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Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats★

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Abstract

Riddelliine is a naturally occurring pyrrolizidine alkaloid that induces liver hemangiosarcomas in rats and mice. We previously reported higher levels of DNA adducts in liver endothelial cells than in liver parenchymal cells of riddelliine-treated mice and rats [*Cancer Lett.* 193 (2003) 119], suggesting that the tumor specificity is due to higher levels of DNA damage in the cells that form hemangiosarcomas. In the present study, we evaluated the cell-specificity of riddelliine mutagenicity in rat liver. Female transgenic Big Blue rats were treated by gavage with 0.3 mg riddelliine per kg body weight, 5 days a week for 12 weeks. One day after the last treatment, the rats were sacrificed and liver parenchymal and endothelial cell fractions were isolated and purified. DNA was extracted from the cell fractions and used to assay for mutant frequency (MF) in the *cII* transgene. While there was no difference in the *cII*MFs of liver parenchymal cells in control and riddelliine-treated rats, the *cII*MF of liver endothelial cells from treated rats was significantly greater than the *cII*MF of endothelial cells from control rats. Molecular analysis of the mutants in liver endothelial cells indicated that G:C→T:A transversion, a mutation that is characteristically induced by riddelliine, accounted for only 9% of all mutations in control rats, but made up 17% of mutations in treated rats. In contrast, G:C→A:T transition, the major mutation in control rats where it made up 54% of all mutations, was reduced to 40% of mutations in riddelliine-treated rats. These results suggest that the relatively high mutagenicity of riddelliine in rat liver endothelial cells may be partially responsible for the tumorigenic specificity of this agent.

Keywords

Riddelliine; Pyrrolizidine alkaloid; Transgenic rat; *cII* gene; Endothelial cell; Hemangiosarcomas

1. Introduction

Pyrrolizidine alkaloids are probably the most common plant constituents that poison livestock, wildlife, and humans worldwide. Riddelliine, a prototype genotoxic and tumoric

★The views presented in this article do not necessarily reflect those of the Food and Drug administration.

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pyrrolizidine alkaloid, has been isolated from plants of the genera *Crotalaria*, *Amsinckia*, and *Senecio* that grow in the western United States [1]. The riddelliine in these plants appears to find its way into the human food chain since riddelliine residues have been detected in meat, milk, and honey [2]. Due to the potential for human exposure, riddelliine was nominated by the US Food and Drug Administration to the National Toxicology Program (NTP) for genotoxicity and carcinogenicity testing [3].

Riddelliine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α , β -unsaturated double bond linked to the ester group at the C-7 position of the necine base. Riddelliine is metabolized to the major metabolites, 6,7-dihydro-1-hydroxymethyl-5*H*-pyrrolizine (DHP) and riddelliine N-oxide, by mammalian microsomes [1]. ³²P-Postlabeling-HPLC analysis has identified a set of DHP-derived DNA adducts from rat and human liver microsomal metabolism of riddelliine in vitro [4] and in the livers of rats treated in vivo [5]. Riddelliine is also mutagenic and produces a unique mutational spectrum in the liver *cH* gene of Big Blue rats [6]. G:C→T:A transversion was the major type of mutation in rats treated with riddelliine. In the NTP carcinogenicity studies, riddelliine was tumorigenic in both rats and mice, with a major target tissue being the liver. Specifically riddelliine induced a high incidence of liver hemangiosarcomas, which originate from the endothelial cells, and lower incidences of hepatocellular adenoma and carcinoma [3,7].

These observations on the metabolism and target-tissue specificity for riddelliine tumorigenesis suggested that active metabolites of riddelliine interact with endothelial cells in the liver, which causes cell toxicity, followed by compensatory proliferation of DNA-damaged endothelial cells, 'fixation' of the adducts into mutations in these cells, and eventual development of hemangiosarcoma [8]. Consistent with liver endothelial cells being a specific target for riddelliine toxicity, we previously found that riddelliine-treated mice and rats had higher and more persistent DNA adduct levels in liver endothelial cells than in parenchymal cells [9]. However, the comparative mutagenicity of riddelliine in endothelial and parenchymal cells has not been studied.

Transgenic animals have been widely used to study tissue-specific mutagenicity in relationship to the carcinogenicity of chemical agents. Separation of the cell types that make up tissues can also be used to relate cell-specific mutagenicity with carcinogenicity [10,11]. In this study, we treated transgenic Big Blue rats with riddelliine and isolated the liver parenchymal and endothelial cells. We found that mutants were induced in the endothelial cells, the cells from which hemangiosarcomas are derived, but not in the parenchymal cells.

