Carcinogenesis vol.25 no.3 pp.309–315, 2004
DOI: 10.1093/carcin/bgh017

Susceptibility to vascular neoplasms but no increased susceptibility to renal carcinogenesis in Vhl knockout mice

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The von Hippel-Lindau (VHL) tumor suppressor gene plays a prominent role in the development of renal cell carcinoma (RCC) in humans. VHL functions as a ubiquitin E3 ligase, controlling the stability of hypoxia inducible factor (HIF) and tumor angiogenesis. Alterations in this tumor suppressor gene are rarely observed in spontaneous or chemically induced RCC that arise in conventional strains of rodents and Vhl knockout mice ($Vhl^{+/-}$) do not develop spontaneous RCC. We tested whether Vhl knockout mice exhibited increased susceptibility to renal carcinogenesis using the well-characterized renal carcinogen streptozotocin. No differences were observed between wild-type and $Vhl^{+/-}$ animals in the frequency or type of renal lesions induced by 50–200 mg/kg streptozotocin. Carcinogen-induced RCC that developed in Vhl heterozygotes and wild-type mice did not contain mutations in the wild-type Vhl, as determined by direct sequencing of the primary tumors. While $Vhl^{+/-}$ mice exhibited no increase in renal lesions in response to streptozotocin, heterozygous animals did develop vascular proliferative lesions of the liver, uterus, ovary, spleen and heart. These lesions, ranging from angiectasis to hemangiosarcoma, were most prominent in the livers of $Vhl^{+/-}$ mice, where they were found in high incidence and high multiplicity. Wild-type mice developed a low-frequency of liver angiectasis (7–15%) only at the highest doses of carcinogen used (150 and 200 mg/kg, respectively) while $Vhl^{+/-}$ mice exhibited angiectasis, hemangioma and hemangiosarcomas with a frequency ranging from 19 to 46% at 50–200 mg/kg streptozotocin. Untreated $Vhl^{+/-}$ mice had a spontaneous incidence of hepatic vascular lesions of 21%. Furthermore, vascular lesions of the uterus, ovary, spleen and heart were observed only in $Vhl^{+/-}$ mice, with an incidence of (5–28%). Taken together, the data indicate that heterozygosity at the Vhl locus predisposes mice to a vascular phenotype ranging from angiectasis to hemangiosarcoma, consistent with the ability of this tumor suppressor gene to control the stability of HIF and regulate key proteins that participate in angiogenesis.

Abbreviations: HIF, hypoxia inducible factor; LCM, laser capture microdissection; RRC, renal cell carcinoma; Tsc-2, tuberous sclerosis complex-2; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

Introduction

Renal cell carcinoma (RCC) arises from the epithelial cells of the renal nephron and is characterized by its many different cytological and histological variants (1). Alterations in the von Hippel-Lindau (VHL) tumor suppressor gene are associated with the clear cell variant of this disease in humans, accounting for ~80% of all clear cell tumors (2–4). In contrast to the human disease, rodent RCC are predominantly solid tumors with a chromophilic rather than clear cell cytology (5). The incidence of spontaneous RCC in laboratory strains of mice and rats is very low (<5%), although many chemical carcinogens target the kidney of rodents and induce these tumors. The kidney is, in fact, a major site for tumor induction by chemicals screened in National Toxicology Program bioassays (6).

While the VHL gene plays a prominent role in human RCC, the involvement of this gene in rodent RCC appears to be more limited. Vhl knockout mice for example, do not develop spontaneous RCC (7). Rather, the primary target for RCC in rodents appears to be the tuberous sclerosis complex-2 (Tsc-2) tumor suppressor gene. Germ-line inactivation of the Tsc-2 gene predisposes rats to high-frequency spontaneous RCC (8–10), which develop in these animals subsequent to loss of the wild-type Tsc-2 allele. Tsc-2 knockout mice similarly develop spontaneous RCC (11,12). In addition, carcinogen-induced rat and mouse RCC exhibit mutations in the Tsc-2 gene without detectable alterations in Vhl (11,13–19). Vhl mutations have been reported in rat RCC, although they were induced by transplacental carcinogenesis and are associated with the clear cell histological variant (20), which is rarely seen in these animals.

In addition to RCC, individuals with VHL syndrome also develop vascular lesions such as benign hemangioblastomas of the cerebellum, spine, brain stem and retina (21,22). The presence of vascular lesions in these individuals has been attributed to the important role of pVHL in the regulation of hypoxia inducible factor (HIF) (23). Under normoxic conditions HIF is targeted for degradation by the E3 ubiquitin ligase activity of pVHL and is a short-lived protein (24–29). Loss of function of pVHL in human RCC results in accumulation of HIF (25,30–34). HIF is a transcription factor that regulates many genes involved in maintaining O_2 homeostasis and the physiologic response to O_2 deprivation, such as erythropoietin, glucose transporters, glycolytic pathway enzymes, heme oxygenase, inducible nitric oxide synthase and vascular endothelial growth factor (VEGF) (24). VEGF in particular is a potent endothelial cell-specific mitogen that promotes the growth and maintenance of vascular endothelial cells and is the major angiogenesis inducer *in vivo* (35,36).

Although Vhl heterozygous mice do not develop spontaneous kidney tumors, the susceptibility of these animals to carcinogen-induced RCC has not been investigated. Streptozotocin is a well-characterized murine renal carcinogen (37–39) and a nitrosamine with the ability to induce high-frequency RCC in CBA/T6J mice (37). These tumors have been found to share some ultrastructural features with human clear-cell tumors (40). We have examined the relative susceptibility of Vhl heterozygous mice to this carcinogen. While there was no statistically significant difference in the susceptibility to the induction of renal tumors between wild-type and $Vhl^{+/-}$ mice, $Vhl^{+/-}$ animals had significantly higher incidence of vascular proliferative lesions compared with wild-type mice ranging from angiectasis to hemangiosarcoma.

