An Exploratory Inhalation Toxicity Study with Cigarette Mainstream Smoke in Two Transgenic Mouse Strains, rasH2 and p53$^{+/-}$

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Background and Objective

- The ILSI/HESI Collaborative Evaluation Program on Alternative Models for Carcinogenicity Assessment from 1996-2001 has demonstrated the feasibility of using short- or medium-term *in vivo* rodent test systems, such as transgenic and knockout animal models, in place of a second 2-year rodent bioassay (ILSI/HESI Alternatives to Carcinogenicity Testing Project, 2001).
- Establishing reproducible and validated animal models for lung cancer induced by tobacco has proven difficult, despite the causality between lung tumors and cigarette smoking in humans (IARC, 2004).

We investigated the suitability of the transgenic mouse model expressing the human c-Has-ras proto-oncogene (rasH2) and the heterozygous tumor suppressor p53$^{+/-}$ knockout mouse model (p53$^{+/-}$) for studying cigarette-mainstream-smoke-induced carcinogenicity.

Test Atmosphere Characterization

- Diluted mainstream smoke (MS) from the University of Kentucky Reference Cigarette 2R4F
- Samples collected at the breathing zone of the animals

Parameter	Unit	Exposure Group Sham	2R4F_Low (3 h/day)	2R4F_High (2 x 3 h/day)
TPM	(µg/l)	below DL	240.0 ± 10.3 (N = 128)	244.9 ± 6.9 (N = 106)
CO	(ppm)	below DL	307.0 ± 10.9 (N = 129)	303.2 ± 5.8 (N = 106)
nicotine	(µg/l)	below DL	6.36 ± 0.36 (N = 3)	6.36 ± 0.36 (N = 3)
formaldehyde	(µg/l)	n.d.	0.29 ± 0.01 (N = 2)	0.29 ± 0.01 (N = 2)
acetaldehyde	(µg/l)	n.d.	20.70 ± 0.19 (N = 2)	20.70 ± 0.19 (N = 2)
acrolein	(µg/l)	n.d.	1.81 ± 0.02 (N = 2)	1.81 ± 0.02 (N = 2)

Materials and Methods

MS Generation

- Cigarettes smoked according to ISO protocol (35 ml/puff in 2 s, each cigarette puffed once every minute, butt length 35 mm; Vanscheeuwijck *et al.*, 2002)
- Dilution to 240 µg total particulate matter (TPM)/l with conditioned fresh air, continuous flow of aerosol

Mice and Treatment

- Young adult, female mice from Taconic Farms Inc. Madison, Connecticut 06443, USA:
  - rasH2 mice (1178-T), created by micro-injection of a human hybride c-Ha-ras gene construct with a point mutation, finally resulting in an enhanced expression of the transgene (Gulezian *et al.*, 2000)
  - TSG-p53$^{+/-}$ heterozygote knock-out mice (P53N5-T) (Gulezian *et al.*, 2000)
- Whole-body exposure, 5 days per week over 6 months followed by a 3-month post-exposure period
  - 6 hours/day to conditioned fresh air (sham)
  - 3 hours/day to 2R4F MS at 240 µg TPM/l (2R4F_Low)
  - 2 x 3 hours/day to 2R4F MS at 240 µg TPM/l (2R4F_High)
- Urethane treatment (tumor initiation) of satellite groups prior to exposure (250 mg/kg body-weight i.p.) to verify tumor promotion by MS.

End Points, Sample Collection, and Determinations

- Body weight development
- Micronucleated reticulocytes with high expression of CD71
  - sample collection and fixation 24, 48, and 72 hours post-urethane treatment, according to µicroFlow Mouse Micronucleus kit™ (Litron Labs) protocol
  - flow cytometric analysis (Litron LabsRochester, NY)
- Determination of the percentage of CD71-positive reticulocytes out of approximately 10^5 erythrocytes
- Determination of the percentage of micronucleated CD71-positive reticulocytes out of approx. 20,000 CD71-positive reticulocytes
- Macroscopic lung tumor multiplicity and incidence
  - macroscopic counting of nodules after instillation with Tellyesniczky's fixative for 1 day followed by fixation in ethanol
- Histopathological evaluation of hematoxylin/eosin (HE)-stained lung sections (3 sections at 200 µm distance)

Statistics

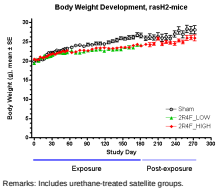
- Body weight and organ weight data: one-way analysis of covariance (using pre-exposure weights as covariate) and variance respectively, followed by individual pairwise comparisons using Dunnett's multiple comparison method
- Incidence of lung nodules and histopathological changes: Fisher's exact probability test
- Multiplicity of lung nodules: analysis of variance for unequal variances (Welch test)
- Results considered statistically significant at p <0.05

Care and use of the animals was in accordance with AALAS policy (1991).

