Increased Susceptibility of Mice Lacking Clara Cell 10-kDa Protein to Lung Tumorigenesis by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butaneone, a Potent Carcinogen in Cigarette Smoke

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Animals, Exposure, and Tissue Collection—CC10-KO mice (21) were generated by gene targeting in embryonic stem cells. The strain- and age-matched C57BL/6 WT mice (Jackson Laboratory, Bar Harbor, ME) were housed under standard conditions in a National Institutes of Health animal facility. All procedures were approved by the NCI Animal Care and Use Committee. Starting at 8 weeks of age, the mice received NNK intraperitoneally (104 mg/kg of body weight, three times, given every other month) or physiological saline, respectively. The

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\$ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

\$ The abbreviations used are: CC10, Clara cell-specific 10-kDa protein; WT, wild type; KO, knock-out; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone; SSCP, single-strand conformational polymorphism; MAPK, mitogen-activated protein kinase; Erk (and ERK), extracellular signal-regulated kinase; PBS, phosphate-buffered saline.

Ninety percent of all human lung cancers are related to cigarette smoking. Both tobacco smoke and lung tumorigenesis are associated with drastically reduced levels of Clara cell 10-kDa protein (CC10), a multifunctional secreted protein, naturally produced by the airway epithelia of virtually all mammals. We previously reported that the expression of CC10 in mice is markedly reduced in animals exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone, NNK, a potent carcinogen in tobacco smoke. Furthermore, it has been reported that CC10 expression, induced in certain tumor cells, reverses the transformed phenotype. We demonstrate here that NNK exposure of CC10-knock-out (CC10-KO) mice causes a significantly higher incidence of airway epithelial hyperplasia and lung adenomas compared with wild type (WT) littermates (30% CC10-KO versus 5% WT, \(p = 0.041\)). We also found that compared with NNK-treated WT mice, CC10-KO mice manifest increased frequency of K-ras mutation, elevated level of Fas ligand (FasL) expression, and increased MAPK/Erk phosphorylation, all of which are considered predisposing events in NNK-induced lung tumorigenesis. We propose that CC10 has a protective role against NNK-induced tumorigenesis mediated via down-regulation of the above-mentioned predisposing events.

Lung cancer is the leading cause of cancer deaths in both men and women in the United States, and 90% of all human lung cancers are related to cigarette smoking (1, 2). Both tobacco smoke (3) and lung tumorigenesis (4, 5) are associated with reduced levels of Clara cell 10-kDa (CC10) protein, a steroid-inducible, multifunctional, secreted polypeptide that accounts for ~7% of the total protein in bronchial alveolar lavage fluid (6, 7). CC10, first identified as blastokinin (8) or uteroglobin (9), is the founding member of a newly formed superfamily of proteins called Secretoglobins (10). Most of the proteins of this superfamily are tissue-specifically expressed in the secretory epithelia of virtually all organs. The human CC10 gene is mapped to chromosome 11q12.2-13.1 (11) and encodes a 16-kDa homodimeric protein in which the two identical 70-amino acid subunits are covalently linked by two disulfide bonds (6). The altered expression of CC10 (3) or single nucleotide polymorphism in the CC10 gene (9) is associated with a variety of pulmonary diseases in humans (9, 11–13). Previous studies have suggested a potential protective role of CC10 in suppressing inflammation or modulating the immune response in the lungs following pulmonary injury or infection (6, 11–13).

It has been reported that CC10 expression is rarely detectable in human non-small cell lung cancers, despite the fact that it is abundantly produced by the progenitor cells in normal airways (4, 14). Its expression is drastically reduced in SV40-induced carcinogenesis (15, 16), and it has been reported that CC10 expression induced in certain cancer cells leads to diminished invasiveness and anchorage-independent growth, characteristic of these cells (17, 18). Moreover, the overexpression of CC10 in immortalized bronchial epithelial cells delayed the induction of anchorage-independent growth in response to a potent carcinogen in cigarette smoke, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK; Refs. 5 and 17–20). Thus, it is suggested that CC10 may have a protective role against lung tumorigenesis, induced by NNK. To test this hypothesis, we exposed wild type (WT) and CC10-knockout (KO) mice to NNK and compared the rate of lung adenoma formation in these two groups of animals. In addition, we determined whether compared with WT mice NNK treatment of the CC10-KO mice caused: (a) epithelial hyperproliferation, (b) a higher incidence of mutations in the proto-oncogene, K-ras, (c) a higher level of FasL expression, and (d) increased phosphorylation of MAPK/Erk1, all of which are associated with lung tumorigenesis. Our results show that compared with the lungs of NNK-treated WT mice, those of the NNK-treated CC10-KO mice manifest: (i) a markedly higher incidence of airway epithelial hyperplasia and the formation of adenomas, (ii) a markedly increased frequency of K-ras mutations, (iii) a significantly higher level of FasL expression, and (iv) an increased phosphorylation of MAPK/Erk1. We propose that CC10 plays a critical role in protecting the lungs against NNK-induced hyperplasia and adenoma formation, most likely by suppressing the events that are known to precede tumorigenesis in this organ.