Materials and methods

Animals

C57Bl/6;129 $Vhl^{+/-}$ mice (7) were maintained as a closed colony at the Science Park-Research Division, University of Texas MD Anderson Cancer Center (UTMDACC). The animals were housed in polycarbonate cages and provided *ad libitum* with 8640 Teklad rodent diet (Harlan Teklad, Madison, WS) and deionized tap water. The study was conducted under the National Institute's of Health guidelines and approved by the Institutional Animal Care and Use Committee, UTMDACC. At 8 weeks after birth, streptozotocin was administered to mice at 50, 100, 150 and 200 mg/kg by a single i.p. injection. Each dose group had 26 female mice. Animals were killed at 14–15 months of age, and the kidney, liver, lung, heart, ovary and uterus were collected from each animal. Left kidneys were fixed in OCT and the remaining organs were fixed in 10% neutral buffered formalin. Large tumors were divided for fixation and for snap-freezing. Histopathology was performed using routine methods. Two-sided Fisher exact test ($P < 0.05$) was used to compare tumor incidence in $Vhl^{+/-}$ versus wild-type mice after streptozotocin treatment, and in untreated $Vhl^{+/-}$ versus treated $Vhl^{+/-}$ mice.

Vhl gene analysis

To obtain DNA from microscopic renal tumors, laser capture microdissection (LCM) was performed using PixCell II system (Arcturus, Mtn View, CA) using OCT-embedded tissues. DNA was extracted from either snap-frozen renal tumors or microdissected lesions using Qiagen (Valencia, CA) and Arcturus DNA extraction kits, respectively. Primers for amplification of VHL were exon 1: MVHL1-FCCATTTCCGCCTTCAAGTTGC and MVHL1-R GTGGGTAGGGACAAGATGCTCG; exon 2: MVHL2-F 5′-AAACAGGTGCCATGCCCAGAC and MVHL2-R 5′-TACATAGCTCCAGGACAACC; and exon 3: MVHL3-F 5′-TCATGTGACCAGAGTCTGCC and MVHL3-R 5′-GTGACCTGTCCTATGTGTAG. PCR was performed using AmpliTaq™ Gold PCR Master Mix (Applied Biosystems, Foster City, CA) 50 μM of primer, and 40 ng of DNA according to the manufacturer's instructions. The 9600 Gene Amp PCR System was used with the following cycle conditions: 95°C, 10 min; 95°C, 45 s, 60°C, 30 s, 72°C, 45 s, 30 cycles; 72°C, 4 min. PCR products were gel-purified using a Wizard PCR purification kit (Promega, Madison, WI). PCR products were sequenced from both strands using an ABI Prism Big DyeTerminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on the ABI Prism 377 DNA Sequencer (Applied Biosystems) and analyzed with AutoAssembler DNA Sequence Assembly 2.0 Software (Applied Biosystems). RNA was extracted from snap-frozen tumors using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's instructions. Quantitative RT–PCR was performed using Sybr Green PCR Master Mix according to the manufacturer's protocol (Applied Biosystems) on an ABI Prism 7700 Sequence Detection System. Primer Express 1.5 (Applied Biosystems) was used to design primers for quantitative RT–PCR and mouse Vhl exon 1.

Results

Carcinogen-induced renal lesions in wild-type and $Vhl^{+/-}$ mice

The highest dose of streptozotocin tested (200 mg/kg) exceeded the LD_{50}, and animals in this dose group had only a 45% survival rate. Exposure to 150 mg/kg also caused significant morbidity, and these animals had a 77% survival rate. Morbidity associated with the two higher dose groups, wild-type and carriers, was due to the diabetogenic effects of this compound, which resulted in death of the animals $\leq$5 months of age, prior to tumor onset. No gross kidney lesions were noted in those animals ($n = 5$) that died between 6 and 13 months of age. Animals in 100 and 50 mg/kg dose groups had 98 and 100% survival rates, respectively.

At 14–15 months, carcinogen-exposed Vhl heterozygotes and control animals (untreated $VHL^{+/-}$ and wild-type mice exposed to streptozotocin) were examined for the presence of renal lesions. As shown in Table I, only a single animal from the unexposed group of $Vhl^{+/-}$ mice ($n = 28$) developed a renal lesion, indicating that the spontaneous kidney tumor incidence in these animals is $<5\%$, similar to the background frequency of renal proliferative lesions in the C57Bl/6;129 strain (41). The incidence of pre-neoplastic lesions, adenomas and carcinomas of the kidney was significantly increased with carcinogen exposure in both wild-type and Vhl heterozygotes. Tumor incidence was maximum in the 150 mg/kg dose group, with a 47 and 39% incidence of renal lesions observed in wild-type and $Vhl^{+/-}$ mice, respectively. However, within each dose of streptozotocin, no significant difference was observed in the incidence of renal lesions between wild-type and Vhl heterozygotes.

Proliferative renal lesions

Renal tumors were found in the outer cortex as raised focal or multifocal 2–5 mm spherical gray–white solid or cystic masses that contained clear serous or blood-tinged fluid. Histologically, the lesions consisted of proliferations of tubular epithelial cells ranging from solid and cystic dysplasias (so-called atypical hyperplasias) to neoplastic proliferations of cuboidal to columnar renal tubular epithelial cells arranged in a cystpapillary or tubular-solid appearance (Figure 1). Cytologically, the proliferative cells were chromophilic with dark acidophilic or basophilic granular cytoplasm. No clear cell RCC variants were found.

Vhl gene analysis

Two frozen renal carcinomas and four microscopic lesions of renal cell adenomas were examined for alterations in the Vhl gene. Table II summarizes the results of the Vhl gene analysis in streptozotocin-induced renal lesions. In both carcinomas, Vhl gene expression was detected by quantitative RT–PCR (data not shown). Direct sequencing of Vhl exons 1–3 that were PCR-amplified from DNA revealed no mutations in these tumors. To ensure that the wild-type Vhl allele was not amplified from any normal cells present in tumors, PCR was repeated using DNA obtained from LCM material from these carcinomas. Direct sequencing confirmed the lack of Vhl mutations in these tumors. Sequencing of exons 2 and 3 by LCM of an additional four renal cell adenomas also revealed no mutations. The lack of streptozotocin-induced alterations in the mouse Vhl gene was consistent with the absence of any increased susceptibility to carcinogen-induced renal cell tumors in $Vhl^{+/-}$ compared with wild-type mice.

