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Acute damage by naphthalene triggers expression of the neuroendocrine marker PGP9.5 in airway epithelial cells

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Abstract

Protein Gene Product 9.5 (PGP9.5) is highly expressed in nervous tissue. Recently PGP9.5 expression has been found to be upregulated in the pulmonary epithelium of smokers and in non-small cell lung cancer, suggesting that it also plays a role in carcinogen-inflicted lung epithelial injury and carcinogenesis. We investigated the expression of PGP9.5 in mice in response to two prominent carcinogens found in tobacco smoke: Naphthalene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). By immunostaining, we found that PGP9.5 protein was highly expressed throughout the airway epithelium in the days immediately following a single injection of naphthalene. In contrast, PGP9.5 was exclusively confined to neurons and neuroendocrine cells in the control and NNK-exposed lungs. Furthermore, we investigated the expression of PGP9.5 mRNA in the lungs by quantitative RT-PCR (qPCR). PGP9.5 mRNA expression was highly upregulated in the days immediately following naphthalene injection and gradually returning to that of control mice 5 days after naphthalene injection. In contrast, exposure to NNK did not result in a significant increase in PGP9.5 mRNA 10 weeks after exposure. No increased expression of two other neuroendocrine markers was found in the non-neuroendocrine epithelial cells after naphthalene exposure. In contrast, immunostaining for the cell cycle regulator p27^{Kip1}, which has previously been associated with PGP9.5 in lung cancer cells, revealed transient downregulation of p27^{Kip1} in naphthalene exposed airways compared to controls, indicating that the rise in PGP9.5 in the airway epithelium is related to downregulation of p27^{Kip1}. This study is the first to specifically identify the carcinogen naphthalene as an inducer of PGP9.5 expression in non-neuroendocrine epithelium after acute lung injury and further strengthens the accumulating evidence of PGP9.5 as a central player in lung epithelial damage and early carcinogenesis.

Keywords

PGP9.5; UCHL1; naphthalene; NNK; lung; neuroendocrine

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Introduction

Protein Gene Product 9.5 (PGP9.5 aliases Ubiquitin Carboxy Hydrolase –L1, UCHL1 and PARK5) is a de-ubiquitinating enzyme involved in the stabilization of ubiquitin and inhibition of protein degradation (Wilkinson et al., 1989). The L1 iso-enzyme is abundantly expressed in neurons and neuroendocrine (NE) cells. Lack of PGP9.5 is also associated with neurodegenerative disorders such as Parkinsons disease (Liu et al., 2002; Osaka et al., 2003). In the lungs, PGP9.5-expression was long considered to be confined to neurons and pulmonary NE cells (PNECs), but a number of studies have lately demonstrated PGP9.5-expression in non-small cell lung cancer (NSCLC) cells and tumors of non-NE origin (Chen et al., 2002; Hibi et al., 1998; Hibi et al., 1999). Furthermore, a recent microarray analysis identified consistent up-regulation of PGP9.5 mRNA and protein in the normal non-NE pulmonary epithelium of healthy cigarette smokers (Carolan et al., 2006). Mechanistically, PGP9.5 has been found to associate with the Jun activation domain binding protein JAB1 and the cell cycle inhibitor p27^{Kip1} in lung cancer cells (Caballero et al., 2002). JAB1 is known to downregulate p27^{Kip1} by translocating it from the nucleus to the cytoplasm, suggesting that PGP9.5 could contribute to p27^{Kip1} degradation via interaction with JAB1.

PNECs define a rare subpopulation of mammalian pulmonary epithelial cells constituting less than 0.5% of the total cell population in the adult lung (Boers et al., 1996). PNECs are present either as solitary cells or congregated in innervated cell clusters defined as neuroepithelial bodies (NEBs). NEBs are often positioned at airway bifurcations and thought to play a role in the pulmonary response to hypoxia (Fu et al., 1999; O'Kelly et al., 1999). Hyperplasia of PNECs is frequently observed in response to acute and chronic lung damage suggesting that they play a role in the regeneration of pulmonary epithelium after injury (Gosney et al., 1989; Peake et al., 2000; Stevens et al., 1997). However, accumulating evidence shows that the NEB compartment also harbors naphthalene-resistant non-NE cells responsible for epithelial regeneration and that the repair is not directly dependent on PNEC-hyperplasia (Hong et al., 2001; Linnoila et al., 2007). PNECs are characterized by the synthesis, storage and secretion of a variety of growth stimulatory and regulatory compounds such as serotonin, calcitonin gene-related peptide (CGRP) and PGP9.5.

