Aberrant Expression of Cyclin D1 in Pulmonary Proliferative Lesions Induced by High Doses of Urethane in Transgenic Mice Carrying the Human Prototype c-H-ras Gene

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ABSTRACT. In our previous study, when rasH2 mice and non-transgenic (non-Tg) littermates were injected intraperitoneally with 1,000 mg/kg of urethane once or three times at two-day intervals, the incidence of lung proliferative lesions in rasH2 mice given triple doses of urethane was significantly increased, compared to that in rasH2 mice given a single dose of urethane, and the mutation frequency of the transgene in lung tumors in rasH2 mice given triple doses was lower than that in rasH2 mice given a single dose of urethane. In the present study, differential immunohistochemical expressions of Cyclin D1 and PCNA, that lead to abnormal cell proliferation and tumor development due to uncontrolled G1-S transition in the cell cycle, as well as *p53* tumor suppressor gene in pulmonary proliferative lesions obtained from our previous study were investigated. Over-expression of Cyclin D1 in hyperplasias in rasH2 mice given triple doses was significantly increased, compared to that in the single-injection group, but no significant differences in Cyclin D1 between the single and triple injection groups were observed in hyperplasias in non-Tg mice or lung tumors in either rasH2 or non-Tg mice. There were no differences in the PCNA labeling index of hyperplasias in rasH2 or non-Tg mice between the triple-injection and single-injection groups, while the PCNA labeling index tended to be increased in the tumor, compared with that in hyperplasias. There was neither mutation of *p53* nor an increase in immunoreactivity of wild type *p53* in these proliferative lesions. These results suggest that cyclin D1 over-expression in alveolar/bronchiolar hyperplasias in rasH2 mice in the triple-injection group is not only indicative of a high cell proliferation rate but also of an important role in the process of malignant transformation.

KEY WORDS: alveolar/bronchiolar tumor, cyclin D1, immunohistochemistry, rasH2 mouse, urethane.

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A transgenic (Tg) mouse carrying the human c-H-ras proto-oncogene, a rasH2 mouse, has the genetic background of C57BL/6J × BALB/cByJ F1 [27]. This Tg mouse is thought to offer promise for use as an animal model in the development of a rapid carcinogenesis testing system [5, 23, 31–33]. Urethane is a well-known mutagen and complete carcinogen that causes lung tumor at relatively large dosage such as 1,000 mg/kg in experimental animals [4, 13, 18, 24, 29]. It has been shown that rasH2 mice are more susceptible than their non-transgenic (non-Tg) littermates to lung proliferative lesions induced by urethane [20]. In addition, Ogawa *et al.* reported that the transgene in lung tumors in rasH2 mice induced by a single dose of urethane contained a point mutation at the second base of codon 61 in 80% of cases [23]. On the other hand, our previous study demonstrated that the incidence and multiplicity of lung proliferative lesions in rasH2 mice given triple-injection of 1,000 mg/kg of urethane at two-day intervals was significantly increased, compared to that in rasH2 mice given a single dose 1,000 mg/kg of urethane, and the mutation frequency of the transgene in lung tumors in rasH2 mice given triple-injection of urethane was lower than that in rasH2 mice given a single dose [21]. However, we could not clarify the reason why the mutation frequency of the oncogene was decreased in lung proliferative lesions in rasH2 mice by the

administration of triple versus single dose of urethane.

Cyclin D1, one of the cell cycle regulatory genes [9, 10, 22], is emerging as a potentially significant oncogene in lung tumors [8, 28]. By acting in conjunction with protein kinase cdk4, cyclin D1 may initiate its own inactivation due to phosphorylation of the retinoblastoma gene product (*Rb* protein) and consequently to unhindered progression of the cell to the S phase [7, 11, 28, 35]. Over-expression of cyclin D1 found in several types of experimental animals and human neoplasms suggests that the inactivation of the *Rb* pathway may be an important step for tumor development [6]. In addition, cyclin D1 forms complexes with the proliferating cell nuclear antigen (PCNA) [39], which is an auxiliary protein of DNA polymerases required for DNA replication and repair.

An extremely high incidence of mutation and aberrant expression of the *p53* tumor suppressor gene is observed in several experimental animals and human neoplasms [3, 15, 30, 34]. These abnormalities found in tumors at the restriction checkpoint result finally in the loss or suppression of *pRb* function. Therefore, it is reasonable to infer that multiple disruptions of the growth-regulatory mechanisms defined by these proteins would be related to tumor proliferation and progression [35].