2. Materials and methods

2.1. Chemical, animals, and treatments

Riddelliine (>97% pure by reversed-phase HPLC analysis) was obtained from the NTP and dissolved in 0.9% sodium chloride at a concentration of 0.075 mg/ml. Female Big Blue Fisher 344 transgenic rats (6-weeks-old) were obtained from Taconic (Germantown, NY) through purchase from Stratagene (La Jolla, CA). All animal handling, maintenance, treatment, and sacrifice followed the recommendations of the NCTR Institutional Animal Care and Use Committee. Three Big Blue rats were treated with riddelliine at a concentration

of 0.3 mg/kg body weight by gavage five times a week for 12 weeks. Three vehicle control rats were gavaged with 0.9% sodium chloride using the same schedule as for the ridelliine-treated rats.

2.2. Separation of liver parenchymal and endothelial cells

The separation and purification of the liver parenchymal and endothelial cells of Big Blue rats were conducted according to the procedures described previously [9]. Briefly, treated and control rats were anesthetized with 450 μ l of Nembutal (50 mg/ml) 1 day after the last treatment, and the livers were perfused with collagenase (80 mg/500 ml Hanks' Balanced Salt Solution with calcium and magnesium). The perfused liver was collected into a 100 ml beaker and stirred to disrupt the liver fragments. The cell suspension was filtered using a 150 ml Falcon bench-top filter through a nylon gauze (mesh width, 100 μ m) to remove debris and cell aggregates. The parenchymal (hepatocytes) and non-parenchymal (mainly endothelial) cells were separated by a series of low-speed centrifugations and purified by Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation. The cells pellets were stored at -80°C .

2.3. cII mutant frequency analysis

High-molecular-weight genomic DNA was extracted from rat liver endothelial and parenchymal cells using the RecoverEase DNA Isolation Kit (Stratagene) and stored at 4°C until DNA packaging was performed. The packaging of the phage, plating the packaged DNA samples, and determination of mutant frequency (MF) were carried out following the instructions for the λ Select-*cII* Mutation Detection System for Big Blue Rodents (Stratagene). The shuttle vector containing the *cII* target gene was rescued from total genomic DNA with phage packaging extract (Transpack; Stratagene), and total and mutant phage titers were determined using *Escherichia coli* host strain G1250. To determine the total titer of packaged phages, G1250 bacteria were mixed with 1:3000 dilutions of phage, plated on TB1 plates, and incubated overnight at 37°C (nonselective conditions). For mutant selection, the packaged phages were mixed with G1250, plated on TB1 plates, and incubated at 24°C for approximately 42 h (conditions for *cII*- selection). Assays were repeated until a minimum of 2×10^5 plaque-forming units (pfus) from each sample were examined for mutation. The *cII*MF is defined as the total number of mutant plaques (determined at 24°C) divided by the total number of plaques screened (determined at 37°C).

2.4. Sequence analysis of the cII mutants

The mutants were sequenced using a modification of the methods of Chen et al. [12]. The *cII* mutant plaques were isolated from the selection plates and replated at low density to verify the mutant phenotype. Single, well-separated plaques were cored from these plates and transferred to a microcentrifuge tube containing 100 μ l of sterile distilled water. The tube was heated at 100°C for 5 min and centrifuged at $12,000g$ for 3 min. The *cII* target DNA for sequencing then was amplified by PCR using primers 5'-AAAAAGGGCATCAAATTAACC-3' (upstream) and 5'-CCGAAGTTGAGTATTTTTCCTG-3' (downstream). For PCR amplification, 10 μ l of the supernatant were added to 10 μ l of a PCR Master Mix (Promega, Madison, WI) and the primers. The final concentrations of the reagents were $1 \times$ Taq polymerase reaction buffer, 0.2 μ mol of each primer, 200 μ M of

each dNTP, 1.5 mM MgCl₂, and 0.25 U of *Taq* DNA polymerase. The PCR reaction was performed using a PCR System 9700 (Applied Biosystems, Foster City, CA), with the following cycling parameters: a 3 min denaturation at 95 °C; followed by 35 cycles of 30 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C; with a final extension of 10 min at 72 °C. The PCR products were isolated using a PCR purification kit (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced with a CEQ Dye Terminator Cycle Sequencing Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The primer for *cII* mutation sequencing was the upstream primer used for the PCR.

2.5. Statistical analyses

Analyses were performed using SigmaStat 3.0 (SPSS, Chicago, IL). All of the MF data are expressed as the mean±standard deviation (SD) from three rats per group. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by the Holm-Sidak test. Mutational spectra were compared using the computer program written by Cariello and colleagues [13] for the Monte Carlo analysis developed by Adams and Skopek [14].