Naphthalene is an aromatic hydrocarbon present in tobacco smoke (Schmeltz et al., 1976). In mice, exposure to naphthalene constitutes a well-characterized model for acute airway injury by causing specific exfoliation of the non-ciliated bronchial epithelial cells (Clara cells) (Mahvi et al., 1977). Naphthalene requires metabolic activity by the Cytochrome P450 enzymes to be converted into the toxic compound causing cell death and Clara cells are specifically sensitive to naphthalene because they retain an active Cytochrome P450 system. Mechanistically, the cytochrome P450-2F2-isozyme catalyzes the oxidation of naphthalene into the highly cytotoxic naphthalene-1R, 2S epoxide (Plopper et al., 1992). In the days following naphthalene exposure, the pulmonary epithelium rapidly regenerates and this process is accompanied by hyperplasia of PNECs 3 to 5 days after naphthalene exposure (Linnoila et al., 2007; Peake et al., 2000).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is an aromatic nitrosamine component of tobacco smoke, which induces lung tumors in mice, rats and hamsters upon systemic exposure (Hecht, 1998) and has been commonly used in chemical lung carcinogenesis studies. NNK-mediated carcinogenesis is a complex process requiring the activation of NNK by metabolic enzymes including components of the Cytochrome P450 system (Weng et al., 2007). Metabolic activation of NNK converts it to a DNA-binding compound capable of forming adducts and leading to genetic mutations ultimately resulting in tumorigenesis (Akopyan and Bonavida, 2006). In contrast to the acute damage and regeneration process of the lung epithelium observed after naphthalene exposure, NNK exerts a more chronic effect

on the lung epithelium, which results in formation of tumors in rodents in the months following exposure (Weng et al., 2007; Yang et al., 2004).

The recently reported up-regulation of PGP9.5 in non-NE pulmonary epithelium in smokers (Carolan et al., 2006) suggests a role for this protein in the non-malignant lung tissue repair response to tobacco-smoke induced injury. However, the function of PGP9.5 in lung epithelial cells remains to be determined. In the current study, we examined the expression of PGP9.5 in the pulmonary epithelium of naphthalene- and NNK-exposed mice, which represent two well-established models for acute and chronic tobacco-related carcinogen exposures. We found that naphthalene induces a significant increase in PGP9.5 expression in the days immediately following the exposure, whereas no significant effect on PGP9.5 expression was detected on a longer term in mice that were exposed to NNK when compared to control animals. Furthermore, a transient downregulation of the cell cycle regulator and tumor suppressor p27^{Kip1} was observed in the Clara cells immediately following naphthalene treatment, indicating an inverse relationship between PGP9.5 and p27^{Kip1} after naphthalene exposure. These results suggest a role for PGP9.5 in the repair response of normal lung epithelium to acute epithelial injury, but not to chronic injury exerted by carcinogens in mice. It is plausible that naphthalene as a tobacco smoke component is also involved in triggering the elevated levels of PGP9.5 in the lungs of smokers.

Materials and Methods

Animals and treatment

Male FVB mice were purchased from Charles River Laboratories, Wilmington, MA, USA. Naphthalene (Sigma, St. Louis, MO, USA) was dissolved to a concentration of 30mg/ml in Mazola corn oil and a single injection (10 ml per kg of body weight) was administered intraperitoneally when the mice were two months old. Analogously, control animals received similar injections of corn oil alone. Mice were sacrificed 1, 2, 3, 4 or 5 days after the injections of naphthalene or corn oil. For NNK, the mice were given two monthly injections of 103mg of NNK (Toronto Research Chemicals, Toronto, Canada) per kg body weight intraperitoneally, starting at 2 months of age. Control animals received PBS. Mice were sacrificed at 10 weeks from the first NNK or PBS injection.

Tissue collection and immunohistochemistry

The lungs were surgically removed and 1–2 lobes were fixed overnight (18 hours) in freshly prepared neutral buffered 4% paraformaldehyde (Kiernan, 1990). The paraformaldehyde solution was replaced with 70% ethanol and tissues were embedded in paraffin and cut to 5 micron sections in a routine fashion on sialinated slides. Slides were deparaffinized, hydrated and stained using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the vendors instructions except for previously described modifications (Linnoila et al., 1988). Polyclonal rabbit-anti-CGRP (1:3000, Amersham Biosciences, Piscataway, NJ, USA) antibody was used to identify the PNECs while polyclonal rabbit-anti-PGP9.5 (1:6000, AbD Serotec, Raleigh, NC, USA) antibody was used to stain for PGP9.5 and monoclonal mouse anti p27^{Kip1} (1:1000, BD Transduction Laboratories, Brondby, Denmark) antibody was used to stain for p27^{Kip1}. Following immunohistochemical staining, the level of PNEC hyperplasia was quantified as the NE foci per airway ratio as previously described (Linnoila et al., 2007). Briefly, NE foci were determined by staining for CGRP and counting the number of solitary PNECs and NEBs composed of a cluster of ≥ 3 CGRP positive PNECs divided by the number of airways in lung sections. Differences in mean number of NE-foci between naphthalene and control treated tissues for each day were tested using two-tailed *t*-test for comparing means. To estimate the size of NEBs, digital images of NEBs were collected and the areas of CGRP- or PGP9.5-containing NEBs were measured using Metamorph® software