EXPERIMENTAL PROCEDURES

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animals were serially sacrificed at 5 and 6 months and at 11 and 12 months. These time periods were chosen because it has been reported that aging B6 and 129 mouse strains spontaneously develop cancers in various organs, including the lung, at a high frequency (22). Two hours prior to sacrifice, mice received 100 μg/g 5-bromo-2′-deoxyuridine (BrdUrd, Sigma) intraperitoneally. Representative tissue specimens were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, sectioned and stained, and embedded in Tissue-Tek® OCT compound (Miles Laboratories Inc., Elkhart, IN) before freezing or snap-frozen in dry ice-cooled 2-methylbutane and stored at −140 °C for molecular analysis as described below.

**Immunohistochemistry—**Immunohistochemistry was performed essentially as described previously (4) using the Vectastain ABC kit (Vector, Burlingame, CA). Primary antibodies were rabbit polyclonal antibodies anti-CC10 (diluted 1:10,000; a gift from Dr. Francesco De-Mayo, Baylor School of Medicine, Houston, TX), anti-surfactant-associated protein C (SP-C, diluted 1:1000, Chemicon International, Inc., Temecula, CA), anti-Fas (diluted 1:3000), anti-FasL (diluted 1:1000, both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-iKGF (diluted 1:1000, Novocastra, Laboratories Ltd., Newcastle, UK), and rat anti-BrdUrd monoclonal antibody (diluted 1:300, Accurate Chemical Science, NY). Expression levels of CC10, Fas, and FasL were analyzed with a Nikon Eclipse 400® microscope and Metamorph® software. Proliferation indexes were generated by counting BrdUrd or Ki67-labeled nuclei per 1000–3000 cells per animal lung with a Nikon Eclipse 4000 microscope. Statistical analyses were performed using the Mann Whitney U test.

**Immunoprecipitation and Western Blot Analysis—**Snap-frozen lung tissues were homogenized by ultrasonication in lysis buffer composed of PBS, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 1% proteinase and phosphatase inhibitor mixtures (Sigma). The levels of CC10 in the lung lysates were analyzed by immunoprecipitation as described previously (17). The levels of Erk1/2 were directly immunoblotted with a rabbit anti-ERK1 antibody (diluted 1:500; Santa Cruz Biotechnology, Inc.), and the levels of phospho-MAPK and phospho-Erk1 were assessed, respectively, using a rabbit anti-phospho-MAPK polyclonal antibody (diluted 1:1500; New England Biolabs, Inc., Beverly, MA) and a Phospho Plus Elk1 Antibody Kit (New England Biolabs, Inc.) as described in the manufacturer's procedures. The levels of CC10, Erk1, phospho-MAPK and phospho-Erk1 were quantified with a Densitometer (Pharmaceutical Biotech).

**DNA Isolation and Laser Capture Microdissection—**About 500 cells from adenomas and airway epithelia were acquired from deparaffinized sections using PixCell I (Arcturus Engineering, Mountain View, CA) laser capture microdissection. DNA was purified according to manufacturer's procedures and used for amplification of K-Ras DNA by polymerase chain reaction (PCR) as described below.

