Elevated Expression of Interleukins in Lung Adenocarcinomas Induced by N-Nitrosobis(2-hydroxypropyl)amine in Rats

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The expression of interleukins (ILs) in lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP) in rats was investigated using a multiprobe RNase protection assay (RPA) followed by densitometric quantification. Male Wistar rats, 6 weeks old, were given 2000 ppm BHP in their drinking water for 12 weeks and maintained without further treatment until they were killed at week 25. Total RNAs were extracted from 14 individual adenocarcinomas and 2 specimens of normal lung tissue of untreated rats. In adenocarcinomas, elevated expression of IL-1α (6/14), IL-1β (14/14), IL-3 (7/14), IL-4 (11/14), IL-5 (9/14), IL-6 (11/14) and IL-10 (8/14) was observed, compared with normal lung tissues. In contrast, no expression of IL-2 was detected in any case. The results suggest that preferential expression of these ILs and their complex networks may contribute to the development and progression of lung adenocarcinomas induced by BHP in rats.

Key words: Interleukin — Lung adenocarcinoma — Rat — Nitrosamine — RPA

It is considered that complex interactions between tumor cells and host inflammatory cells occur during the progression of carcinogenesis.[1–6] These involve pro-inflammatory cytokines and non-protein factors.[7, 8] The former are peptides active in signaling between cells, which are produced mainly by lymphocytes and mononuclear phagocytes.[7, 9] They are divided into several categories, such as interleukins (ILs), interferons (IFNs), the tumor necrosis factor (TNF) family and transforming growth factor βs (TGFβs). Cytokines are not only important in immune responses, but also play roles in tumor pathogenesis and progression.[10–17] Recently, it has been reported that a variety of lung tumor-derived factors, including ILs and TGFβs, may either regulate tumor growth or alter the antitumor immune response.[10–17]

Previously, we described a model for the development of non-small cell lung carcinomas (NSCLCs) in rats given N-nitrosobis(2-hydroxypropyl)amine (BHP) in drinking water; high yields of adenomatous lesions, including adenocarcinomas, were obtained.[18, 19] This model is useful for investigation of the molecular mechanisms involved in the development of lung adenocarcinomas. So far in this model it has been demonstrated that mutations of the Ki-*ras* gene but not Ha-*ras* and *p53* genes, are frequent early events in lung carcinogenesis induced by BHP,[20] and that the lesions overexpress vascular endothelial growth factor (VEGF)[21] and midkine.[22] Recently, we have found elevated expression of TGFβs and TNF family members in lung adenocarcinomas induced by BHP in rats, using a multi RNase protection assay (RPA), which has advantages for simultaneous investigation of mRNA expression of several genes (unpublished results). However, to our knowledge no systematic investigation of the expression of ILs in rat lung tumors has been performed. Therefore, in the present study, we investigated the involvement of specific ILs in the development and progression of BHP-induced rat lung adenocarcinomas by RPA.

Male Wistar rats, 5 weeks old, were purchased from Japan SLC Inc. (Shizuoka) and housed 3–5 to a plastic cage in an air-conditioned room, with a constant temperature of 25°C with a 12-h light-dark cycle. Food and water were given *ad libitum* throughout the study. After a 1-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Co., Ltd., Tokyo), the animals were given 2000 ppm BHP (Nacalai Tesque Co., Ltd., Kyoto) in their drinking water for 12 weeks and then drinking water without BHP. The animals were killed under ether anesthesia 25 weeks after the beginning of the experiment. At sacrifice, the lungs were immediately excised and grossly apparent tumors were dissected from surrounding tissue. Samples were frozen in liquid nitrogen, and stored at −80°C until analysis. Portions of the tumors were also fixed in 10% formalin for routine processing and staining of sections with hematoxylin and eosin (H&E) for histological examination.

Total RNAs were extracted from 14 individual adenocarcinomas and 2 specimens of normal lung tissue of untreated rats as controls using an ISOGENE kit (Nippon Gene, Toyama). A panel of cytokine mRNA species was

detected using a multiprobe protection assay system with the rCK-1 rat cytokine multi-probe template set (Ribo-Quant, PharMingen, San Diego, CA). Radiolabeled probes were synthesized from DNA templates containing a T7 RNA polymerase promoter (PharMingen), and transcribed in the presence of 100 μCi of [α-^{32}P]UTP to yield radioactive probes of defined sizes. Probes were hybridized with 3 μg of total RNA and resolved on 5% polyacrylamide/7 *M* urea gels at 50 W for 70 min. Dried gels were analyzed to determine band locations and phosphostimulating luminescence (PSL) values with a BAS 1000 Phospho Imaging Analyzer (Fuji Photo Film Co., Ltd., Tokyo). Within each sample, the intensity of each cytokine mRNA band was divided by the sum of the L32+glyceraldehyde 3-phosphate dehydrogenase (GAPDH) bands.[23] The size of each band was analyzed in terms of its migration distance against a plotted standard curve of migration distance vs. log nucleotide length for each undigested probe (Ribo-Quant, Instruction manual, 6th Ed., August 1999, PharMingen). The resulting value for each mRNA species was then expressed as a percentage for the parameter. Adenocarcinoma which expressed more than twice the level in normal lung tissue was defined as having elevated expression. Finally, dried gels were redeveloped overnight by traditional autoradiography.