and differences in medians of the measurements were tested using the Mann-Whitney Rank sum test.

Tissue RNA extraction

Lung tissue for RNA-extraction was snap frozen immediately on dry ice and stored at -80°C for RNA-extraction. A combined TRIzol® (Invitrogen, Carlsbad, CA, USA) and RNeasy® (Qiagen, Valencia, CA, USA) based extraction method was used as follows. The frozen lung tissue was pulverized on dry ice using a hammer and the powdered tissue was immediately transferred to TRIzol solution on ice and lysed using a polytron homogenizer. Chloroform was added to the lysate followed by centrifugation according to instructions provided by Invitrogen. The upper aqueous phase was recovered, 1 volume 70% ethanol was added and the sample was transferred to an RNeasy-mini column and purified according to the protocol provided by Qiagen. To ensure complete removal of genomic DNA, each sample was treated with RNase-free DNase (Qiagen) during the column purification according to manufacturers instructions.

cDNA-preparation and qPCR

From each sample, cDNA was synthesized from 3µg extracted RNA with random hexamer and oligo (dT)-primers in a ratio of 2:1 using the Superscript™ III Platinum® First-strand synthesis supermix (Invitrogen).

For qPCR, the Quantitect SYBR green system (Qiagen) was used with primers specific for mouse PGP9.5 transcript (GenBank Accession no. NM_011670 Sense: 5'-ACGGCCATCTGTACGAGCTC, Antisense: 5'-CGGCAGAGAAGCGGACCTCC) or mouse β -actin transcript (GenBank Accession no. NM_007393 Sense: 5'-GGCTGTATTCCCCTCCATCG, Antisense: 5'-CCAGTTGGTAACAATGCCATGT). Both primer sets were designed to span one or more introns in order to detect contaminating genomic DNA in the samples. All reactions were performed at an annealing temperature of 55°C according to the standard protocol recommended by Qiagen. The relative expression values were determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with β -actin as endogenous control and the average value for oil/saline treated mice as the calibrator for the fold change after naphthalene/NNK treatment respectively. Differences in fold change means between days were tested using one way ANOVA with Dunnett's post hoc test, setting the fold change mean at day 5 as the reference control group.

Results

Exposure to naphthalene induces PNEC hyperplasia

In order to investigate the expression of PGP9.5 in response to acute and chronic damage by the components of tobacco smoke, pulmonary injury was inflicted by administration of either naphthalene or NNK. Control mice were treated with either corn oil (naphthalene controls) or PBS (NNK controls). The naphthalene-exposed mice were sacrificed 1, 2, 3, 4 or 5 days after injection and the number of PNECs and NEBs (collectively defined as NE foci) per airway was quantified after immunostaining for CGRP. While the number of NE foci was somewhat variable on days 1–3, exposure to naphthalene resulted in a statistically significant increase in the number of NE-foci per airway on day 4 ($p<0,05$) and day 5 ($p<0,01$) as shown in Figure 1A. In addition to the increase in the number of NE-foci, the NEBs were generally larger and contained more cells than those detected in epithelium from corn oil treated animals (data not shown). In comparison, NNK-exposed mice at 10 weeks revealed a similar number of NE-foci as their controls, which received PBS and the corn oil treated mice (naphthalene controls; Figure 1A). The NEBs in NNK-exposed mice appeared slightly larger than those in control mice, but the difference was not statistically significant (Figure 1B). Moreover, CGRP- and PGP9.5-containing NEBs were about the same size.