In the present study, we examined differential immuno-

histochemical expression of Cyclin D1, PCNA and P53 in the pulmonary proliferative lesions in rasH2 mice induced by the administration of a single or triple-injection of urethane in our previous study [19-21].

MATERIALS AND METHODS

Male and female rasH2 mice and non-Tg littermates were injected intraperitoneally with 1,000 mg/kg of urethane once or three times at two-day intervals. Details of the experimental procedure have been published previously [21]. Briefly, five rasH2 and non-Tg mice of each sex in the single- and triple-injection groups were killed under ether anesthesia for an interim kill 10 weeks after the urethane treatment. All the surviving mice were subjected to terminal kill 15/20 weeks after single- and triple-injection urethane treatments, respectively. Lungs were embedded in paraffin, sectioned at a thickness of 4 μ m, and subjected to routine hematoxylin and eosin staining.

Immunohistochemical staining was performed to detect expression of Cyclin D1, PCNA and P53. Formalin-fixed paraffin-embedded serial sections containing pulmonary lesions were pretreated twice by microwaving at 500 W in an antigen retrieval solution (BioGenex Labs., San Ramon, CA, U.S.A.). After routine inhibition of endogenous peroxidase activity with 0.3% H₂O₂, non-specific proteins were blocked with normal goat serum. Specimens were incubated with one of the following primary antibodies overnight at 4°C: monoclonal antibody against cyclin D1 protein (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.), monoclonal antibody against PCNA (PC10, DAKO Corporation, Carpinteria, CA, U.S.A.), and polyclonal antibody reactive to wild type and mutant p53 protein (CM1, Novocastra Labs., Newcastle, UK). Immunolocalization was demonstrated using the streptavidin-biotin immunoperoxidase method (Nichirei Corporation, Tokyo, Japan) with diaminobenzidine as the substrate and Mayer's hematoxylin for counter staining. Selected formalin-fixed paraffin-embedded sections of follicular cell carcinomas from rat

thyroid glands, which are known to show over-expression of Cyclin D1 and P53 were used as positive controls. The mutation patterns of the P53 positive controls were certified by PCR single-strand conformation polymorphism (SSCP) analysis. Negative control tissues were prepared in exactly the same way as described above for each antibody, except the primary antibody was omitted and only the primary antibody-diluting buffer was used. The numbers of Cyclin D1 and PCNA positive nuclei in each lung proliferative lesion, including hyperplasias, adenomas and carcinomas, were measured using image analyzing software (KS400, Zeiss, Germany) to give percentage (%) values.

For electron microscopy, selected tumor specimens from rasH2 mice killed at 15 weeks after triple injections were fixed in isotonic buffered (pH 7.4) glutaraldehyde, post-fixed in 1.0% osmium tetroxide and embedded in epoxy resin. Ultra-thin sections were doubly stained with uranyl acetate and citrate and observed under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

The data on the percentage of Cyclin D1 and PCNA positive cells in lung proliferative lesions were analyzed by Student's *t*-test. Intergroup differences were compared between the single- and triple-injection groups.

RESULTS

The incidences of lung proliferative lesions in rasH2 mice treated with urethane are summarized in Table 1. Alveolar/bronchiolar hyperplasias and adenomas of the lung were observed in all animals in each group from week 10, and carcinomas were observed in male and female rasH2 mice in the triple-injection group from week 10 and in female non-Tg mice in the single-injection group at 15/20 weeks.

Histopathologically, tumors induced in rasH2 mice by the treatment with urethane were classified as alveolar/bronchiolar hyperplasias, adenomas or carcinomas. Alveolar/bronchiolar hyperplasias were composed of cuboidal to columnar epithelial cells arranged in papillary or glandular patterns overlying a delicate fibrovascular stroma (Fig. 1).