Vhl heterozygotes display a vascular phenotype exacerbated by carcinogen exposure

In contrast to the absence of susceptibility to kidney tumorigenesis, Vhl heterozygotes did exhibit a susceptibility to proliferative vascular lesions ranging from angiectasis to

Table I. Incidence of organ-specific lesions developed in the $Vhl^{+/-}$ mice treated with streptozotocin

Tissue	Lesion	Untreated	50 mg/kg		100 mg/kg		150 mg/kg		200 mg/kg	
		Vhl	WT	Vhl	WT	Vhl	WT	Vhl	WT	Vhl
	Pre-neoplastic		1	1	1	2	1	2	1	3
	Adenoma	1		1		1	4	4	2	3
Kidney	Carcinoma			1		1		1	1	
	Gross cysts	1				1	4	6	1	4
	Incidence (%)	1/28 (4)	1/22 (5)	2/21 (10)	1/21 (5)	5/21 (24)	7/15 (47)	7/18 (39)	2/7 (29)	4/13 (31)
	Angiectasia	6		2		3	1	2	1	5
Liver	Angioma	8		2		2		3		3
	Hemangiosarcoma	1		2				1		2
	Incidence (%)	6/28 (21)	0/22 (0)	6/21 (29)[a]	0/21 (0)	4/21 (19)	1/15 (7)	6/18 (33)	1/7 (15)	6/13 (46)
	Angiectasia									
Uterus	Angioma			1		2		1		1
	Hemangiosarcoma					1				
	Incidence (%)			1/21 (5)		3/21 (14)		1/18 (6)		1/13 (8)
	Angiectasia					1				
	Angioma									
Spleen	Hemangiosarcoma									
	Incidence (%)					1/21 (5)				
	Angiectasia			1						
Ovary	Angioma			1		2				
	Hemangiosarcoma					1				
	Incidence (%)			2/21 (10)		3/21 (14)				
	Angiectasia	2				1				
Heart	Angioma	2		1						1
	Hemangiosarcoma	2						1		
	Incidence (%)	6/28 (21)		1/21 (5)		1/21 (5)		1/18 (6)		1/13 (8)
Other tissues	Mesenteric									
	Hemangioma						1			
	Intestine									
	Hemangiosarcoma		1							
	Incidence (%)		1/22 (5)				1/15 (7)			
Total number of animals		80	22	21	21	21	15	18	7	13

[a]Significant ($P < 0.05$) difference between wild-type and $Vhl^{+/-}$ mice.

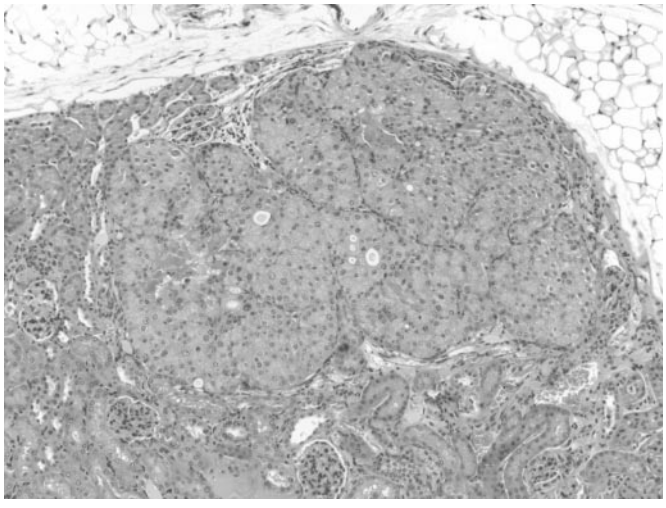

Fig. 1. Kidney cortex of STZ-treated $VHL^{+/-}$ mouse showing renal cell adenoma comprised of a solid nest of neoplastic tubular epithelial cells. The eosinophilic chromophilic cells are typical of the cytology commonly noted in spontaneous and chemically induced murine renal cell tumors. H&E ×200. A color version is available as supplementary material online.

hemangiosarcomas (Table I) with the liver being the primary affected organ. These vascular lesions were noted in hepatic parenchyma of both untreated and treated Vhl heterozygotes but only in streptozotocin-treated wild-type mice. Proliferative lesions were noted macroscopically as focal to multifocal irregular red raised firm nodular masses ranging in size from 2–3 mm in diameter to 1 cm. They were noted on the capsular surface extending down into the hepatic parenchyma (Figure 2A). In some instances, the hepatic lesions manifested as irregular red depressed areas with a fluctuant consistency. They were found in all regions of the liver but were often noted at the free margins of hepatic lobes. Microscopically, the vascular lesions were represented by a biological continuum ranging from angiectasis (also termed peliosis hepatis in the pathology literature) to overt hemangiomas and hemangiosarcomas. Hepatic angiectasis consisted of dilatation of hepatic sinusoids lined by prominent endothelial cells with hyperchromatic nuclei (Figure 2B). Hepatic hemangiomas (Figure 2C) were typical of those noted in rodents and were difficult to distinguish from angiectasia since they were often large cavernous hemangiomas. In some instances hemangiosarcomas were diagnosed based on proliferation and anaplasia of

Table II. Status of the Vhl gene in streptozotocin-induced murine renal tumors

Animal Vhl status	STZ dose, mg/kg	Tumor type	DNA/RNA source	Vhl expression	Exons 1–3 mutations
$Vhl^{+/-}$	100	Adenoma	LCM cells	n.d.	No[a]
$Vhl^{+/-}$	100	Carcinoma	Frozen tumor, LCM cells	Yes	No
$Vhl^{+/-}$	150	Adenoma	LCM cells	n.d.	No[a]
$Vhl^{+/-}$	150	Adenoma	LCM cells	n.d.	No[a]
$Vhl^{+/-}$	150	Carcinoma	Frozen tumor, LCM cells	Yes	No
Wild-type	200	Adenoma	LCM cells	n.d.	No[a]

[a]Exons 2–3 only.
n.d., not determined.

lining endothelial cells. Lesion multiplicity was difficult to discern although many animals had distinct lesions within several hepatic lobes suggesting separate entities. In several animals multiple stages of vascular proliferation were noted in the same livers.