**Single-strand Conformational Polymorphism (SSCP)—**The SSCP analysis was based on the methods described previously (23). Briefly, a 143-base pair K-ras exon 1 DNA fragment was yielded by PCR using primers RasE1F (5′-TTA TTG TAA GGC CTG CTG AA-3′) and RasE1R (5′-GCA GCG TTA CCT CTG TA-3′). In addition, a 192-base pair K-ras exon 2 DNA fragment was generated with primers RasE2F (5′-TTCTCAGGACTCTACAGGA-3′) and RasE2R (5′-ACC CAC CTA TAA TGG TGA AT-3′). PCR was carried out in a 20-μl PCR reaction mixture containing 3 μl of DNA prepared by laser capture microdissection, 2 μl of 10× PCR buffer, 0.4 μl of 10 mM dNTP, 0.4 μl of a 10 μM concentration of each primer, 0.4 μl of [32P]dCTP (20 μCi/μl) (PerkinElmer Life Sciences) and 1 unit of Taq polymerase (Invitrogen). Each sample was subjected to 35 cycles, and each cycle consisted of denaturing at 94 °C for 45 s, annealing at 56 °C for 45 s, and extending at 72 °C for 90 s, with a final step at 72 °C for 8 min. An equal amount of PCR product was mixed with 2× SSCP loading buffer (98% formamide, 20 mM EDTA, pH 8, 0.1% bromphenol blue, 0.1% xylene cyanol) and denatured at 95 °C for 5 min. Four μl of each sample was loaded onto the SSCP gel (FMC BioProducts, Rockland, ME) and run at 60 volts for 16 h or 0.6× TBE at room temperature. The SSCP gels were dried and exposed to autoradiography film.

**DNA Sequencing—**Mutant DNA derived from a variant SSCP band was amplified by PCR with the primers RasE1F and RasE1R. The amplified DNA was then purified with QiAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced with a BigDye Terminator Cycle Sequencing Kit and an ABI PRISM DNA sequencer (PE Applied Biosystems, Foster City, CA).

**RESULTS**

**Morphology of Epithelial Cells in the Airways of WT and CC10-KO Mice before and after NNK Treatment—**To assess the effects of NNK on WT and CC10-KO mouse lungs, we first examined the morphology of airway epithelium and CC10 level (Fig. 1). As expected, while high levels of CC10 expression in the lungs of WT mice are readily detectable (Fig. 1A), none of the airway epithelial cells of CC10-KO mice were positive for CC10 (Fig. 1B). The Clara cells of CC10-KO mice showed a marked reduction in apical cytoplasm, normally the storage cytoplasm (hematoxylin-eosin staining). CC10-deficient epithelial cells (arrow) in a CC10-KO mouse, which demonstrates reduction in the apical cytoplasm (hematoxylin-eosin staining). E and F, high magnification (×200) of C and D. Following 5 months of NNK exposure of WT mice the airway epithelia show markedly reduced levels of CC10 immunoreactivity (G), which was further confirmed by immunoblot analysis (H). Note the reduction of CC10 protein in the lung lysates of NNK-treated WT mice (lanes 3 and 4). Photomicrographs A–D and G are of the same magnification (×100).
Susceptibility of CC10-KO Mice to NNK-induced Lung Tumorigenesis

Development of lung epithelial hyperplasia and adenomas in NNK-treated CC10-KO mice

| Treatment | Genotype (n) | Duration of treatment (months) | Mice with hyperplasia | Mice with adenomas |
|-----------|--------------|--------------------------------|-----------------------|-------------------|
| NNK       | KO(12)       | 5–8                            | 3 (33%)               | 3 (25%)           |
| NNK       | WT(12)       | 5–8                            | 0                     | 0                 |
| PBS       | KO(6)        | 5–8                            | 0                     | 0                 |
| PBS       | WT(12)       | 5–8                            | 0                     | 0                 |
| NNK       | KO(8)        | 11–12                          | 0                     | 0                 |
| NNK       | WT(10)       | 11–12                          | 0                     | 3 (38%)           |
| PBS       | KO(6)        | 11–12                          | 0                     | 1                 |
| PBS       | WT(10)       | 11–12                          | 0                     | 0                 |

n, number of mice used. One half of the lung tissue from each mouse was used to determine the tumor incidence and the other half was used for molecular studies.

Table I

TABLE I
Development of lung epithelial hyperplasia and adenomas in NNK-treated CC10-KO mice

- The susceptibility of CC10-KO mice to NNK-induced lung tumorigenesis was assessed by examining lung tissues from mice treated with NNK. The table shows the incidence of hyperplasia and adenomas in both NNK-treated KO and WT mice, as well as untreated KO and WT mice.
- At 5–8 months after NNK treatment, histopathology revealed focal airway epithelial hyperplasia in 33% (4 of 12 mice) and solitary lung adenomas in 25% (3 of 12 mice) of the CC10-KO animals (Table 1). At 11–12 months after NNK exposure, epithelial hyperplasia and small solitary lung adenomas were induced in 38% (3 of 8) of the CC10-KO mice (Table 1).
- All NNK-induced lung adenomas in CC10-KO mice were solitary, less than 1 mm in diameter, and of alveolar type II cell lineage (Fig. 2, A–C). Moreover, compared with the cells surrounding the alveoli proliferating cells in adenomas are markedly increased as suggested by an elevated BrdUrd index (Fig. 2, D and E). Only one out of 22 NNK-treated WT mice developed a solitary lung adenoma (p = 0.041). These results indicate that CC10-KO mice are more prone to developing hyperplasia and lung adenomas than their WT counterparts following exposure to NNK.

