stage of chemical exposure is of particular importance, since it may suggest that p53 assessment can serve as biomarker for carcinogenesis (Batheja *et al.*, 2000; Castelli *et al.*, 2001; Downing *et al.*, 2001; Kawasaki *et al.*, 2001). The present study demonstrated that p53 mutation occurred early, after 8days of exposure to riddelliine.

Recent studies demonstrated the formation of riddelliine-derived DNA adducts (Yang *et al.*, 2001). Our data suggest that, following riddelliine DNA-adduct formation, mutations, including p53, may have promoted hemangiosarcoma development through the proliferation of endothelial cells that have undamaged and/or damaged DNA. Compensatory DNA synthesis or regenerative hyperplasia may create the opportunity to "fix" the DNA adducts into stable mutations (Dorchies *et al.*, 2001; Masuhara *et al.*, 1996; Tombolan *et al.*, 1999).

Our study demonstrated the presence of mild to moderate endothelial cell enlargement, or cytomegaly, and irregularly shaped, karyomegalic nuclei. Wilson *et al.* (2000) reported that monocrotaline induced continued DNA synthesis and concentration-dependent cell-cycle arrest in cultures of human pulmonary artery endothelial cells. The exposed cells underwent a process of multiplication of chromosomal copies, defined as endopolyploidy, with nuclear and cytoplasmic gigantism. A direct correlation between cytoplasmic volume and nuclear DNA content was suggested. Our data indicated that similar events might have occurred with riddelliine exposure.

VEGF is a secreted endothelial cell-specific mitogen, induced by hypoxia (Shweiki *et al.*, 1992). Investigators have proposed that endothelial enlargement causes local perfusion impairment, resulting in hepatocytic hypoxia, triggering VEGF induction and angiogenesis stimulation (Rosmorduc *et al.*, 1999). Thrombi may be formed at the site of endothelial cell damage induced by pyrrolyzidine alkaloids (Lalich *et al.*, 1977; Meyrick and Reid, 1982; Reindel *et al.*, 1991). Thrombin and platelet activation causes the release of VEGF, bFGF, and other growth factors, e.g., platelet-derived endothelial cell growth factor, thrombospondin, and platelet factor 4 (Maragoudakis *et al.*, 2000; Verheul and Pinedo, 1998; Waltham *et al.*, 2000). Induction of systemic hypoxia in a mouse model was associated with redistribution rather than general induction of VEGF within the hepatocytes, increased expression around central veins, and diminished occurrence around periportal fields (Marti and Risau, 1998). Transplantation of VEGF-expressing myoblasts into the myocardial walls of mice results in the formation of hemangioma (Carmeliet, 2000; Lee *et al.*, 2000). We suggest that the riddelliine-associated endothelial karyo- and cytomegaly may have induced hepatocytic hypoxia, triggering VEGF induction and further promotion of endothelial proliferation, although no increase in the serum levels of VEGF was detected. This notion was supported by data from NTP 13-week studies of riddelliine (NTP, 1993). In the 10 mg/kg-dosed animals, coagulative changes or dropout of hepatocytes, primarily in centrilobular areas, with associated congestion and/or hemorrhage were noted. Hepatocytic necrotic and congestive changes in association with enlarged endothelial cells may suggest that sinusoidal circulatory disturbances