Enhanced expression of PGP9.5 in non-NE airway epithelial cells following naphthalene exposure

In light of the recent finding, that the expression of the NE marker PGP9.5 is also elevated in the non-NE epithelial cells in the airways of cigarette smokers (Carolan et al., 2006), we examined the pattern of PGP9.5 immunoreactivity in the airway lining cells following the exposure to naphthalene and NNK in mice. In order to identify PNECs and NEBs, sections were stained for CGRP, which is the most abundant neuropeptide in the rodent pulmonary NE system (Linnoila, 2006). Adjacent sections were stained for PGP9.5. Representative staining patterns in naphthalene-exposed mice are illustrated in Figure 2. On day 3, CGRP immunoreactivity specifically detects a solitary PNEC and NEB (Figure 2A and D). In the adjacent section both the NEB and non-NE epithelium stain positive for PGP9.5 following naphthalene exposure on day 3 (Figure 2B) while the expression is limited to a solitary PNEC and NEB in a control mouse (Figure 2E). A typical pattern of strong epithelial staining for PGP9.5 is shown also on day 4 (Figure 2C). In a corn oil treated control mouse on day 4, PGP9.5 immunoreactivity is again limited to PNECs (Figure 2F). Similar patterns for PGP9.5 expression were observed 1, 2 and 5 days post-naphthalene for both oil and naphthalene treated mice (data not shown). A different pattern was typically seen for NNK-exposed mice at 10 weeks, where the expression of PGP9.5 was restricted to PNECs and NEBs (Figure 3). Adjacent sections demonstrated immunoreactivity for both CGRP (Figure 3A) and PGP9.5 (Figure 3B) in PNECs, while the non-NE cells are negative. Similar patterns were observed in control mice that received PBS only (Figures 3C and E).

Transient increase in the expression of PGP9.5 mRNA following exposure to naphthalene

In an effort to quantify the changes in PGP9.5 gene expression after carcinogen exposure, total RNA was extracted from resected lungs of mice treated with naphthalene and NNK and the corn oil and PBS treated control animals, cDNA was synthesized and qPCR was performed. In the days immediately following treatment (days 1 and 2), PGP9.5 mRNA levels were significantly upregulated in lungs from naphthalene treated mice compared to oil treated controls ($p < 0.001$ Figure 4). On day 1 there was a five-fold increase in the PGP9.5 mRNA. By day 5 the mRNA level had returned to the control level. There was no change in the PGP9.5 mRNA expression at 10 weeks following the exposure to NNK (Figure 4).

Lower levels of p27^{Kip1}-immunoreactivity in Clara cells following exposure to naphthalene

It has been suggested that PGP9.5 forms a complex with JAB1 and p27^{Kip1} and may contribute to the regulation of p27^{Kip1}. In order to determine the status of p27^{Kip1} during the naphthalene-induced damage to airway epithelium we performed immunohistochemical staining for p27^{Kip1}. One day after naphthalene exposure, nuclear immunoreactivity was observed in the airway epithelium with stronger intensity in the NE cells (Figure 5 A and B). The reactivity in non-NE (Clara) cells was transiently reduced at day 1 following the administration of naphthalene, and gradually restored at 2 and 4 days after naphthalene (data not shown) suggesting a reverse correlation with PGP9.5 expression, while the level remained unchanged in NE cells. In contrast, immunostaining of oil-exposed epithelium demonstrated similar p27^{Kip1} staining in both NE-cells and Clara cells (Figure 5 C and D).

Discussion

Naphthalene and NNK are significant components of tobacco smoke (Hecht, 1999; Schmeltz et al., 1976), and both compounds cause injury to pulmonary epithelium. One of the responses of the airway epithelium to injury is the hyperplasia of PNECs, which are characterized by high expression of a variety of NE-markers including the ubiquitin hydrolase PGP9.5 (Lauweryns and Van Ranst, 1988; Linnoila, 2006). In the normal lung, PGP9.5 has traditionally been considered a NE and neuronal marker and also a marker for the NE malignancy SCLC,

but recent studies have discovered high expression of PGP9.5 also in non-NE pulmonary epithelium of smokers (Carolan et al., 2006; Harvey et al., 2007). Furthermore, a number of studies have identified PGP9.5 expression in tumors and serum from patients with NSCLC (Brichory et al., 2001; Chen et al., 2002; Hibi et al., 1999). These findings prompted us to evaluate the changes in PGP9.5 expression in the pulmonary epithelium of mice after exposure to naphthalene and NNK.