Fourteen adenocarcinomas induced by BHP in 14 rats were used for the analysis. Two normal lung tissues of untreated rats were used as controls, because of the possibility that non-cancerous portions from BHP-treated rats may include small microscopic lesions which are undetectable macroscopically, such as not only adenocarcinomas, but also hyperplasias and adenomas. The results of RPA and the densitometric analysis data are shown in Fig. 1 and Table I. Elevated expression of IL-1α (6/14; 42.9%),

IL-1β (14/14; 100%), IL-3 (7/14; 50.0%), IL-4 (11/14; 78.6%), IL-5 (9/14; 64.3%), IL-6 (11/14; 78.6%) and IL-10 (8/14; 57.1%) was found in adenocarcinomas, as compared with normal lung tissues. Levels in adenocarcinomas were approximately 2–30 fold higher than those in normal lung tissues. In contrast, no expression of IL-2 was detected in any of the samples. The present results also confirmed the elevated expression of TNFα (13/14; 92.9%) and TNFβ (10/14; 71.4%), and the lack of expression of IFNγ in adenocarcinomas found previously (unpublished results).

It has been reported that human NSCLCs and their cell lines feature a distinct type 2 cytokine pattern, involving IL-4, IL-5 and IL-10.[13] IL-5 and IL-10 were found to be expressed in all NSCLC cell lines and IL-4 in 60%.[13] Whereas type 1 cytokines promote cell-mediated responses, type 2 cytokines stimulate immunoglobulin production and inhibit the differentiation of type 1 cells and the release of type 1 cytokines, such as IL-2 and IFNγ.[8, 24–27] IL-10 possesses several properties that may inhibit the generation of antitumor immunity,[28] including proinflammatory cytokine production by macrophages,[29, 30] T-lymphocyte proliferation[31] and type 1 cytokine production.[27] It has been suggested that IL-4-producing tumor-infiltrating lymphocytes may promote tumor production of IL-10.[13] In the present study, the lack of expression of IL-2 and IFNγ might thus have been due to the inhibitory effects of IL-4, IL-5 and IL-10 in adenocarcinomas. However, IL-4 is also known to inhibit the production of TNFα, IL-1 and IL-8,[32–34] and the present results showed clear elevation of the expression of IL-1α, IL-1β and TNFα. The findings are in line with high expression of IL-1 in NSCLC specimens, with the cellular source probably being mononuclear cells rather than tumor
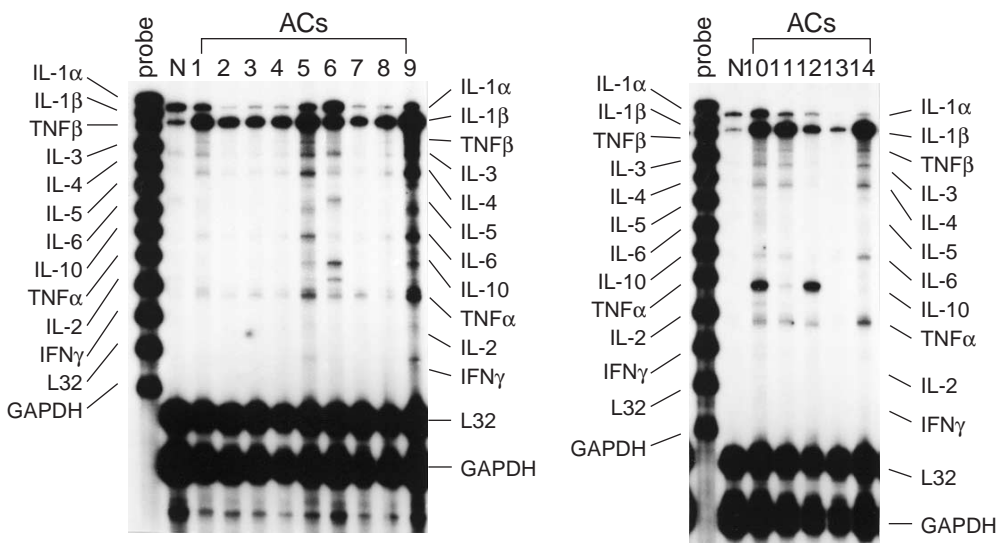

Fig. 1. A representative autoradiogram with multi probe RPA. ACs, adenocarcinomas; N, normal lung tissue; L32, L32 rRNA.