Table 1. Incidence of lung proliferative lesions induced by urethane in rasH2 and non-Tg mice

			10 weeks				15/20 weeks			
			No. of mice examined	Incidence (%)			No. of mice examined	Incidence (%)		
				HP	AD	CA		HP	AD	CA
Male	Tg(+)	UR-1	5	5 (100)	5 (100)	0 (0)	3	3 (100)	3 (100)	0 (0)
		UR-3	5	5 (100)	5 (100)	4 (80)	7	7 (100)	7 (100)	6 (85)
	Tg (-)	UR-1	5	5 (100)	0 (0)	0 (0)	5	5 (100)	1 (20)	0 (0)
		UR-3	5	5 (100)	4 (80)	0 (0)	6	6 (100)	6 (100)	0 (0)
Female	Tg (+)	UR-1	5	5 (100)	5 (100)	0 (0)	4	4 (100)	4 (100)	0 (0)
		UR-3	5	5 (100)	5 (100)	4 (80)	8	8 (100)	8 (100)	8 (100)
	Tg (-)	UR-1	5	5 (100)	5 (100)	0 (0)	5	5 (100)	5 (100)	1 (20)
		UR-3	5	5 (100)	4 (80)	0 (0)	9	9 (100)	9 (100)	0 (0)

Tg(+): Transgenic mice carrying human c-H-ras genes, Tg(-): non-transgenic mice.

HP: Alveolar/bronchiolar hyperplasia, AD: adenoma, CA: carcinoma

UR-1: Subjected to a single injection of 1,000 mg/kg urethane.

UR-3: Subjected to triple-injection of 1,000 mg/kg urethane.

Fig. 1. Histopathological appearance of an alveolar/bronchiolar hyperplasia characterized by papillary/glandular growth of cuboidal to columnar epithelial cells that was observed in a male rasH2 mouse killed 10 weeks after a single injection of

The cuboidal cells were relatively uniform with round to oval nuclei and moderately abundant cytoplasm. In alveolar/bronchiolar adenomas showing papillary growth, prominent projections into alveolar spaces were characteristic (Fig. 2). With alveolar/bronchiolar adenomas showing glandular patterns, tumor cells lining the alveolar walls exhibited prominent tubular profiles. Alveolar/bronchiolar carcinomas demonstrated a mixed cellular pattern, showing papillary and glandular as well as solid growth at different sites (Fig. 3). Tumor cells in areas showing papillary growth grew on a fine fibrovascular stroma, and those showing glandular patterns were evident as nests or acini. The solid tumors were characterized by solid growth of cuboidal or rounded cells with little stroma. Pleomorphic and anaplastic spindle-shaped cells were also observed in these tumors.

Immunohistochemically, over-expression of Cyclin D1 was localized within the nuclei of cells forming lung proliferative lesions. Representative cases are shown in Figs. 1B, 2B and 3B. Normal lung tissue such as that made up of broncho-alveolar cells was not stained, while a few broncho-epithelial cells were stained, corresponding to a Cyclin D1 index of less than 1%. The positive expression rates of hyperplasias in the single- and triple-injection groups were 19.7 ± 12.8 and 46.3 ± 12.2 in male rasH2 mice and 18.4 ± 9.8 and 43.6 ± 13.3 in female rasH2 mice at week 10, respectively, and 17.8 ± 14.5 and 42.1 ± 13.1 in male rasH2 mice and 15.8 ± 8.2 and 40.1 ± 12.4 in female rasH2 mice at week 15/20, respectively. The positive expression rates of hyperplasias in rasH2 mice of both sexes at week 10 and 15/20 in the triple-injection group were significantly higher than these in the single-injection group. No significant differences were observed in hyperplasias in non-Tg mice or adenomas in either rasH2 or non-Tg mice of either sex (Table 2).

A positive immunoreaction for PCNA was obtained in each proliferative lesion (Figs. 1C, 2C and 3C). The PCNA labeling index tended to be increased with tumor transformation from hyperplasias (Table 3). These lung proliferative lesions in rasH2 and non-Tg mice of both sexes given single- and triple-injection were evaluated for immunoreactivity against *p53* proteins, but there were no immunoreactive cells in any of the lesions examined.

Electron microscopic observation of alveolar/bronchiolar carcinomas in male rasH2 mice given triple-injection of urethane revealed that most of the tumor cells had microvilli on their free surface and numerous large lamellar bodies in their cytoplasm which are characteristics of alveolar type 2 cells (Fig. 4).

urethane. H&E stain, magnification, $\times 25$ (A); Immunohistochemical staining for Cyclin D1, magnification, $\times 25$ (B); Immunohistochemical staining for PCNA, magnification, $\times 25$ (C).