As expected, naphthalene was found to induce PGP9.5 positive hyperplasia of PNECs in mice. Interestingly, PGP9.5-protein was also detected in the non-NE pulmonary epithelium (Clara cells) of mice treated with naphthalene compared to controls. A transient rise in PGP9.5 mRNA expression was observed in the lungs of naphthalene treated animals in the days immediately following exposure and the expression gradually returned to control levels 5 days post-naphthalene. Minimal impact on pulmonary PGP9.5 expression or PNECs in mice was demonstrated by the exposure to NNK at 10 weeks. Naphthalene causes acute damage to the mouse airway epithelium with massive Clara cell death and epithelial exfoliation followed by days of recovery and regeneration (Mahvi et al., 1977). Here we demonstrate that PGP9.5 is highly expressed in the epithelial cells during 2 days in the immediate recovery phase following naphthalene injury. While it has previously been suggested that NE-differentiation plays an important role in the recovery of pulmonary epithelium after injury, the impact of PNECs in this process has become somewhat controversial. In the current study we observed PNEC hyperplasia at 4–5 days post-naphthalene, which is in agreement with previous studies. Traditionally, PNECs have been thought to contribute to the epithelial regeneration process, mainly because PNEC-hyperplasia has been observed after epithelial injury (Gosney et al., 1989; Peake et al., 2000; Stevens et al., 1997). However, recent research points to a potentially more complex relationship of PNECs to epithelial cells (Hong et al., 2001; Linnoila et al., 2007).

The marked up-regulation of PGP9.5 in naphthalene-exposed mouse pulmonary epithelium indicates that this NE-related protein may be involved in the early response to injury. In comparison, no difference was detected in the mRNA levels or cellular patterns of expression of other NE-markers such as Achaete-scute homologue 1 and synaptophysin (data not shown). These data point to a unique role of PGP9.5 in the epithelium, which is not limited to NE response. It is also conceivable that it is not possible to detect changes in the mRNA of other NE markers because of the very sparse population of PNECs. Our results are in agreement with human studies. Using microarray analysis with TaqMan confirmation Carolan et al. showed that out of 11 genes considered to be NE related, only PGP9.5 was consistently up-regulated in smokers compared with non-smokers (Carolan et al., 2006). Moreover, they also noted that PGP9.5 was expressed in the non-NE epithelium in smokers, while it was limited to PNECs in the non-smokers. An independent microarray study of changes in pulmonary epithelial gene expression after tobacco smoke exposure included a group of ex-smokers that were found to exhibit normal low levels of PGP9.5 in the lung (Spira et al., 2004 as discussed in Carolan et al., 2006). These results are in agreement with our findings of PGP9.5 mRNA returning to control levels 5 days after naphthalene exposure suggesting that the up-regulation might be reversible and transient in normal tissues in response to epithelial cell stress or injury.

Another study on human gene expression in the small airway epithelium in response to cigarette smoking by Harvey *et al.* reports PGP9.5 up-regulation in smokers with early airway disease. The levels of PGP9.5 were comparable to the levels of known response genes to oxidants and xenobiotics (Harvey et al., 2007). It appears that the stress exerted by the continuous exposure to tobacco smoke is a sufficient trigger to permanently elevate epithelial PGP9.5-expression. Furthermore, there is frequent overexpression of PGP9.5 in NSCLC (Chen et al., 2002; Hibi et al., 1998) with higher expression correlating with advanced stage malignancy (Hibi et al., 1999), which suggests involvement of the protein in lung carcinogenesis. It can therefore be

hypothesized, that the general increase in PGP9.5 exerted by the chronic exposure to tobacco smoke components such as naphthalene may play a pivotal role in the early stages of lung carcinogenesis. In light of our reported findings, it could be of interest to further explore the effects of lower naphthalene doses or repeated/chronic naphthalene exposure on PGP9.5-levels, especially since repeated exposure to the drug can render the lung epithelium resistant to induced injury via downregulation of naphthalene metabolism (O'Brien et al., 1989).

Unlike naphthalene, the exposure to NNK had minimal impact on PNECs or pulmonary expression of PGP9.5 in mice. We chose 10 weeks of exposure to mimic chronic exposure, but still to be able to harvest the tissues before tumor formation. However, from the present data we cannot exclude an acute stimulatory effect of NNK on PGP9.5 expression at earlier time points. NNK exerts multiple effects in the target cell one of which is downregulation of the activity of a number of genes by promoter methylation (Akopyan and Bonavida, 2006). Methylation of the promoter region of the PGP9.5 gene has been demonstrated to downregulate PGP9.5 expression in a variety of cell types (Bittencourt Rosas et al., 2001; Okochi-Takada et al., 2006). It is therefore possible that downregulation of PGP9.5-expression by promoter methylation could occur in the period immediately following NNK-exposure. In hamsters an approach analogous to the one used for NNK in the current study has been applied as a model for PNEC hyperplasia (Linnoila et al., 1981; Sunday et al., 1995) that resembles the increase in number of PNECs seen in smokers (Aguayo et al., 1989; Gosney et al., 1989). It is obvious that there are species differences, but in mice up-regulated PGP9.5 is a distinct feature of acute response to naphthalene injury and repair.