3. Results

3.1. *cII* MF in liver parenchymal and endothelial cells

The results of *cII*MF analyses in the different cell populations from the riddelliine-treated and control rats are shown in Table 1. The analyses were repeated one to three times either to confirm the MF or to obtain a minimum of 2×10^5 pfus from each sample examined for mutation. There was no significant difference in the *cII*MFs of liver parenchymal cells from control ($35.2 \pm 5.7 \times 10^{-6}$) and riddelliine-treated rats ($37.5 \pm 9.3 \times 10^{-6}$), while the MF of endothelial cells from riddelliine-treated rats ($67.0 \pm 17.1 \times 10^{-6}$) was significantly higher ($P < 0.05$) than the MF of endothelial cells from control rats ($39.5 \pm 3.8 \times 10^{-6}$). In addition, the mutagenicity of riddelliine was greater in rat liver endothelial cells than in parenchymal cells ($P < 0.01$).

3.2. Types of mutations in the *cII* gene of liver endothelial cells

Riddelliine-induced *cII* mutations in liver endothelial cells were evaluated by DNA sequence analysis of 47 mutants isolated from three treated rats (Table 2). Mutations that were found more than once among the mutants isolated from a single animal were assumed to be siblings resulting from the amplification of a single independent mutation. Accordingly, 42 independent mutations were identified from riddelliine-treated endothelial cells. Table 3 summarizes the types of riddelliine-induced *cII* mutations observed in the liver endothelial cells in comparison to a previously reported mutation spectrum generated by sequencing *cII* mutants from the livers of control female Big Blue rats [6]. Over 80% of the independent mutations from riddelliine-treated liver endothelial cells were base pair substitutions. The percentage of mutations with G:C→A:T transition, the major mutation in both spectra, was lower in the cells from treated rats (40%) than in the control spectrum (54%), and the percentages of mutations with G:C→T:A and A:T→T:A transversion were higher in cells from the treated rats (17 and 10%, respectively) than in livers from control rats (9 and 5%).

The overall pattern of mutations, however, did not differ significantly between the liver endothelial cells from treated rats and the control rats.

4. Discussion

Results from a 2-year NTP carcinogenicity study indicated that riddelliine induced liver tumors in rats and male mice, lung tumors in female mice, and leukemia in rats; liver tumors were the cause of death for the most of these animals [3,7]. Besides tissue specificity, many carcinogens exhibit cell specificity within the target organs [15–17]. Most organs are comprised of several different cell types, which have differences in their biochemistry and biology. Cell specificity is evident in the liver tumorigenicity of riddelliine. For instance, rats administered riddelliine at a dose of 1.0 mg/kg body weight by gavage had a 76–86% incidence of liver hemangiosarcomas, which are derived from endothelial cells, and an 8–14% incidence of hepatocellular adenomas, which are derived from parenchymal cells. Hemangiosarcoma may occur in many tissues, including heart, liver, skeletal muscle, and skin. Hemangiosarcomas typically cause death due to necrosis and hemorrhage in the primary tumors and/or metastases.

The sensitivity of tissues and cell types to the mutagenicity of carcinogens may be an important factor in the tissue- and cell-specificity of tumorigenesis. Parenchymal cells comprise 60–70% of the cells in the liver; the remaining nonparenchymal cell population consists almost entirely of endothelial and Kupffer cells. Previous studies indicate that the toxicity of riddelliine is specifically directed toward liver endothelial cells [9]. Riddelliine is activated by the rat and human hepatic P450 enzymes [4,5], and its active metabolites cause a disproportionate amount of DNA damage [9] and a disproportionate level of S-phase arrest [8] in liver endothelial cells. Following 6-week treatment with 1.0 and 2.5 mg/kg/day riddelliine, endothelial cells showed karyomegaly, cytomegaly, decreased apoptosis, more S-phase nuclei, and p53-positive nuclei, while parenchymal cells exhibited reduced mitosis, fewer S-phase nuclei, increased hypertrophy, and fatty degeneration [8]. Malignant endothelial cells from liver hemangiosarcomas of rats treated with riddelliine exhibited strong nuclear staining for the p53 protein [18]. It was hypothesized that riddelliine-induced DNA-adduct formation in liver endothelial cells, followed by cellular proliferation and fixation of mutations, including mutations in *p53*, may be responsible for hemangiosarcoma development in treated animals. The results of this present study indicate that the mutagenicity of riddelliine in rat liver is greater in endothelial cells than in the parenchymal cells, which is in accord with the more extensive S-phase nuclei in the endothelial cells observed by Naska et al. [8].