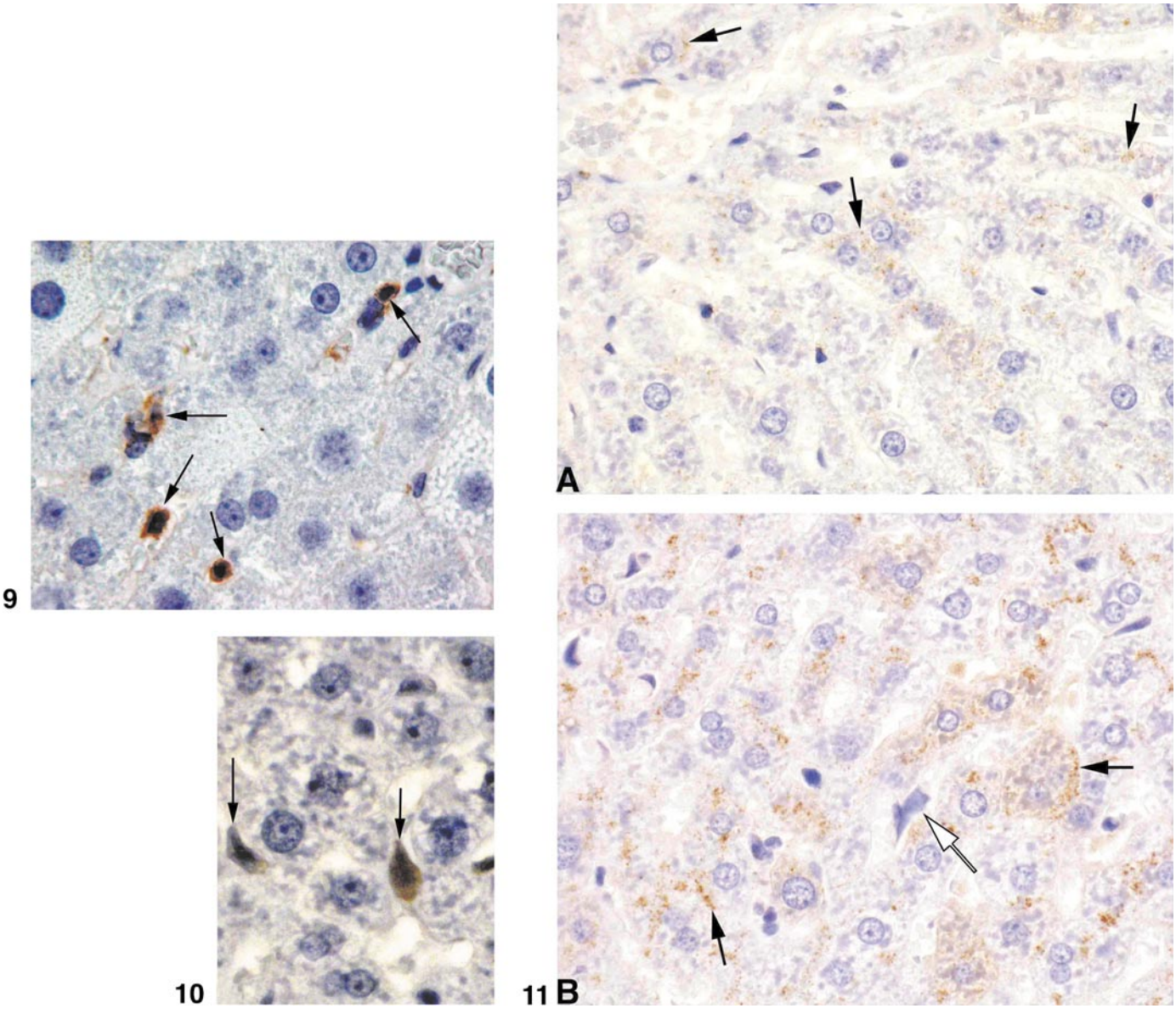

FIG. 9. VEGF-receptor 2 (VEGFR2) cytoplasmic immunohistochemical positivity of karyomegalic endothelial cell (arrows) in the liver following 30 consecutive daily doses of 2.5 mg/kg/day riddelliine. Magnification 600×.

FIG. 10. p53 Nuclear immunohistochemical positivity of karyomegalic endothelial cell (arrow) in the liver following eight consecutive daily doses of 2.5 mg/kg/day riddelliine. Magnification 600×.

FIG. 11. Vascular endothelial growth factor (VEGF) cytoplasmic immunohistochemical positivity in the (A) liver of a control rat and (B) liver of a rat following 30 daily doses (excluding weekends) of 2.5 mg/kg/day riddelliine. Note that the hepatocytes express immunopositive granules (dark arrows) in the cytoplasm–minimal in the control rat in contrast to mild in the riddelliine-treated rat. Open arrow indicates endothelial cell karyomegaly and cytomegaly. Magnification 400× (H & E).

caused hypoxemia, affecting the viability of the hepatocytes, with eventual up-regulation of VEGF synthesis in neighboring unaffected hepatocytes.

Glutathione *S*-transferases (GSTs) are multiple enzymes involved in phase II detoxification and chemical activation. GSTs decrease reactivity of electrophiles and detoxify endogenous products of lipid peroxidation by catalyzing the nucleophilic conjugation with glutathione (Armstrong, 1997; Eaton and Bammler, 1999). The specific toxicity of monocrotaline to hepatic sinusoidal cells was suggested to be caused by profound glutathione depletion (DeLeve *et al.*, 1996). The lack of any change in GSTπ expression in our experiment suggests that this pathway was not involved in this relatively short-term exposure.