Multiple functions of PGP9.5 have been reported in different organs pointing to an increasingly complex role of the protein in normal tissues. PGP9.5 was originally characterized in neurons as a deubiquitinating enzyme involved in the release of ubiquitin monomers from target proteins, thereby mediating protein stabilization (Wilkinson et al., 1989). Conversely, PGP9.5 has more recently been shown to function as a ubiquitin ligase and a mono-ubiquitin binding protein in brain resulting in ubiquitin stabilization possibly altering several aspects of the proteasome pathway (Osaka et al., 2003; Setsuie and Wada, 2007). In mammalian cells, ubiquitination is involved in many key regulatory processes including the targeting of proteins for degradation, internalization and intracellular vesicular transport and release (Mukhopadhyay and Riezman, 2007). It remains to be established how and whether the ubiquitin modulating functions of PGP9.5 observed in neuronal cells translate into a functional role of the protein in naphthalene exposed lung epithelium. Considering the high and immediate induction of PGP9.5 gene expression post-naphthalene it can be speculated that the protein contributes to increasing the metabolic conversion of the xenobiotic drug, for instance by directly or indirectly stabilising the metabolic enzymes (including Cytochrome P450-2F2) involved in converting naphthalene into its cytotoxic counterpart. A candidate target pathway for ubiquitination by PGP9.5 in this setting could involve Keratinocyte growth factor (KGF), which was recently found to protect Clara cells from naphthalene induced injury by down-regulation of CYP450-2F2 activity (Yildirim et al., 2008) and the receptor of which is downregulated in a ubiquitin dependent manner (Belleudi et al., 2007).

In lung cancer cells, PGP9.5 has been demonstrated to bind and form a heteromeric complex with the *c-jun* co-activator JAB1 and the cyclin kinase inhibitor p27^{Kip1} (Caballero et al., 2002). Decreased expression of the tumor suppressor p27^{Kip1} is a common alteration in human cancers often due to increased ubiquitin-mediated degradation (Nicleleit et al., 2007). Moreover, JAB1 has been found to facilitate phosphorylation and translocation of p27^{Kip1} from the nucleus to the cytoplasm thereby targeting it for proteosomal degradation (Tomoda et al., 1999). Thus, in lung cancer cells PGP9.5 may be associated with increased p27^{Kip1}-degradation, resulting in increased cell cycle progression and tumorigenesis although the exact mechanism remains to be elucidated. To investigate this possible relationship further in the

lung epithelium after naphthalene induced injury, we stained for p27^{Kip1} protein in naphthalene and oil exposed lung. Interestingly, our study revealed a reverse, albeit somewhat limited, correlation with PGP9.5 and p27^{Kip1} expression in the Clara cells following naphthalene exposure. Nevertheless, in NSCLC low levels of p27^{Kip1} expression together with high expression of Ki67, which is a marker of cellular proliferation, have been associated with poor prognosis (Hommura et al., 2000). Moreover, it has been also shown that lung adenocarcinomas (NSCLC) from never smokers are associated with high levels of p27^{Kip1} (Dutu et al., 2005). In contrast to p27^{Kip1}, PGP9.5 expression is increased in lung cancer, and can be induced by smoking. Our results now suggest that this may happen through repeated exposures to naphthalene in the cigarette smoke. Taken together, it is conceivable that PGP9.5 contributes to the function of p27^{Kip1} in tumorigenesis, which in itself is a complex balance of tumor suppressor and oncogenic activities (Besson et al., 2007; Sicinski et al., 2007).

An investigation of differentially expressed genes in NSCLC by Serial Analysis of Gene Expression (SAGE) identified the transcription factor B-Myb as notably overexpressed in the majority of NSCLC together with PGP9.5 (Hibi et al., 1998), pointing to a possible regulatory role of B-myb in PGP9.5 expression. To investigate, whether the two proteins are in fact mechanistically related, Long et al. (2003) explored activation of the PGP9.5-promoter and gene expression in the presence and absence of B-myb (Long et al., 2003). They showed that B-Myb was capable of activating a 1.6 kb PGP9.5-promoter region although the mechanism of this activation appeared independent of Myb-consensus binding sites in the PGP9.5 promoter. Furthermore, transgenic mice expressing constitutively active B-Myb specifically in type II pneumocytes exhibited elevated expression of PGP9.5-protein in the lungs. Finally, co-transfection of PGP9.5 resulted in potentiation of B-Myb mediated activation of PGP9.5-promoter activity suggesting that PGP9.5 may act to stabilize B-Myb allowing for the latter to further activate PGP9.5-expression in a positive feed-back loop (Long et al., 2003). Investigations into B-myb expression and its relation to PGP9.5 expression in airway epithelium post-naphthalene await further studies. However, it can be further speculated that PGP9.5, via stabilisation of B-Myb could enhance the potentially oncogenic effects of B-Myb including promotion of cell cycle progression and resistance to apoptosis (Sala, 2005).