The incidence of spontaneous proliferative vascular lesions in the liver of $Vhl^{+/-}$ mice was 21% (untreated), and a similar frequency of these lesions was observed in the 50 (29%) and 100 mg/kg (19%) groups. At higher doses of carcinogen, liver lesions were observed in 33 (150 mg/kg) and 46% (200 mg/kg) of the streptozotocin-exposed animals. However, because of the limited survival of animals in the high dose groups, the increased frequency of liver lesions in the 150 and 200 mg/kg dose groups did not reach statistical significance. Wild-type animals did not develop spontaneous vascular neoplasms in the liver. However, higher doses of streptozotocin (150 and 200 mg/kg) induced a low frequency of angiectasis (7 and 15%) in wild-type animals, consistent with the potential for this carcinogen to enhance the development of these types of vascular lesions in the $Vhl^{+/-}$ mice.

Although proliferative vascular lesions in the Vhl heterozygotes were of highest incidence in the liver, they were also found in a variety of other organs. Cardiac vascular lesions (21% incidence) were observed in the myocardium of untreated Vhl heterozygotes, but the frequency of these lesions was not significantly increased with streptozotocin treatment (5–8%). Reproductive tract lesions occurred in streptozotocin-treated animals with a frequency ranging from 5 to 14% for the uterine lesions and 10–14% for the ovarian lesions, but were not dose-dependent. Microscopically, the vascular changes ranged from angiectasia to overt neoplasia with proliferation of endothelial cells exhibiting cytomegaly and pleomorphism. Both cavernous (Figure 3) and capillary (Figure 4) histologic patterns were noted in the uterus and myocardial tissue although the cavernous subtype predominated. Thus, germline heterozygosity at the mouse Vhl locus predisposed to the development of vascular neoplasms in multiple organs that occurred with a frequency of 5 to almost 50%, depending on the target organ and carcinogen exposure.

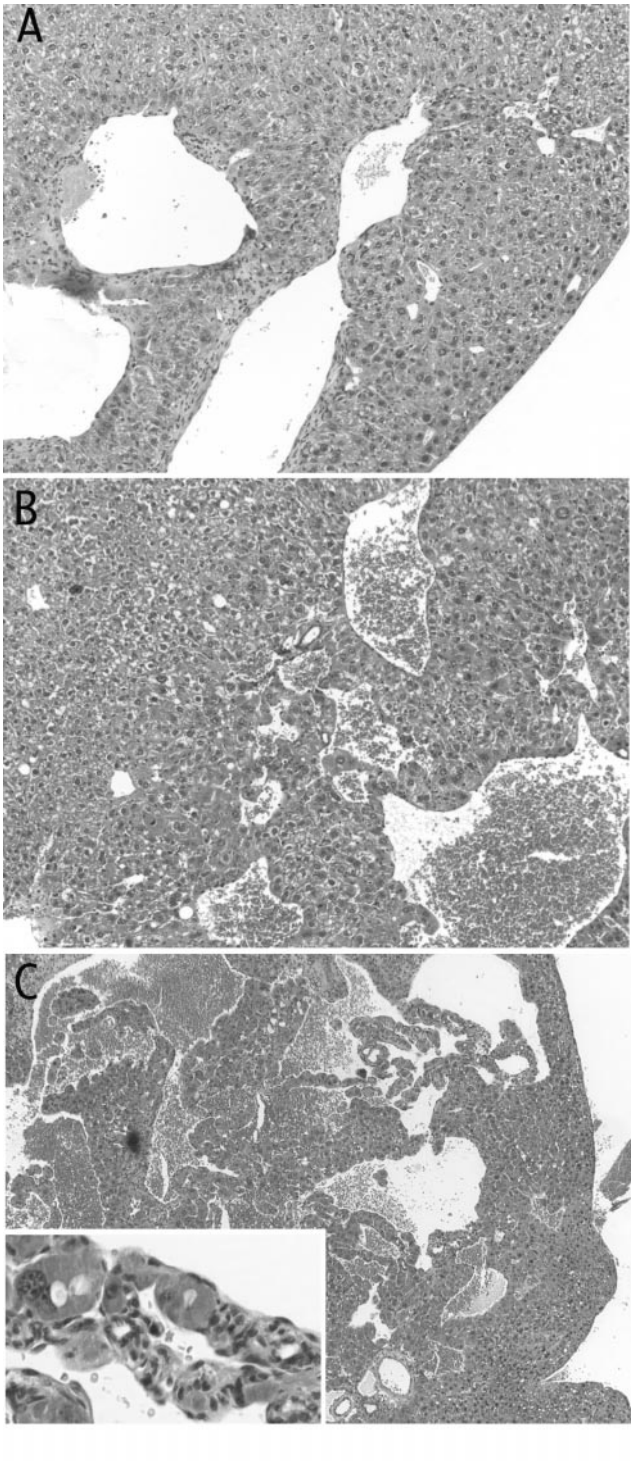

Fig. 2. (**A**) Section of hepatic parenchyma of untreated $VHL^{+/-}$ mouse with angiectasis consisting of dilatation of endothelial lined hepatic sinusoids. (**B**) Hepatic hemangioma with loss of normal liver trabecular structure and replacement by irregular endothelial lined vascular channels. (**C**) Hemangiosarcoma distorting hepatic architecture. High magnification inset (×400) shows atypical neoplastic endothelial cells lining remnants of hepatic cords. H&E ×100. A color version is available as supplementary material online.