Fig. 2. Histopathological appearance of an alveolar/bronchiolar adenoma characterized by papillary/glandular growth of cuboidal tumor cells and slight compression of surrounding alveolar parenchyma that was observed in a male rasH2 mouse killed 15

DISCUSSION

Tg mice carrying the human c-H-ras proto-oncogene, rasH2 mice, are generally much more susceptible to both mutagenic and nonmutagenic carcinogens than control non-Tg mice [31]. In our previous study, rasH2 mice were found to be very susceptible to lung proliferative lesion induction by urethane [20], and after triple-injection with urethane, lung proliferative lesions developed in all of the treated rasH2 mice. However, the mutation frequencies of both the transgene and mouse c-Ki-ras gene were not increased [21]. Lung proliferative lesions have been frequently induced in rasH2 mice after administration of a variety of carcinogens, including *N,N'*-diethylnitrosamine, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, phenacetin, 4,4'-thiodianiline, vinyl carbamate and benzene [19, 20, 31–33]. The incidence of alveolar/bronchiolar adenomas and/or carcinomas in these chemically treated rasH2 mice is significantly higher than that in corresponding non-Tg mice. However, incidences of transgene and murine c-Ki-ras gene mutations in these carcinogen-induced lung lesions are very low [33]. These data suggest that point mutations of the transgene and murine c-Ki-ras gene do not play a major role in lung tumor induction by various carcinogens.

Cyclin D1 forms complexes with other proteins including proliferating cell nuclear antigen (PCNA) [39], which is an auxiliary protein of DNA polymerases required for DNA replication and repair. In our study, the PCNA labeling index in alveolar/bronchiolar hyperplasias in rasH2 mice in the triple-injection group was increased with the malignant transformation, and the Cyclin D1 labeling index was also significantly increased. Over-expression of Cyclin D1 in pre-malignant lesions has been reported in rodents [16, 25, 26, 38] and humans [1, 12]. It thus appears that over-expression of Cyclin D1 is a common and presumably important event that occurs in the early stages of carcinogenesis in many tissues including the lung of rasH2 mice. In immunohistochemical examinations, aberrant over-expression of Cyclin D1 was observed in alveolar/bronchiolar hyperplasias in rasH2 mice in the triple-injection group, and aberrant high Cyclin D1 and PCNA labeling indices were noted in alveolar/bronchiolar adenomas and carcinomas in rasH2 and non-Tg mice, irrespective of the frequency of the treatment of urethane.

It has been reported that a good correlation between the presence of *p53* missense mutations and nuclear reactivity in the majority of tumor cells exists for various tumors in humans and that polyclonal antibodies to *p53* provide a more consistent indicator of the presence of mutant protein than monoclonal antibodies [15, 36]. However, the present study indicates that there is neither mutation of *p53* nor an increase in immunoreactivity of wild type *p53* in these pro-

weeks after triple-injection of urethane. H&E stain, magnification, $\times 25$ (A); Immunohistochemical staining for Cyclin D1, magnification, $\times 25$ (B); Immunohistochemical staining for PCNA, magnification, $\times 25$ (C).

Fig. 3. Histopathological appearance of an alveolar/bronchiolar carcinoma, characterized by solid/papillary growth of pleomorphic and basophilic tumor cells, observed in a male rasH2 mouse killed 15 weeks after triple-injection of urethane. H&E stain; magnification, $\times 25$ (A), Immunohistochemical staining

liferative lesions associated with tumor development. The *p53* gene product exerts its regulatory function on the cell cycle through the *p21/WAF1* protein, which has an inhibitory effect on the Cyclin D1/Cyclin dependent kinase 4 (cdk4) complex [7]. This complex is responsible for the G1/S transition by phosphorylation of the *Rb* protein. High levels of Cyclin D1 result in a shorter G1 phase and may also contribute to carcinogenesis [14]. Amplification and over-expression of Cyclin D1 are observed in some experimental animals and human tumors and are associated with poor prognosis in patients with these tumors [2, 17, 37]. However, our results suggest that the *p53* protein is not important for the modulation of the Cyclin D1 protein in the lung carcinogenesis observed in rasH2 mice.

In conclusion, the data from the present study suggest that Cyclin D1 over-expression in alveolar/bronchiolar hyperplasias in rasH2 mice in the triple-injection group is not only indicative of a high cell proliferation rate but also of an important role in the process of malignant transformation.

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for Cyclin D1, magnification, $\times 25$ (B); Immunohistochemical staining for PCNA; magnification, $\times 25$ (C).