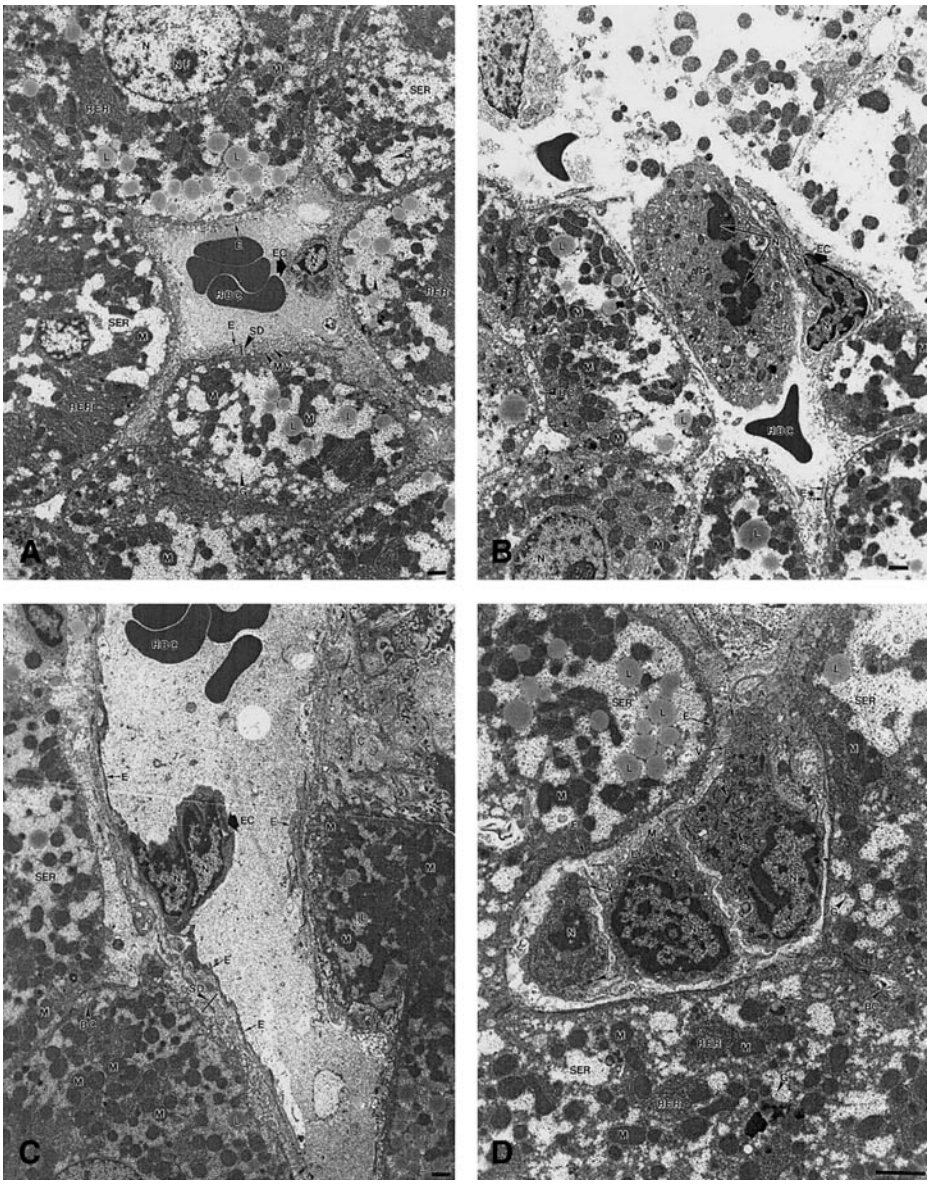

FIG. 12. Electron micrographs. (A) Liver section of a control rat following 30 daily doses (excluding weekends) of the vehicle control. In the center of this micrograph, hepatocytes surround a sinusoid lined by an endothelial cell. Original magnification 7530×. (B) Liver section of a rat following eight consecutive daily doses of 2.5 mg/kg/day of riddelliine. A large sinusoid is near the center of this micrograph. Along the right edge of the sinusoid, an endothelial cell is distinguished by a crescent-shaped nucleus. Adjacent this endothelial cell within the sinusoidal lumen is a large endothelial cell containing a nucleus that is electron dense and fragmented; along the cell's plasma membrane, there are a number of micropinocytotic vesicles that are distinguishing features of endothelial cells. Original magnification 7530×. (C) Liver section of a rat following 30 doses (excluding weekends) of 1.0 mg/kg/day of riddelliine. A binucleated endothelial cell lines the sinusoid; the nuclei are somewhat irregular in size and shape. Original magnification 10,200×. (D) Liver section of a rat; the same treatment as described in C. In the lumen of a large sinusoid, three cells show features consistent with those of endothelial cells. *The larger cell on the right contains numerous electron-dense, rod-shaped microtubulated bodies. The cell on the left has a shrunken nucleus containing coarsely clumped chromatin. Original magnification 7530×. AP, apoptotic cell; BC, bile canaliculus; D, degenerate cell; E, endothelium; EC, endothelial cell; G, glycogen; L, lipid droplet; LY, lysosome; M, mitochondria; MV, microvilli; N, nucleus; NU, nucleolus; RBC, erythrocyte; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; SD, space of Disse; V, micropinocytotic vesicles (caveolae).

