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 K-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 (RiboQuant, 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-32P]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. 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 (RiboQuant, 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.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 proliferation31) 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...
cells \textit{per se}.\textsuperscript{12} IL-1 promotes angiogenesis and favors a prometastatic environment.\textsuperscript{35} Therefore, elevated expression of IL-1\(\alpha\) and IL-1\(\beta\) 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.\textsuperscript{36–38} Growth stimulation occurs in melanoma cells and renal cell carcinomas,\textsuperscript{36} while inhibitory effects are exerted in breast cancer and lymphoma/leukemia cell lines.\textsuperscript{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.\textsuperscript{15} IL-3 acts on the development and maturation of many hematopoietic cells\textsuperscript{39} and has been reported to affect the antitumor response to mouse lung carcinomas.\textsuperscript{40} In contrast, it was not found to cause significant and reproducible growth modulation in SCLC cell lines.\textsuperscript{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 IFN\(\gamma\) was seen in biopsy specimens of human NSCLCs, while the expression levels of IL-4 and IL-2 were moderate and low, respectively.\textsuperscript{42} In human NSCLC and SCLC cell lines, the levels of several cytokines, such as IL-1\(\beta\), IL-6 and TNF\(\alpha\), varied among different cell lines.\textsuperscript{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 \textit{per se}.\textsuperscript{12–15, 17, 42, 43} Further studies to examine which cells mainly produce which cytokines should be conducted, using methods such as \textit{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.\textsuperscript{44} Histologically, macrophages, lymphocytes and neutrophils were seen in the alveolar walls and alveolar spaces within and around adenocarcinomas.\textsuperscript{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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REFERENCES

1) Fidler, I. J. Macrophages and metastasis—a biological approach to cancer therapy; Presidential address. Cancer Res., 45, 4714–4726 (1985).

2) Evans, R. The immunological network at the site of tumor rejection. Biochem. Biophys. Acta, 865, 1–11 (1986).

3) Balkwill, F. R. “Cytokines in Cancer Therapy” (1991). Oxford University Press, Oxford.

4) North, R. J., Awward, J. M. and Dunn, P. The immune response to tumors. Transplant. Proc., 21, 575–577 (1989).

5) Prehn, R. T. Tumor immunogenicity: how far can it be pushed? Proc. Natl. Acad. Sci. USA, 90, 4332–4333 (1993).

6) Yamamura, M., Modlin, R. L., Ohmen, J. D. and Moya, R. Local expression of antiinflammatory cytokines in cancer. J. Clin. Invest., 91, 1005–1010 (1993).

7) Paul, W. E. Pleiotropy and redundancy: T cell-derived lymphokines in the immune response. Cell, 57, 521–524 (1989).

8) Hursting, S. D., Slaga, T. J., Fischer, S. M., DiGiovanni, J. and Phang, J. M. Mechanism-based cancer prevention approaches: targets, examples, and the use of transgenic mice. J. Natl. Cancer Inst., 91, 215–229 (1999).

9) Paul, W. E. and Seder, R. A. Lymphocyte responses and cytokines. Cell, 76, 241–251 (1994).

10) Damstrup, L., Ryagaard, K., Spang-Thomsen, M. and Poulsen, H. S. Expression of transforming growth factor β (TGFβ) receptors and expression of TGFβ1, TGFβ2 and TGFβ3 in human small cell lung cancer cell lines. Br. J. Cancer, 67, 1015–1021 (1993).

11) Jakowlew, S. B., Mathias, A., Chung, P. and Moddy, T. W. Receptor expression of transforming growth factor β ligand and receptor messenger RNAs in lung cancer cell lines. Cell Growth Differ., 6, 465–476 (1995).

12) Colasante, A., Mascetta, N., Brunetti, M., Lattanzio, G., Diodoro, M., Caltagirone, S., Musiani, P. and Aiello, F. B. Transforming growth factor β1, interleukin-8 and interleukin-1, in non-small-cell lung tumors. Am. J. Respir. Crit. Care Med., 156, 968–973 (1997).