- NNK-treated CC10-KO Manifest a Markedly Elevated Level of Proliferation of Airway Epithelial Cells—We next determined whether lack of CC10 protein had changed the cellular dynamics in the pulmonary epithelia following NNK treatment by studying cell proliferation. Again, at 5–6 months after treatment, about 1.5 and 0.91% of BrdUrd-positive epithelial cells appeared in the airways and alveoli, respectively, of the NNK-treated KO mice (Fig. 3, A and B). This reflected a 3–10-fold increase in the bronchiolar epithelium, as compared with PBS-treated WT mice (data not shown). These results were confirmed by immunostaining using the cellular proliferation marker Ki-67. There was a 2–6-fold increase in the Ki-67 labeling index in the lungs of NNK-treated CC10-KO, as compared with NNK-treated WT mice, PBS-treated CC10-KO, and WT mice (data not shown). These data suggest that hyperproliferation induced by NNK in KO mice may be responsible for higher incidence of lung adenomas.

- Increased K-ras Mutation in CC10-KO Lungs—Recent reports indicate that most chemically induced lung tumors (~90%) in A/J mice carry an activating point mutation in the K-ras proto-oncogene (25, 26). To determine whether there is an increase in K-ras mutations in the lung tumors of NNK-treated KO mice, we microdissected the tissues and carried out PCR-SSCP and sequencing assays to detect mutations in exons 1 and 2 of this gene. This area contains clusters of the known transformation mutations. We found that three out of six lung adenomas (50%) in NNK-treated CC10-KO mice had K-ras mutations in codon 12 of exon 1 (supplemental Fig. 1), in which GGT was transversed to GCT, resulting in an amino acid change from glycine to alanine. A K-ras mutation at codon 32 was found in a lung adenoma of an NNK-treated WT mouse, in which TAC was transitioned to TAT, resulting in a silent mutation. All three K-ras mutations in NNK-exposed KO mice were early phase alveolar adenomas, suggesting that CC10 deficiency confers susceptibility to NNK-mediated genomic instability such as K-ras mutations, which may contribute to carcinogenesis.

- Elevated Levels of MAPK/Erk1 Phosphorylation in NNK-treated CC10-KO Mouse Lungs—Since the MAPK cascade, situated downstream from activating ras mutations, plays a critical role in regulating cell growth, proliferation, and responses to extracellular signals (27, 28), we examined the activation

Fig. 2. Photomicrographs of NNK-induced lung tumors in CC10-KO mice. A, a small solitary adenoma in the peripheral lung of a NNK-treated CC10-KO mouse. All of the NNK-induced adenomas in KO mice were solitary and less than 1 mm in diameter. Note the location in the alveolar compartment without any apparent connection with the airways (hematoxylin-eosin stain, ×15). B, higher magnification (×200) reveals a tubular adenoma with minimal atypia. C, tumor cells are positive for a type II pneumocyte marker SP-C, suggesting alveolar type II cell lineage of the tumor (immunoperoxidase stain, ×700). D, BrdUrd index in the adenomas was increased 5-fold, compared with the surrounding alveoli, while there was no significant difference in tumors or surrounding alveoli between NNK-treated KO and WT mice (immunoperoxidase staining, ×100). E, numerous tumor cells displayed Ki-67 immunoreactivity (immunoperoxidase staining, ×100). Throughout the lung tissue including adenomas, Ki-67-positive cells were observed at higher numbers than BrdUrd-positive cells.

2 Y. Yang, Z. Zhang, A. B. Mukherjee, and R. I. Linnoila, unpublished results.
Susceptibility of CC10-KO Mice to NNK-induced Lung Tumorigenesis