In conclusion, the active metabolite of riddelliine, dehydroretronecine, interacts with endothelial DNA (Yang *et al.*, 2001), causing damage to hepatic endothelial cells, including apoptosis, karyomegaly, cytomegaly, increases in their mitoses and numbers of *S*-phase nuclei, and p53 mutation. These enlarged cells expressed immunopositivity for specific markers of endothelial cells. Hepatocytes of riddelliine-treated animals expressed higher VEGF immunopositivity than controls. We

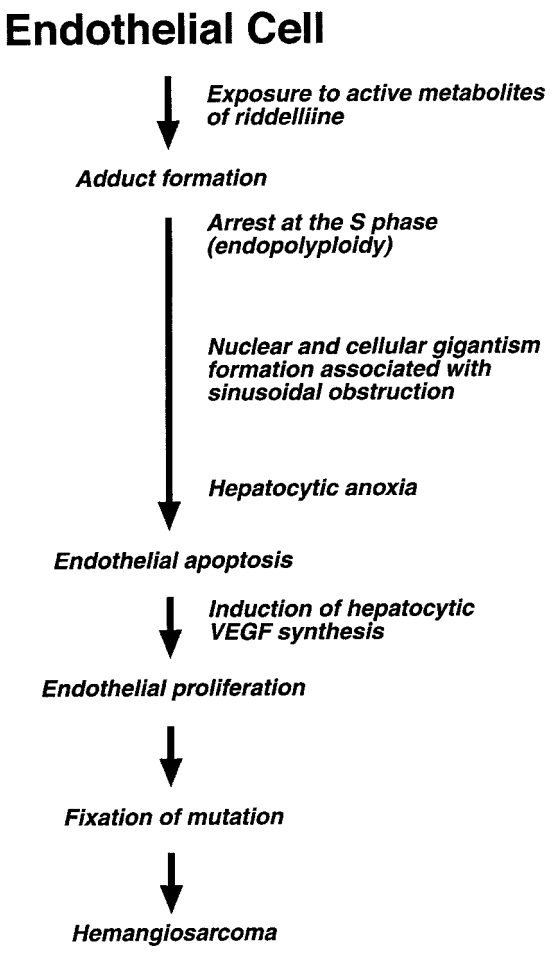

FIG. 13. Proposed mechanism of liver hemangiosarcoma induction following riddelliine gavage-exposure in rats. We propose that the active metabolite of riddelliine, dehydroretronecine, interacts with endothelial DNA, causing damage that, at a certain threshold, leads to apoptosis. Our study demonstrated that riddelliine exposure is associated with specific damage to hepatic endothelial cells, including apoptosis, karyomegaly, cytomegaly, increases in their mitoses and numbers of *S*-phase nuclei, and p53 mutation. Hepatocytes expressed higher VEGF immunopositivity than controls. We propose that the endothelial proliferation and eventual mutation and hemangiosarcoma development were also promoted through VEGF induction.

suggest that the observed changes may have promoted hemangiosarcoma development upon long-term exposure (Fig. 13). We further propose that endothelial proliferation was also promoted through endothelial *S*-phase arrest, which was associated with endothelial karyo- and cytomegaly, resulting in hepatocytic hypoxia, triggering VEGF induction (Fig. 13). The decrease in hepatocytic proliferation is consistent with the known antimitotic effect of pyrrolyzidine alkaloids (Wilson *et al.*, 2000) and may explain the fewer hepatocellular tumors in mice and only modest increase in numbers of these tumors in rats observed in the 2-year exposure.

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