There are two possible pathways that lead to riddelliine-induced DNA damage. One is that dehydroriddelliine, a potent electrophile, covalently binds to cellular DNA to form dehydroriddelliine-derived DNA adducts which are subsequently hydrolyzed to DHR-derived DNA adducts. Another is that dehydroriddelliine is unstable and easily hydrolyzed by esterases and/or other hepatic enzymes to form DHR which subsequently binds to DNA [5]. The DNA adduct profiles in purified preparations of endothelial and parenchymal cells are similar to that obtained with DNA from the whole liver and are consistent with the activation of riddelliine through a DHR intermediate [9]. It is not clear that why riddelliine

induces more DNA damage and a higher MF in liver endothelial cells than in parenchymal cells. However, it was demonstrated that the different cell populations in the rat liver possess different capacities for DNA repair after administration of 1,2-dimethylhydrazine, which induces malignant haemangioendotheliomas, due to a preferential accumulation of O^6 -methylguanine in liver endothelial cells and a higher rate of endothelial cell division [15]. Recently, Smith et al. [19] also reported that the replication rates are significantly higher for endothelial cells when a statistical dynamic model was applied to investigate the short-term cellular kinetics induced by riddelliine.

We previously reported that the mutation spectrum in whole liver of rats treated with 1 mg/kg riddelliine was significantly different from the control spectrum, with the greatest change being an increase in G:C→T:A transversion in mutations from treated rats [6]. In the present study, the overall spectra of mutants from endothelial cells of rats treated with 0.3 mg/kg riddelliine was not significantly different from the control spectrum (Table 3), presumably because of the relatively modest increase in MF in the cells from treated rats and the relatively small number of mutants available for analysis. However, cells from rats treated with riddelliine did have an increase in the percentage of G:C→T:A transversion, a mutation that is consistent with the types of DNA adducts formed by riddelliine [6]. In the carcinogenicity bioassay, riddelliine increased the incidence of hemangiosarcoma, but the increase in rats treated with 0.3 mg/kg was small: only three of 50 (6%) female rats treated with this dose had hemangiosarcoma, compared with 38 of 50 (76% incidence) for the 1.0 mg/kg dose group [7]. We hypothesize that the DNA damage induced in the endothelial cells of rats treated with 0.3 mg/kg riddelliine may be repaired or the damaged cells removed with a greater efficiency than at higher doses, resulting in a non-linear induction of liver hemangiosarcoma.

Female rats are considered more suitable than male rats for investigating the low-dose effects of riddelliine [3]. Analysis by ^{32}P -postlabeling/HPLC of DNA adducts from the liver of female F344/N rats treated with 0.01–1.0 mg/kg riddelliine for 3 or 6 months yielded eight DHR-derived DNA adducts that varied in a dose-related manner [5]. The *cII* mutation assay using liver from female Big Blue rats treated with 0.1–1.0 mg/kg riddelliine for 3 months also increased in a linear dose-responsive manner [6]. The DHR-derived DNA adducts, if not repaired prior to DNA synthesis, might produce replication errors and mutations, which eventually could result in the development of neoplasms in the treated rats. However, the formation of DNA adducts and the induction of *cII* mutation was not directly correlated with the incidence of liver tumors, which were not observed in female rats gavaged with less than 0.33 mg/kg riddelliine. This suggests that factors other than DNA damage and mutation are necessary for tumor induction, such as cell proliferation, apoptosis, and the rate of progression from initiated cells to neoplasms [3].

Separation of different cell types from bulk tissue allows the examination of cell-specific, as well as tissue-specific, mutation induction [20]. In the present study, the analysis of *cII* MFs in the liver parenchymal and endothelial cells of rats treated with riddelliine was used to determine whether or not the high incidence of liver hemangiosarcomas is associated with a high frequency of mutation in liver endothelial cells. Our results indicate that riddelliine is

mutagenic in rat liver, and mainly mutagenic in the endothelial cells of the liver, confirming that riddelliine is a genotoxic carcinogen.