13) Huang, M., Wang, J., Lee, P., Sharma, S., Mao, J. T., Meissner, H., Uyemura, K., Modlin, R., Wollman, J. and Dubinett, S. M. Human non-small cell lung cancer cells express a type 2 cytokine pattern. Cancer Res., 55, 3847–3853 (1995).

14) Smith, D. R., Kunkel, S. L., Burdick, M. D., Wilke, C. A., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A. and Strieter, R. M. Inhibition of interleukin-8 attenuates angiogenesis in bronchogenic carcinoma. J. Exp. Med., 179, 1409–1415 (1994).

15) Wang, J., Huang, M. and Dubinett, S. M. Autocrine production of interleukin-8 decreases lung cancer proliferation and augments lymphocyte-tumor adhesion. J. Immunol., 150, 89 (1993).

16) Konishi, Y., Denda, A., Kondo, H. and Takahashi, S. Lung carcinomas induced by oral administration of N-bis(2-hydroxypropyl)nitrosamine in rats. Jpn. J. Cancer Res., 67, 773–780 (1976).

17) Konishi, Y., Kondoh, H., Denda, A., Takahashi, S. and Inui, S. Lung carcinomas induced by oral administration of N-bis(2-hydroxypropyl)nitrosamine in rats. Mol. Carcinog., 15, 276–283 (1996).

18) Takahama, M., Tsutsujiuchi, T., Takahama, M., Fukuda, T., Narita, N. and Konishi, Y. Frequent mutations of Ki-ras but no mutations of Ha-ras and p53 in lung lesions induced by N-nitrosobis(2-hydroxypropyl)amine in rats. Mol. Carcinog., 24, 287–293 (1999).

19) Sakitani, H., Tsutsumi, M., Tsujiuchi, T., Kido, A., Sakitani, H., Iki, K., Taniguchi, S., Kitamura, S. and Konishi, Y. Expression of vascular endothelial growth factor and its receptors during lung carcinogenesis by N-nitrosobis(2-hydroxypropyl)amine in rats. Mol. Carcinog., 24, 287–293 (1999).

20) Takahama, M., Tsutsumi, M., Tsujiuchi, T., Ishikawa, K., Iki, K., Taniguchi, S., Kitamura, S. and Konishi, Y. Overexpression of midkine in lung tumors induced by N-nitrosobis(2-hydroxypropyl)amine and its increase with progression. Carcinogenesis, 20, 465–469 (1999).

21) Takahama, M., Tsutsumi, M., Tsujiuchi, T., Ishikawa, K., Ishikawa, K., Taniguchi, S., Kato, Y. and Konishi, Y. Elevated expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell carcinomas. Clin. Cancer Res., 5, 2506–2510 (1999).

22) Romagnani, S. Type 1 T helper and type 2 helper cells: functions, regulation and role in protection and disease. Int. J. Clin. Lab. Res., 21, 152–158 (1991).

23) Lederer, J. A., Liou, J. S., Todd, M. D., Glimcher, L. H. and Lichtman, A. H. Regulation of cytokine gene expression in T helper cell subsets. J. Immunol., 152, 77–87 (1994).

24) Spagnoli, G. C., Juretic, A., Schultz-Thater, E., Dellabona, P., Filgueira, L., Hörig, H., Zuber, M., Garotta, G. and Heberer, M. On the relative roles of interleukin-2 and interleukin-10 in the generation of lymphokine-activated killer cell activity. Cell. Immunol., 146, 391–405 (1993).

25) Mosmann, T. R. and Moore, K. W. The role of IL-10 in crossregulation of Th1 and Th2 responses. Immunol.
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28) Holland, G. and Zlotnik, A. I. Interleukin-10 and cancer. *Cancer Invest.*, **11**, 751–758 (1993).

29) DeWaal-Malefyt, R., Abrams, J., Bennet, B., Figdor, C. and DeVries, J. IL-10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.*, **174**, 1209–1220 (1991).