In summary, we find PGP9.5 to be transiently up-regulated in the lungs of naphthalene-exposed mice. Although more studies are necessary, the results presented here can prove important in further elucidation of the function of PGP9.5 in the cellular response to injury and initiation of tumorigenesis in tobacco-exposed pulmonary epithelium.

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References

- Aguayo SM, Kane MA, King TE Jr, Schwarz MI, Grauer L, Miller YE. Increased levels of bombesin-like peptides in the lower respiratory tract of asymptomatic cigarette smokers. *J. Clin. Invest* 1989;84:1105–1113. [PubMed: 2794048]
- Akopyan G, Bonavida B. Understanding tobacco smoke carcinogen NNK and lung tumorigenesis. *Int. J. Oncol* 2006;29:745–752. [PubMed: 16964372]
- Belleudi F, Leone L, Nobili V, Raffa S, Francescangeli F, Maggio M, Morrone S, Marchese C, Torrisi MR. Keratinocyte growth factor receptor ligands target the receptor to different intracellular pathways. *Traffic* 2007;8:1854–1872. [PubMed: 17944804]

- Besson A, Hwang HC, Cicero S, Donovan SL, Gurian-West M, Johnson D, Clurman BE, Dyer MA, Roberts JM. Discovery of an oncogenic activity in p27Kip1 that causes stem cell expansion and a multiple tumor phenotype. *Genes Dev* 2007;21:1731–1746. [PubMed: 17626791]
- Bittencourt Rosas SL, Caballero OL, Dong SM, da Costa Carvalho MG, Sidransky D, Jen J. Methylation status in the promoter region of the human PGP9.5 gene in cancer and normal tissues. *Cancer Lett* 2001;170:73–79. [PubMed: 11448537]
- Boers JE, den Brok JL, Koudstaal J, Arends JW, Thunnissen FB. Number and proliferation of neuroendocrine cells in normal human airway epithelium. *Am. J. Respir. Crit Care Med* 1996;154:758–763. [PubMed: 8810616]
- Brichory F, Beer D, Le Naour F, Giordano T, Hanash S. Proteomics-based identification of protein gene product 9.5 as a tumor antigen that induces a humoral immune response in lung cancer. *Cancer Res* 2001;61:7908–7912. [PubMed: 11691811]
- Caballero OL, Resto V, Patturajan M, Meerzaman D, Guo MZ, Engles J, Yochem R, Ratovitski E, Sidransky D, Jen J. Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1). *Oncogene* 2002;21:3003–3010. [PubMed: 12082530]
- Carolan BJ, Heguy A, Harvey BG, Leopold PL, Ferris B, Crystal RG. Up-regulation of expression of the ubiquitin carboxyl-terminal hydrolase L1 gene in human airway epithelium of cigarette smokers. *Cancer Res* 2006;66:10729–10740. [PubMed: 17108109]
- Chen G, Gharib TG, Huang CC, Thomas DG, Shedden KA, Taylor JM, Kardia SL, Misek DE, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG. Proteomic analysis of lung adenocarcinoma: identification of a highly expressed set of proteins in tumors. *Clin. Cancer Res* 2002;8:2298–2305. [PubMed: 12114434]
- Dutu T, Michiels S, Fouret P, Penault-Llorca F, Validire P, Benhamou S, Taranchon E, Morat L, Grunenwald D, Le CT, Sabatier L, Soria JC. Differential expression of biomarkers in lung adenocarcinoma: a comparative study between smokers and never-smokers. *Ann. Oncol* 2005;16:1906–1914. [PubMed: 16219624]
- Fu XW, Nurse CA, Wang YT, Cutz E. Selective modulation of membrane currents by hypoxia in intact airway chemoreceptors from neonatal rabbit. *J. Physiol* 1999;514(Pt 1):139–150. [PubMed: 9831722]
- Gosney JR, Sissons MC, Allibone RO, Blakey AF. Pulmonary endocrine cells in chronic bronchitis and emphysema. *J. Pathol* 1989;157:127–133. [PubMed: 2921673]
- Harvey BG, Heguy A, Leopold PL, Carolan BJ, Ferris B, Crystal RG. Modification of gene expression of the small airway epithelium in response to cigarette smoking. *J. Mol. Med* 2007;85:39–53. [PubMed: 17115125]
- Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol* 1998;11:559–603. [PubMed: 9625726]
- Hecht SS. Tobacco smoke carcinogens and lung cancer. *J. Natl. Cancer Inst* 1999;91:1194–1210. [PubMed: 10413421]
- Hibi K, Liu Q, Beaudry GA, Madden SL, Westra WH, Wehage SL, Yang SC, Heitmiller RF, Bertelsen AH, Sidransky D, Jen J. Serial analysis of gene expression in non-small cell lung cancer. *Cancer Res* 1998;58:5690–5694. [PubMed: 9865724]
- Hibi K, Westra WH, Borges M, Goodman S, Sidransky D, Jen J. PGP9.5 as a candidate tumor marker for non-small-cell lung cancer. *Am. J. Pathol* 1999;155:711–715. [PubMed: 10487828]
- Hommura F, aka-Akita H, Mishina T, Nishi M, Kojima T, Hiroumi H, Ogura S, Shimizu M, Katoh H, Kawakami Y. Prognostic significance of p27KIP1 protein and ki-67 growth fraction in non-small cell lung cancers. *Clin. Cancer Res* 2000;6:4073–4081. [PubMed: 11051259]
- Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am. J. Respir. Cell Mol. Biol* 2001;24:671–681. [PubMed: 11415931]
- Kiernan, JA. *Histological & Histochemical Methods: Theory & Practice*. Oxford, UK: Pergamon Press; 1990.