Table 2. Percentage of Cyclin D1 positive cell of lung proliferative lesions induced by urethane in rasH2 and non-Tg mice

		10 weeks						15/20 weeks					
		No. of mice examined	No. of mice with lesions	Mean(%) ± SD			No. of mice examined	No. of mice with lesions	Mean(%) ± SD				
				HP	AD	CA			HP	AD	CA		
Male	Tg(+)	UR-1	5	5	19.7±12.8	32.6±17.8	0	3	3	17.8±14.5	31.8±22.1	0	
		UR-3	5	5	46.3±12.2**	43.8±21.7	41.5±6.2	7	7	42.1±13.1**	46.7±20.7	43.4±7.8	
	Tg(−)	UR-1	5	5	19.1± 7.9	0	0	5	5	15.3± 8.8	41.2± 0	0	
		UR-3	5	5	28.4± 6.1	53.8±17.9	0	6	6	26.2± 4.1	54.7±13.8	0	
Female	Tg(+)	UR-1	5	5	18.4± 9.8	34.8±19.2	0	4	4	15.8± 8.2	31.0±19.4	0	
		UR-3	5	5	43.6±13.3**	41.3±16.9	42.2±8.6	8	8	40.1±12.4**	41.7±21.2	42.7±9.0	
	Tg(−)	UR-1	5	5	19.4±12.5	39.6±13.6	0	5	5	14.1±11.5	40.2±13.1	38.6±0	
		UR-3	5	5	24.6±11.7	51.2±10.7	0	9	9	26.2±16.7	53.0±12.9	0	

Tg(+): Transgenic mice carrying human c-H-ras genes, Tg(-): non-transgenic mice.

HP: Alveolar/bronchiolar hyperplasia, AD: adenoma, CA: carcinoma

UR-1: Subjected to a single injection of 1,000 mg/kg urethane.

UR-3: Subjected to triple-injection of 1,000 mg/kg urethane.

*, ** Significantly different from UR-1 group at $p < 0.05$ and $p < 0.01$, respectively.

Table 3. Percentage of PCNA positive cell of lung proliferative lesions induced by urethane in rasH2 and non-Tg mice

		10 weeks							15/20 weeks					
				No. of mice with lesions	Mean(%) ± SD			No. of mice with lesions		Mean(%) ± SD				
					HP	AD	CA			HP	AD	CA		
Male	Tg(+)	UR-1	5	5	6.3±2.8	16.6± 6.9	0	3	3	5.8±1.5	21.8±12.0	0		
		UR-3	5	5	8.3±2.1	23.8±10.9	21.5±6.2	7	7	7.1±1.1	26.7±10.7	23.4±7.8		
	Tg(−)	UR-1	5	5	9.1±1.9	0	0	5	5	5.3±1.2	21.3± 0	0		
		UR-3	5	5	8.2±2.8	24.8±13.1	0	6	6	6.2±1.1	24.7±13.6	0		
Famale	Tg(+)	UR-1	5	5	6.2±2.1	19.2± 5.9	0	4	4	5.6±1.3	16.0 ±6.5	0		
		UR-3	5	5	8.6±2.8	23.3±10.7	22.2±5.6	8	8	7.2±1.4	26.7 ±9.2	22.7±4.0		
	Tg(−)	UR-1	5	5	8.4±2.0	17.6± 8.6	0	5	5	4.1±0.8	17.2 ±8.1	22.8±0		
		UR-3	5	5	9.6±3.9	21.2±10.7	0	9	9	6.1±1.9	20.10±8.8	0		

Tg(+): Transgenic mice carrying human c-H-ras genes, Tg(-): non-transgenic mice.

HP: Alveolar/bronchiolar hyperplasia, AD: adenoma, CA: carcinoma

UR-1: Subjected to a single injection of 1,000 mg/kg urethane.

UR-3: Subjected to triple-injection of 1,000 mg/kg urethane.

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Fig. 4. Electron microscopic appearance of a lung alveolar/bronchiolar cell tumor in a rasH2 Tg mouse killed at 15 weeks after triple-injection of urethane. Most of the tumor cells have microvilli on their free surface and numerous large lamellar bodies in their cytoplasm, which are characteristics of alveolar type 2 cells. Uranyl acetate and lead citrate stain, Bar = 2 μ m.

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