Results

Body Weight Development

- rasH2: MS-related effect during exposure period; not dose-related.

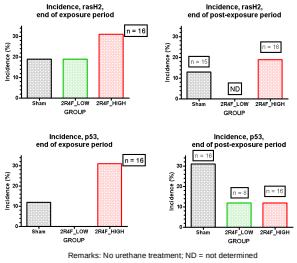


Remarks: Includes urethane-treated satellite groups.

- p53$^{+/-}$: No MS-related effect (data not shown).

Lung Nodule Incidence

- No statistically significant MS-mediated increases.



Remarks: No urethane treatment; ND = not determined

Lung Nodule Multiplicity

- No statistically significant MS-mediated increases.

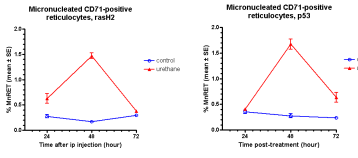
Strain	Exposure Group	End of Exposure Period	N	End of Post-exposure Period	N
RasH2	sham	0.31 ± 0.20	16	0.13 ± 0.09	16
	2R4F_Low	0.19 ± 0.10	16	-	-
	2R4F_High	0.31 ± 0.12	16	0.31 ± 0.20	16
P53$^{+/-}$	Sham	0.25 ± 0.19	16	0.56 ± 0.27	16
	2R4F_Low	0 ± 0	16	0.12 ± 0.12	8
	2R4F_High	0.31 ± 0.12	16	0.07#	16

= excluding one animal with 81 lung nodules, metastases from primary tumor in preputial glands

Remarks: Total number of nodules divided by total number of mice (nodule-bearing and non-nodule-bearing) per group; no urethane treatment.

Urethane Treatment

- Micronuclei in peripheral blood induced in both strains, most pronounced 48 hours after urethane treatment.



Remarks: 250 mg urethane per kg body weight i.p.

Lung Nodule Incidence and Multiplicity in Urethane-Treated Mice

- Increase in lung nodule incidence, but no statistically significant differences between sham and smoke groups.

Strain	Exposure Group	End of Exposure Period Incidence	Multiplicity	End of Post-exposure Period Incidence	Multiplicity
RasH2	sham	5/6	1.67 ± 0.62	6/6	3.67 ± 0.49
	2R4F_Low	6/6	9.33 ± 6.94	-	-
	2R4F_High	3/6	2.33 ± 1.61	4/6	1.67 ± 0.80
P53$^{+/-}$	sham	0/6	0 ± 0	2/6	0.67 ± 0.49
	2R4F_Low	3/6	1.00 ± 0.52	3/8	0.62 ± 0.38
	2R4F_High	0/6	0 ± 0	0/6	0 ± 0

Histopathological Evaluation of Lungs

- RasH2: Presence of broncho-alveolar adenomas and carcinomas, irrespective of urethane treatment and time point.

Type of Neoplastic Lesion	Exposure Group	End of Exposure Period without Urethane	with Urethane	End of Post-exposure Period without Urethane	with Urethane
BA-adenoma	sham	0/16	0/6	1/16	3/6
	2R4F_Low	1/16	2/6	-	-
	2R4F_High	3/16	2/6	1/16	1/6
multiple BA-adenoma	sham	0/16	4/6	0/16	0/6
	2R4F_Low	0/16	3/6	-	-
	2R4F_High	0/16	2/6	0/16	2/6
BA-carcinoma	sham	0/16	1/6	0	2/6
	2R4F_Low	0/16	0/6	-	-
	2R4F_High	2/16	1/6	2/16	1/6

- P53$^{+/-}$: Single bronchiolar-alveolar adenoma found in one urethane-treated mouse, 2R4F_High group.

Summary and Discussion

- MS did not induce lung tumors in rasH2 and p53$^{+/-}$ mice at the end of the 6-month exposure period or the 3-month post-exposure period.
- MS did not promote lung tumor development in urethane-treated rasH2 and p53$^{+/-}$ mice at the end of the 6-month exposure period or the 3-month post-exposure period.
- The findings for rasH2 are different from those reported by Curtin *et al.* (2004), who observed an increase in lung tumor multiplicity in whole-body-exposed rasH2 mice (with a slightly different exposure/ post-exposure duration*).
- The findings are different from earlier observations by Stinn *et al.* (2006), who reported an increase in tumor multiplicity in A/J and SWR mice (with a slightly different exposure/post-exposure duration*).

*5 months exposure / 4 months post-exposure

Conclusion

Under the conditions of this study, the ILSI/ HESI alternative models for carcinogenicity testing, rasH2 and p53$^{+/-}$ mice, were not suitable as a model for MS-induced lung tumors.

References:

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