Table I. Data from Densitometric Analysis with Multi Probe RPA

Samples	Cytokines[a]										
	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-10	TNFα	TNFβ	TFNγ
N 1	6.02	2.85	ND	0.98	ND	0.27	0.15	ND	0.18	0.27	ND
2	6.61	3.31	ND	0.51	0.46	0.31	0.31	0.46	0.65	0.56	ND
AC 1	13.74	65.76	ND	3.48	3.48	1.69	1.98	0.66	3.01	3.76	ND
2	1.31	23.64	ND	0.21	0.55	ND	0.14	ND	1.03	0.62	ND
3	2.14	26.34	ND	0.41	0.33	ND	0.33	ND	2.06	1.15	ND
4	4.71	50.72	ND	1.70	1.96	0.39	1.83	0.26	3.40	2.22	ND
5	13.92	78.50	ND	6.89	7.17	3.34	5.08	3.83	6.82	7.31	ND
6	25.67	26.62	ND	2.82	1.86	2.53	1.15	3.72	2.48	2.72	ND
7	2.77	16.18	ND	0.48	0.28	ND	0.55	0.14	2.63	0.55	ND
8	3.49	38.86	ND	1.62	1.87	0.43	1.96	0.51	2.21	2.72	ND
9	7.18	95.87	ND	7.42	9.04	3.96	4.91	3.23	6.83	9.63	ND
10	24.89	71.40	ND	7.42	6.55	4.80	5.24	20.96	7.21	9.83	ND
11	14.75	118.36	ND	9.51	7.54	4.59	6.23	5.25	11.15	11.15	ND
12	13.33	47.69	ND	4.62	4.62	2.56	5.64	45.13	13.85	5.64	ND
13	3.33	18.57	ND	0.07	0.95	2.38	2.38	2.38	3.81	0.95	ND
14	6.45	105.66	ND	7.03	7.62	2.54	5.86	2.73	7.81	8.79	ND

a) (Photostimulating luminescence − background)$\times 10^2/\mathrm{mm}^2$.
N, normal lung tissue; AC, adenocarcinoma; ND, not detected.

cells *per se*.[12] IL-1 promotes angiogenesis and favors a prometastatic environment.[35] Therefore, elevated expression of IL-1α and IL-1β may contribute to the growth of adenocarcinomas throughout stromal interactions.

IL-6 has dual effects, both stimulating and inhibiting cell proliferation, dependent on the cell type.[36–38] Growth stimulation occurs in melanoma cells and renal cell carcinomas,[36] while inhibitory effects are exerted in breast cancer and lymphoma/leukemia cell lines.[37] In human lung cancer cell lines, IL-6 may also act as a growth inhibitor, although they may have relatively low IL-6 sensitivity as compared to normal bronchial epithelial cell.[15] IL-3 acts on the development and maturation of many hematopoietic cells[39] and has been reported to affect the antitumor response to mouse lung carcinomas.[40] In contrast, it was not found to cause significant and reproducible growth modulation in SCLC cell lines.[41] In the present study, we found elevated expression of IL-6 and IL-3 in adenocarcinomas, but the significance of this remains to be elucidated.

It has been reported that high expression of IL-6, IL-10 and IFNγ was seen in biopsy specimens of human NSCLCs, while the expression levels of IL-4 and IL-2 were moderate and low, respectively.[42] In human NSCLC and SCLC cell lines, the levels of several cytokines, such as IL-1β, IL-6 and TNFα, varied among different cell lines.[43] Therefore, the different patterns of cytokine expression may be dependent on the tumor cell type. It has also been reported that the cells producing the cytokines were not only the tumor-infiltrating immune cells, but also

the tumor cells *per se*.[12–15, 17, 42, 43] Further studies to examine which cells mainly produce which cytokines should be conducted, using methods such as *in situ* hybridization.

Recently, we reported that the combined administration of anti-inflammatory drugs and antibiotics inhibits the development of lung lesions induced by BHP in rats, suggesting that chronic inflammation is important for tumor growth or progression.[44] Histologically, macrophages, lymphocytes and neutrophils were seen in the alveolar walls and alveolar spaces within and around adenocarcinomas.[44] Taken together with the present findings, this suggests that preferential expression of ILs and their complex networks may contribute to the development of lung adenocarcinomas induced by BHP in rats by favoring a state of chronic inflammation.

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