Discussion

The present study demonstrated that mice with a loss of one allele of the Vhl tumor suppressor gene did not show significant predisposition to the development of renal cell tumors following the administration of streptozotocin, a well-studied murine renal carcinogen (37,39). Incidence, multiplicity and cytologic appearance of renal cell tumors did not vary significantly between wild-type and $Vhl^{+/-}$ mice. This study confirmed that proliferative renal lesions are only infrequently found spontaneously in $Vhl^{+/-}$ mice, and further demonstrated that

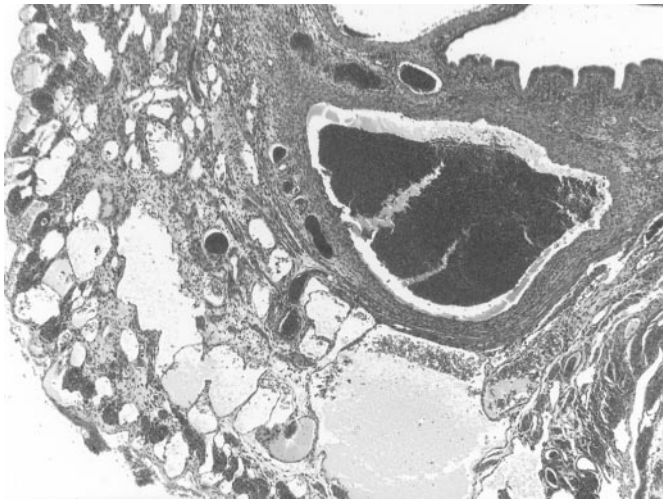

Fig. 3. Cross-section of uterus from untreated VHL$^{+/-}$ mouse showing transmural invasion of cavernous variant hemangiosaroma extending from the serosa to submucosa. H&E ×100. A color version is available as supplementary material online.

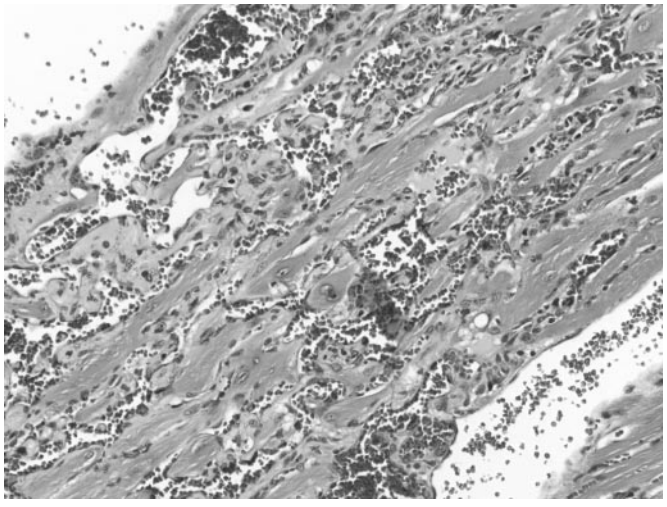

Fig. 4. Myocardium from interventricular septum of untreated VHL$^{+/-}$ mouse showing replacement by vascular channels lined by neoplastic endothelial cells. This is a capillary variant hemangiosarcoma. H&E ×200. A color version is available as supplementary material online.

these animals are no more susceptible to the development of carcinogen-induced renal neoplasms than other murine stocks and strains. Histologic review of streptozotocin-induced renal cell tumors in Vhl$^{+/-}$ mice in the present study did not reveal the presence of any clear cell subtypes, the renal tumor phenotype associated with loss of VHL function in VHL patients [3] and with inactivation of the Vhl tumor suppressor gene in rats (20). The pre-neoplastic renal lesions and renal cell tumors that arose in kidneys of streptozotocin-treated wild-type and Vhl heterozygotes were identical, consisting of chromophilic tubular epithelial cell proliferations histologically and cytologically similar to those reported to arise spontaneously and in carcinogen-treated mice of other stocks and strains (42).

The hepatic vascular lesions in the present study are believed to represent a biological continuum of proliferative changes ranging from early (pre-neoplastic) lesions of angiectasia to overt cavernous hemangiomas and hemangiosarcomas. The lesion that we are terming 'angiectasis' has also been termed peliosis hepatis (43) or peliosis-like syndrome by others (44). Neither the early angiectatic proliferative changes nor the vascular tumors are common spontaneous lesions in aged B6,129 mice (41,45), therefore, these changes appear to be associated with loss of Vhl function as described in similar mice by others (46). These early vascular proliferative lesions have been reported in both humans (47) and mice (43) and have been associated with several chemical carcinogens resulting in overt vascular neoplasia in the liver of rodents (48).

Chemically induced hepatic vascular tumors in rodents have been associated with the monohaloethylenes such as vinyl fluoride and vinyl chloride inhalation that have also been associated with hepatic angiectasis in chronic rodent bioassays (49). Hepatic angiectasia has been reported both as a possible pre-neoplastic state as well as a paraneoplastic syndrome associated with the elaboration of tumor-secreted VEGF (44). In the present study it is presumed that angiectasis represents an early stage in the progression to vascular tumorigenesis. Molecular studies confirm that vascular tumorigenesis in the mouse is probably a multistage process and that genotoxic carcinogens can participate in the pathogenesis of chemically induced vascular neoplasms (50).

In the original description of the C57Bl/6;129 line of Vhl knockout animals used in this study (7), the vascular lesions we have described were not reported. However, in a separate colony of this Vhl line maintained at LSHUSC, we subsequently noted in animals 14–18 months of age spontaneous hepatic vascular lesions at an incidence similar to that described in this report (5/22, 23% in Vhl$^{+/-}$ animals; 0/20 in wild-type littermate controls). In addition, another line of mice with a targeted inactivation of Vhl developed a similar spectrum of vascular proliferative lesions in the liver including hemangiomas (48). Thus, while the differences between the earlier and later reports were thought possibly to be due to genetic differences between the two lines of mice, our data indicate that heterozygosity at the Vhl locus is sufficient to predispose to the development of proliferative vascular lesions. It should be noted that small vascular lesions are difficult to discern macroscopically as they can be confused with hemorrhagic foci. Microscopically, the earliest lesions of vascular proliferation (angiectasia) are not readily apparent unless one is alerted to be aware of them by the presence of more substantial vascular neoplasms. Vascular neoplasia is observed in mice of the background genotypes commonly used in genetic manipulation experiments, but is still a low incidence finding for any target organ (41,45). For all of these reasons the vascular phenotype may not have been adequately appreciated in earlier studies.