30) Wang, P., Wu, P., Siegel, M. I., Egan, R. W. and Billah, M. M. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *J. Immunol.*, **153**, 811–816 (1994).

31) Kazuyuki, T., Mostowski, H. and Tosato, G. Human interleukin-10 can directly inhibit T-cell growth. *Blood*, **81**, 2964–2971 (1993).

32) Essner, R., Rhodes, K., McBride, W. H., Morton, D. L. and Economou, J. S. IL-4 down-regulates IL-2 and TNF gene expression in human monocytes. *J. Immunol.*, **142**, 3857–3861 (1989).

33) Hart, P. H., Vitt, G. F., Burgess, D. R., Whitty, G. A., Piccoli, D. S. and Hamilton, J. A. Potential antiinflammatory effects of IL-4: suppression of human monocyte TNF, IL-1 and PGE2. *Proc. Natl. Acad. Sci. USA*, **86**, 3803–3807 (1989).

34) Standiford, T. J., Strieter, R. M., Chensue, S. W., Westwick, J., Kashara, K. and Kunkel, S. L. IL-4 inhibits the expression of IL-8 from stimulated human monocytes. *J. Immunol.*, **145**, 1435–1439 (1990).

35) Dinarello, C. A. Biologic basis for interleukin-1 in disease. *Blood*, **87**, 2095–2147 (1996).

36) Kawano, M., Hirano, T., Mitsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. Autocrine regulation and essential requirement of BSF-2/IL-6 for human multiple myelomas. *Nature*, **332**, 83–85 (1988).

37) Chen, L., Mory, Y., Zilberstein, A. and Revel, M. Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interferon-β. *Proc. Natl. Acad. Sci. USA*, **85**, 8037–8041 (1988).

38) Huggett, A. C., Ford, C. P. and Thorgerisson, S. S. Effects of interleukin 6 on the growth of normal and transformed rat liver cells in culture. *Growth Factors*, **2**, 83–89 (1989).

39) Ihle, J. N. Interleukin 3 and hematopoiesis. *Chem. Immunol.*, **51**, 65–106 (1992).

40) Berdel, W. E., Zafferani, M., Senekowitsch, R., Kreuser, E. D. and Thiel, E. Effects of interleukin-3 and granulocyte-macrophage colony-stimulating factor on growth of xenotransplanted human tumour cell lines in nude mice. *Eur. J. Cancer*, **28**, 377–380 (1992).

41) Pedrazzoli, P., Bacciocchi, G., Bergamaschi, G., Cazzola, M., Danova, M., Gibelli, N., Giordano, M., Lazzaro, A., Locatelli, F., Pavesi, L., Volpato, G., Zibera, C. and della Cuna, G. R. Effects of granulocyte-macrophage colony-stimulating factor and interleukin-3 on small cell lung cancer cells. *Cancer Invest.*, **12**, 283–288 (1994).

42) Asselin-Paturel, C., Echchakir, H., Carayol, G., Gay, F., Opolon, P., Grunenwald, D., Chouaib, S. and Mami-Chouaib, F. Quantitative analysis of Th1, Th2 and TGF-β cytokine expression in tumor, TIL and PBL of non-small cell lung cancer patients. *Int. J. Cancer*, **77**, 7–12 (1998).

43) Mizuno, K., Sone, S., Orino, E., Nii, A. and Ogura, T. Autonomous expressions of cytokine genes by human lung cancer cells and their paracrine regulation. *Jpn. J. Cancer Res.*, **85**, 179–186 (1994).

44) Tsutsumi, M., Kitada, H., Shiraawa, K., Takahama, M., Tsujitachi, T., Sakitani, H., Sasaki, Y., Murakawa, K., Yoshimoto, M. and Konishi, Y. Inhibitory effects of combined administration of antibiotics and anti-inflammatory drugs on lung tumor development initiated by N-nitrosobis(2-hydroxypropyl)amine in rats. *Carcinogenesis*, **21**, 251–256 (2000).