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Table 1

cII Mutant frequencies in the liver cells of control and riddelline-treated transgenic Big Blue rats

Group	Cells	Rat ID	Total plaques screened ($\times 10^3$)	Mutant plaques	Mutant frequency ($\times 10^{-6}$)
Control	Parenchymal	I-4F	256	10	39.1
		I-5F	238	9	37.8
		I-6F	525	15	28.6
	Endothelial	Mean $\pm$ SD			35.2 $\pm$ 5.7
		I-4F	421	15	35.7
Riddelline	Parenchymal	I-5F	379	15	39.6
		I-6F	254	11	43.3
		Mean $\pm$ SD			39.5 $\pm$ 3.8
	Endothelial	III-4F	392	14	35.7
		III-5F	309	9	29.1
		III-6F	673	32	47.6
	Endothelial	Mean $\pm$ SD			37.5 $\pm$ 9.3
		III-4F	184	13	70.7
	Endothelial	III-5F	372	18	48.4
		III-6F	232	19	81.9
		Mean $\pm$ SD			67.0 $\pm$ 17.1 ^{a,b}

^a Significantly different from MF of control endothelial cells ($P < 0.05$).

^b Significantly different from MF of riddelline-treated parenchymal cells ($P < 0.01$).

Table 2Mutations in the *cII* gene of liver endothelial cells from riddelline-treated Big Blue rats

Position ^a	Mutation ^b	Amino acid change	Sequence context 5'→3' ^c	Number of mutations (independent)
-14	G→T	N/A	ctaaG Gaaa	1
-13	G→T	N/A	ctaaG Gaaa	2 (1)
3	G→A	Met→Ile	catAT Cggtt	1
25	G→T	Glu→Stop	aacGAGgct	1
26	A→G	Glu→Gly	aacGAGgct	1
34	C→T	Arg→Stop	ctaCGAatc	1
40	G→A	Glu→Lys	atcGACgagt	2 (2)
40	G→T	Glu→Stop	atcGACgagt	2 (2)
46	G→C	Ala→Pro	agtGCCgtc	1
50	T→C	Leu→Ser	gcgTTGctt	1
64	G→T	Ala→Ser	atcGCAatg	1
85	A→G	Thr→Ala	aagACAgcg	1
85	A→T	Thr→Ser	aagACAgcg	1
89	C→T	Ala→Val	acaGCGgaa	1
103	G→A	Val→Ile	ggcGTTgat	2 (2)
106	-G	Frameshift	gttGATaag	1
113	C→T	Ser→Leu	aagTCGcag	1
115	C→A	Gln→Lys	tegCAGatc	1
139	T→A	Trp→Arg	gacTGGatt	1
144	T→G	Ile→Met	tggAT Tcca	1
149	A→C	Lys→Thr	ccaAAGtc	1
166	G→A	Ala→Thr	cttGCTgtt	1
175	G→A	Glu→Lys	cttGAAagg	1
178/185	+G	Frameshift	gaaTGGGGGGGTCgtt	4 (3)
179-184	-G	Frameshift	gaaTGGGGGGGTCgtt	3 (2)
180	G→A	Trp→Stop	gaaTG Gggg	1
185	T→G	Val→Gly	gggGTCgtt	1
193	G→A	Asp→Asn	gacGACgac	1

Position ^a	Mutation ^b	Amino acid change	Sequence context 5'→3' ^c	Number of mutations (independent)
196	G→A	Asp→Asn	<i>gacGACatg</i>	2 (2)
199	-A	Frameshift	<i>gacATGgct</i>	1
200	T→A	Met→Lys	<i>gacA7Ggct</i>	1
206	G→A	Arg→Gln	<i>gctCGAttg</i>	1
209	T→A	Leu→Stop	<i>cgaT7Ggcg</i>	1
212	C→T	Ala→Val	<i>tfgGCGcga</i>	2 (1)
214	C→T	Arg→STOP	<i>gcgCGAcaa</i>	2 (1)
Total				47 (42)

Abbreviations: -, deletion; +, insertion.

^aPosition 1 is the first base of the start codon in the *cll* coding sequence.

^bPresented in term of sequence change on nontranscribed DNA strand.

^cUppercase indicates target codon(s) and target bases are italicized.

Table 3

Summary of the independent *cII* gene mutations in control rat liver and liver endothelial cells from riddelliine-treated Big Blue rats

Type of mutation	Riddelliine ^a		Control ^{a,b}	
	Number	%	Number	%
G:C→C:G	1	2	2	4
G:C→A:T	17	40	31	54
G:C→T:A	7	17	5	9
A:T→T:A	4	10	3	5
A:T→C:G	3	7	3	5
A:T→G:C	3	7	3	5
Frameshift	7	17	9	16
Complex mutation	0	0	1	2
Total mutants screened	42	100	57	100

^aThere is no significant difference between control and riddelliine spectra as analyzed by the Adams and Skopek test [14].

^bControl mutations from literature [6].