While the present study demonstrated the development of proliferative vascular lesions and vascular neoplasia in multiple target organs of the Vhl$^{+/-}$ mouse, clearly hepatic neoplasia was the highest incidence finding. The finding of vascular tumors in highest incidence in liver and uterus is not surprising given that these organs are the most frequent target sites for these neoplasms in mice of the commonly utilized background B6;129 genotype (41,51). In rodent bioassays chemically induced vascular lesions are largely restricted to a few selected organs. Interestingly, in the present study the finding of a relatively high incidence of cardiac vascular neoplasia in Vhl$^{+/-}$ mice was unexpected as it is not a common site for spontaneous vascular tumors in the mouse (51). Chemically induced hemangiosarcomas in mice are often

multicentric in origin, although site specificity for the heart has been noted specifically for butadiene exposure (52). The multisite pattern of vascular tumor development in $Vhl^{+/-}$ mice does closely resemble that of humans with VHL syndrome, which develop vascular lesions in multiple sites including the retina, central nervous system and, less frequently, the other organs such as the liver (21).

The absence of a kidney phenotype in Vhl heterozygotes contrasts sharply with mice heterozygous for the Tsc-2 tumor suppressor gene. $Tsc\text{-}2^{+/-}$ mice develop spontaneous renal cell tumors with an incidence of 100% by 10 months of age (11,12). It is not clear why mice are refractory to Vhl-related kidney tumors and susceptible to Tsc-2-related renal cell tumors. Gruys *et al*. also found that cell lines derived from streptozotozin-induced RCC from Balb/c mice lacked Vhl mutations (38). This species-specific preference for Tsc-2 versus VHL in mice and humans, respectively, is especially interesting in light of recent data that similar to loss of VHL function, loss of Tsc-2 gene function also results in stabilization of HIF and increased expression of VEGF (53,54). Consistent with stabilization of HIF2a in Tsc-2-null tumors, Tsc-2 knockout mice also develop hepatic hemangiomas similar to those that occur in Vhl heterozygotes (11). Interestingly, rats carrying the Eker mutation in the Tsc-2 gene ($Tsc\text{-}2^{Ek/+}$) that develop renal cell tumors also develop vascular lesions. Hemangiomas occur in a variety of organs, although these tumors arise predominately in the spleen (55), but unlike the situation in Tsc-2 knockout mice, vascular proliferative lesions have not been noted in the hepatic parenchyma of $Tsc\text{-}2^{Ek/+}$ rats.

In the recent study by Haase *et al*., homozygous inactivation of Vhl in the hepatocytes of these animals was accomplished using an albumin-driven cre-recombinase resulting in the development of foci of increased vascularization in the liver including the development of cavernous hemangiomas (46). Although Vhl inactivation in hepatocytes did not result in the development of hepatocellular carcinoma, the ability to produce vascular lesions of the type we have observed with germline inactivation may have been due to increased paracrine or circulating levels of VEGF produced in the liver. Cre-mediated Vhl inactivation in these animals was estimated to occur with high efficiency (40–60% of hepatocytes) and indeed, these animals had high circulating VEGF levels and elevated HIF expression in their livers (46).

A role for hepatocyte-derived VEGF in the genesis of hepatic proliferative vascular lesions has been suggested recently for chemically induced hepatic vascular neoplasia associated with the pyrrolizidine alkaloid riddelliine (56). Riddelliine induces both angiectasia and vascular tumors in rats and mice in National Toxicology Program bioassays. In conjunction with the finding of dysregulated VEGF synthesis by hepatocytes, riddelliine exposure induced activated VEGFR-2 expression in rat liver endothelium, leading to the induction of endothelial cell proliferation and culminating in the development of hepatic hemangiosarcoma (57). Neither our group nor that of Kobayashi or Haase (11,46) were able to determine if loss of the wild-type Vhl allele occurred in the vascular lesions that develop in Vhl or Tsc-2 knockout mice, due to the high degree of cellular heterogeneity in these tumors. Although these efforts are continuing, the question of whether the vascular lesions that develop in Vhl or Tsc-2 knockout mice are themselves clonal in origin and follow Knudson's 'two-hit' hypothesis remains an open question. Nonetheless, $Vhl^{+/-}$ mice should prove to be a useful model for increasing our understanding of the factors that contribute to vascular tumorigenesis.

Supplementary material

Supplementary material can be found at http://www.carcin.oupjournals.org.

Acknowledgements

The authors thank Dr D.House for performing statistical analysis of the data, and Drs K.Gaido, S.Borghoff and D.Dorman for reviewing this manuscript and valuable suggestions. The work described in this manuscript is supported by National Institutes of Health Grants: CA63613 to C.L.W., CA78335 to J.R.G. and ES07784 and CA16672.