In this study, we provided evidence that CC10-KO mice are significantly more susceptible to developing hyperproliferation of airway epithelial cells and the formation of pulmonary adenomas in response to NNK treatment. Smoking remains a significant health hazard throughout the world, and NNK is a potent carcinogen in cigarette smoke. We discovered that mice exposed to this carcinogen express drastically lower levels of CC10 as does cigarette smoking. The results of previous studies (17–20) suggested an anti-carcinogenic role of this protein in vitro. More specifically, forced overexpression of CC10 in lung cancer cells led to diminished invasiveness and anchorage-independent growth, while the overexpression of CC10 in immortalized bronchial epithelial cells delayed the induction of anchorage-independent growth in response to NNK (17, 19). Using CC10-KO mice, we now demonstrate that CC10 possesses a protective role against NNK-induced lung tumorigenesis. Aging CC10-KO mice often develop multorgan tumors, but NNK-induced lung tumors occurred much earlier (5–12 months after exposure). Structural abnormalities as well as susceptibility to oxygen toxicity of CC10-KO mice have been reported previously (29, 30), and we have noted some of these abnormalities in the present study. Since structural specialization is related to cellular functions, the observed microscopic differences in CC10-KO mice may provide the basis for functional changes where the lack of CC10 protein hampers proper physiological activities.

Our current results further indicate that NNK exposure of CC10-KO mice manifests: (i) increase in cellular proliferation, (ii) elevated expression of FasL, (iii) increased mutation in proto-oncogene K-ras, and (iv) activation of MAPK signaling pathway, all of which are associated with lung tumorigenesis. Our results under score the necessity of further studies to establish a cause and effect relationship of these observations.

It has been reported that nitrosamine-induced lung tumors frequently reveal activated K-ras in A/J mice (25, 26, 31), while NNK-exposed C57BL6 mice, the genetic background of our CC10-KO, have a very low incidence of K-ras mutations (32).
Susceptibility of CC10-KO Mice to NNK-induced Lung Tumorigenesis

Recent studies also suggest that wild type K-ras may have a tumor-suppressive role in resistant mouse strains and MAPK may be activated in only those lung neoplasms that have both K-ras mutations and the loss of the wild type allele (33). On the other hand, activated ERK1/2 has been associated with increased proliferation and lung tumorigenesis (34). How CC10 deficiency might confer NNK-induced hyperproliferation of airway epithelial cells, formation of adenomas, elevated FasL expression, and MAPK/Erk1 phosphorylation is not yet clear. However, it has been reported that CC10 binds to cell surface proteins (putative receptors) with high affinity and specificity and regulates several biological functions of this protein (18, 35). Moreover, we have recently demonstrated that CC10 suppresses allergen-induced phosphorylation of MAPK and inhibits NF-κB activation and the expression of cyclooxygenases-2 (COX-2) in CC10-KO mice (36). Characterizations of its receptor(s) and the signaling pathways may delineate how CC10 exerts its inhibitory effects on NNK-induced lung tumorigenesis.

In summary, our data for the first time clearly demonstrate a protective role of CC10 against NNK-induced lung tumorigenesis and provide some insight into the potential role of CC10 in suppressing some of the concomitants of NNK-induced lung carcinogenesis such as increased Ras mutation, FasL expression, and MAPK phosphorylation. It is likely that the mechanism by which CC10 protects against NNK-induced cellular hyperproliferation and tumorigenesis involves the maintenance of cellular integrity and inhibition of Ras/MAPK signaling pathways.

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REFERENCES

1. Minna, J. D., Roth, J. A., and Gazdar, A. F. (2002) Cancer Cell 1, 49–52
2. Shupland, D. R. (1995) Environ. Health Perspect. 103, Suppl. 8, 131–142
3. Shijubo, N., Itoh, Y., Yamaguchi, T., Shibuya, Y., Morita, Y., Hirasawa, M., Okutani, R., Kawai, T., and Abe, S. (1997) Eur. Respir. J. 10, 1108–1114
4. Linnola, R. I., Jensen, S. M., Steinberg, S. M., Mushline, J. L., Eggleston, J. C., and Gazdar, A. F. (1992) Am. J. Clin. Pathol. 97, 233–243
5. Linnola, R. I., Shoji, E., DeMayo, F., Wittchi, H., Sabourin, C., and Malkinson, A. (2000) Ann. N. Y. Acad. Sci. 923, 249–267
6. Mukherjee, A. B., Kundu, G. C., Mantile-Selvaggi, G., Yuan, C. J., Mandal, A. K., Chattopadhyay, S., Zheng, F., Pattabiraman, N., and Zhang, Z. (1999) Cell Mol. Life Sci. 55, 771–787
7. Singh, G., and Katyal, S. L. (2000) Ann. N. Y. Acad. Sci. 923, 43–58
8. Kruhman, R. S., and Daniel, J. C. (1967) Science 158, 490–492
9. Beier, H. M. (1968) Biochim. Biophys. Acta 160, 289–291
10. Mukherjee, A. B., and Chilton, B. S. (2000) Ann. N. Y. Acad. Sci. 923, 348–354
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