References

1. Thoenes,W., Storkel,S. and Rumpelt,H.J. (1986) Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. *Pathol. Res. Pract.*, **181**, 125–143.
2. Kovacs,G., Akhtar,M., Beckwith,B.J. *et al.* (1997) The Heidelberg classification of renal cell tumours. *J. Pathol.*, **183**, 131–133.
3. Zbar,B. and Lerman,M. (1998) Inherited carcinomas of the kidney. *Adv. Cancer Res.*, **75**, 163–201.
4. Gnarra,J.R., Tory,K., Weng,Y. *et al.* (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nature Genet.*, **7**, 85–90.
5. Tsuda,H. and Krieg,K. (1992) Neoplastic lesions in the kidney. In Mohr,U., Dungworth,D.L. and Capen,C.C. (eds), *Pathobiology of the Aging Rat*. ILSI Press, Washington DC, pp. 227–240.
6. National,T.P. (2002) Chemicals associated with site-specific tumor induction in kidney tubular cell. Toxicology data management system database http://ntp-server.niehs.nih.gov/htdocs/sites/psite_cnt.html.
7. Gnarra,J.R., Ward,J.M., Porter,F.D. *et al.* (1997) Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc. Natl Acad. Sci. USA*, **94**, 9102–9107.
8. Walker,C., Goldsworthy,T.L., Wolf,D.C. and Everitt,J. (1992) Predisposition to renal cell carcinoma due to alteration of a cancer susceptibility gene. *Science*, **255**, 1693–1695.
9. Yeung,R.S., Xiao,G.H., Jin,F., Lee,W.C., Testa,J.R. and Knudson,A.G. (1994) Predisposition to renal carcinoma in the Eker rat is determined by germ-line mutation of the tuberous sclerosis 2 (TSC2) gene. *Proc. Natl Acad. Sci. USA*, **91**, 11413–11416.
10. Kobayashi,T., Hirayama,Y., Kobayashi,E., Kubo,Y. and Hino,O. (1995) A germline insertion in the tuberous sclerosis (Tsc2) gene gives rise to the Eker rat model of dominantly inherited cancer. *Nature Genet.*, **9**, 70–74.
11. Kobayashi,T., Minowa,O., Kuno,J., Mitani,H., Hino,O. and Noda,T. (1999) Renal carcinogenesis, hepatic hemangiomatosis and embryonic lethality caused by a germ-line Tsc2 mutation in mice. *Cancer Res.*, **59**, 1206–1211.
12. Onda,H., Lueck,A., Marks,P.W., Warren,H.B. and Kwiatkowski,D.J. (1999) Tsc2 (+/−) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J. Clin. Invest.*, **104**, 687–695.
13. Kobayashi,T., Urakami,S., Hirayama,Y., Yamamoto,T., Nishizawa,M., Takahara,T., Kubo,Y. and Hino,O. (1997) Intragenic Tsc2 somatic mutations as Knudson's second hit in spontaneous and chemically induced renal carcinomas in the Eker rat model. *Jpn. J. Cancer Res.*, **88**, 254–261.
14. Walker,C., Ahn,Y.T., Everitt,J. and Yuan,X. (1996) Renal cell carcinoma development in the rat independent of alterations at the VHL gene locus. *Mol. Carcinogen.*, **15**, 154–161.
15. Kubo,Y., Mitani,H. and Hino,O. (1994) Allelic loss at the predisposing gene locus in spontaneous and chemically induced renal cell carcinomas in the Eker rat. *Cancer Res.*, **54**, 2633–2635.
16. Urakami,S., Tokuzen,R., Tsuda,H., Igawa,M. and Hino,O. (1997) Somatic mutation of the tuberous sclerosis (Tsc2) tumor suppressor gene in chemically induced rat renal carcinoma cell. *J. Urol.*, **158**, 275–278.
17. Kikuchi,Y., Kobayashi,E., Nishizawa,M., Hamazaki,S., Okada,S. and Hino,O. (1995) Cloning of the rat homologue of the von Hippel-Lindau tumor suppressor gene and its non-somatic mutation in rat renal cell carcinomas. *Jpn. J. Cancer Res.*, **86**, 905–909.

18. Satake,N., Urakami,S., Hirayama,Y., Izumi,K. and Hino,O. (1998) Biallelic mutations of the Tsc2 gene in chemically induced rat renal cell carcinoma. *Int. J. Cancer*, **77**, 895–900.
19. Shiao,Y.H., Diwan,B.A., Perantoni,A.O., Calvert,R.J., Zbar,B., Lerman,M.I. and Rice,J.M. (1997) Polymerase chain reaction-single-strand conformation polymorphism analysis for the VHL gene in chemically induced kidney tumors of rats using intron-derived primers. *Mol. Carcinogen.*, **19**, 230–235.
20. Shiao,Y.H., Rice,J.M., Anderson,L.M., Diwan,B.A. and Hard,G.C. (1998) von Hippel-Lindau gene mutations in *N*-nitrosodimethylamine-induced rat renal epithelial tumors. *J. Natl Cancer Inst.*, **90**, 1720–1723.
21. Friedrich,C. (2001) Genotype-phenotype correlation in von Hippel-Lindau syndrome. *Hum. Mol. Genet.*, **7**, 763–767.
22. Neumann,H.P. (1987) Basic criteria for clinical diagnosis and genetic counselling in von Hippel-Lindau syndrome. *Vasa*, **16**, 220–226.
23. Kondo,K. and Kaelin,W.G.,Jr (2001) The von Hippel-Lindau tumor suppressor gene. *Exp. Cell Res.*, **264**, 117–125.
24. Semenza,G.L. (1999) Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. *Annu. Rev. Cell Dev. Biol.*, **15**, 551–578.
25. Maxwell,P.H., Wiesener,M.S., Chang,G.W., Clifford,S.C., Vaux,E.C., Cockman,M.E., Wykoff,C.C., Pugh,C.W., Maher,E.R. and Ratcliffe,P.J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, **399**, 271–275.
26. Ohh,M., Park,C.W., Ivan,M., Hoffman,M.A., Kim,T.Y., Huang,L.E., Pavletich,N., Chau,V. and Kaelin,W.G. (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nature Cell Biol.*, **2**, 423–427.
27. Ivan,M., Kondo,K., Yang,H., Kim,W., Valiando,J., Ohh,M., Salic,A., Asara,J.M., Lane,W.S. and Kaelin,W.G.,Jr (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science*, **292**, 464–468.
28. Tanimoto,K., Makino,Y., Pereira,T. and Poellinger,L. (2000) Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J.*, **19**, 4298–4309.
29. Jaakkola,P., Mole,D.R., Tian,Y.M. *et al.* (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science*, **292**, 468–472.
30. Wiesener,M.S., Munchenhagen,P.M., Berger,I. *et al.* (2001) Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas. *Cancer Res.*, **61**, 5215–5222.
31. Gnarra,J.R., Zhou,S., Merrill,M.J., Wagner,J.R., Krumm,A., Papavassiliou,E., Oldfield,E.H., Klausner,R.D. and Linehan,W.M. (1996) Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. *Proc. Natl Acad. Sci. USA*, **93**, 10589–10594.
32. Turner,K.J., Moore,J.W., Jones,A. *et al.* (2002) Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel-Lindau gene mutation. *Cancer Res.*, **62**, 2957–2961.
33. Krieg,M., Haas,R., Brauch,H., Acker,T., Flamme,I. and Plate,K.H. (2000) Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. *Oncogene*, **19**, 5435–5443.
34. Iliopoulos,O., Levy,A.P., Jiang,C., Kaelin,W.G.,Jr and Goldberg,M.A. (1996) Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc. Natl Acad. Sci. USA*, **93**, 10595–10599.
35. Ferrara,N. (2002) VEGF and the quest for tumour angiogenesis factors. *Nature Rev. Cancer*, **2**, 795–803.
36. Shibuya,M. (2001) Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Struct. Funct.*, **26**, 25–35.
37. Hard,G. (1985) Identification of a high-frequency model for renal carcinoma by the induction of renal tumors in the mouse with a single dose of streptozotocin. *Cancer Res.*, **45**, 703–708.
38. Gruys,M., Back,T., Subleski,J., Wiltrout,T., Lee,J., Schmidt,L., Watanabe,M., Stanyon,R., Ward,J., Wigginton,J. and Wiltrout,R. (2001) Induction of transplantable mouse renal cell cancers by streptozotocin: *in vivo* growth, metastases and angiogenic phenotype. *Cancer Res.*, **61**, 6255–6263.
39. Horton,L., Fox,C., Corrin,B. and Sonksen,P.H. (1977) Streptozotocin-induced renal tumours in rats. *Br. J. Cancer*, **36**, 692–699.
40. Delahunt,B. and Wakefield,J.S. (1997) Ultrastructure of streptozotocin-induced renal tumours in mice. *Virch. Arch.*, **430**, 173–180.
41. Haines,D.C., Chattopadhyay,S. and Ward,J.M. (2001) Pathology of aging B6;129 mice. *Toxicol. Pathol.*, **29**, 653–661.
42. Lombard,L.S., Rice,J.M. and Vesselinovitch,S.D. (1974) Renal tumors in mice: light microscopic observations of epithelial tumors induced by ethylnitrosourea. *J. Natl Cancer Inst.*, **53**, 1677–1685.
43. Edwards,R., Colombo,T. and Greaves,P. (2002) 'Have you seen this?' Peliosis hepatis. *Toxicol. Pathol.*, **30**, 521–523.
44. Wong,A.K., Alfert,M., Castrillon,D.H., Shen,Q., Holash,J., Yancopoulos,G.D. and Chin,L. (2001) Excessive tumor-elaborated VEGF and its neutralization define a lethal paraneoplastic syndrome. *Proc. Natl Acad. Sci. USA*, **98**, 7481–7486.
45. Booth,C.J. and Sundberg,J.P. (1995) Hemangiomas and hemangiosarcomas in inbred laboratory mice. *Lab. Anim. Sci.*, **45**, 497–502.
46. Haase,V., Glickman,J., Socolovsky,M. and Jaenisch,R. (2001) Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. *Proc. Natl Acad. Sci. USA*, **98**, 1583–1588.
47. Ludwig,J. and Hoffman,H.N. (1975) Hemangiosarcoma of the liver. Spectrum of morphologic changes and clinical findings. *Mayo Clin. Proc.*, **50**, 255–263.
48. Wright,J.A., Marsden,A.M., Willets,J.M. and Orton,T.C. (1991) Hepatocarcinogenic effect of vinyl carbamate in the C57Bl/10J strain mouse. *Toxicol. Pathol.*, **19**, 258–265.
49. Bogdanffy,M.S., Makovec,G.T. and Frame,S.R. (1995) Inhalation oncogenicity bioassay in rats and mice with vinyl fluoride. *Fund. Appl. Toxicol.*, **26**, 223–238.
50. Hong,H.H., Devereux,T.R., Melnick,R.L., Moomaw,C.R., Boorman,G.A. and Sills,R.C. (2000) Mutations of ras protooncogenes and p53 tumor suppressor gene in cardiac hemangiosarcomas from B6C3F1 mice exposed to 1,3-butadiene for 2 years. *Toxicol. Pathol.*, **28**, 529–534.
51. Ward,J.M., Anver,M., Mahler,J. and Devor-Henneman,D. (2000) Pathology of mice commonly used in genetic engineering (C57Bl/6, 129, B6;129 and FVB/N). In Ward,J.M., Mahler,J.F., Maronpot,R.R. and Sundberg,J.P. (eds) *Pathology of Genetically Engineered Mice*. Iowa State University Press, Ames, Iowa, pp. 161–179.
52. Huff,J.E., Melnick,R.L., Solleveld,H.A., Haseman,J.K., Powers,M. and Miller,R.A. (1985) Multiple organ carcinogenicity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation exposure. *Science*, **227**, 548–589.
53. Liu,M.Y., Poellinger,L. and Walker,C.L. (2003) Up-regulation of hypoxia-inducible factor 2alpha in renal cell carcinoma associated with loss of Tsc-2 tumor suppressor gene. *Cancer Res.*, **63**, 2675–2680.
54. Brugarolas,J.B., Vazquez,F., Reddy,A., Sellers,W.R. and Kaelin,W.G.,Jr (2003) TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell*, **4**, 147–158.
55. Everitt,J.I., Goldsworthy,T.L., Wolf,D.C. and Walker,C.L. (1992) Hereditary renal cell carcinoma in the Eker rat: a rodent familial cancer syndrome. *J. Urol.*, **148**, 1932–1936.
56. Allen,D., Moyer,C., Basabe,A., Maronpot,R. and Nyska,A. (2003) Riddelliine exposure induces alterations in VEGF expression in rat liver: a possible mechanism for hemangiosarcoma formation. *Toxicol. Pathol.*, **31** (suppl.), 143.
57. Moyer,C., Basabe,A., Allen,D., Maronpot,R. and Nyska,A. (2003) Riddelliine exposure induces activated VEGFR-2 expression in rat liver endothelium: a possible mechanism for hemangiosarcoma formation. *Toxicol. Pathol.*, **31** (suppl.), 143.

Received June 12, 2003; revised October 17, 2003; accepted October 28, 2003