- Lauweryns JM, Van Ranst L. Protein gene product 9.5 expression in the lungs of humans and other mammals. Immunocytochemical detection in neuroepithelial bodies, neuroendocrine cells and nerves. *Neurosci. Lett* 1988;85:311–316. [PubMed: 2966308]
- Linnoila RI. Functional facets of the pulmonary neuroendocrine system. *Lab Invest* 2006;86:425–444. [PubMed: 16568108]
- Linnoila RI, Jensen-Taubman S, Kazanjian A, Grimes HL. Loss of GF11 impairs pulmonary neuroendocrine cell proliferation, but the neuroendocrine phenotype has limited impact on post-naphthalene airway repair. *Lab Invest* 2007;87:336–344. [PubMed: 17377622]
- Linnoila RI, Mulshine JL, Steinberg SM, Funa K, Matthews MJ, Cotelingam JD, Gazdar AF. Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. *Am. J. Clin. Pathol* 1988;90:641–652. [PubMed: 2848408]
- Linnoila RI, Nettesheim P, DiAugustine RP. Lung endocrine-like cells in hamsters treated with diethylnitrosamine: alterations in vivo and in cell culture. *Proc. Natl. Acad. Sci. U. S. A* 1981;78:5170–5174. [PubMed: 6946463]
- Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* 2002;111:209–218. [PubMed: 12408865]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(Delta Delta C(T))} Method. *Methods* 2001;25:402–408. [PubMed: 11846609]
- Long EM, Long MA, Tsirigotis M, Gray DA. Stimulation of the murine Uchl1 gene promoter by the B-Myb transcription factor. *Lung Cancer* 2003;42:9–21. [PubMed: 14512183]
- Mahvi D, Bank H, Harley R. Morphology of a naphthalene-induced bronchiolar lesion. *Am. J. Pathol* 1977;86:558–572. [PubMed: 842612]
- Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* 2007;315:201–205. [PubMed: 17218518]
- Nickeleit I, Zender S, Kossatz U, Malek NP. p27kip1: a target for tumor therapies? *Cell Div* 2007;2:13. [PubMed: 17488529]
- O'Brien KA, Suverkropp C, Kanekal S, Plopper CG, Buckpitt AR. Tolerance to multiple doses of the pulmonary toxicant, naphthalene. *Toxicol. Appl. Pharmacol* 1989;99:487–500. [PubMed: 2749735]
- O'Kelly I, Stephens RH, Peers C, Kemp PJ. Potential identification of the O₂-sensitive K⁺ current in a human neuroepithelial body-derived cell line. *Am. J. Physiol* 1999;276:L96–L104. [PubMed: 9887061]
- Okochi-Takada E, Nakazawa K, Wakabayashi M, Mori A, Ichimura S, Yasugi T, Ushijima T. Silencing of the UCHL1 gene in human colorectal and ovarian cancers. *Int. J. Cancer* 2006;119:1338–1344. [PubMed: 16642472]
- Osaka H, Wang YL, Takada K, Takizawa S, Setsuie R, Li H, Sato Y, Nishikawa K, Sun YJ, Sakurai M, Harada T, Hara Y, Kimura I, Chiba S, Namikawa K, Kiyama H, Noda M, Aoki S, Wada K. Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum. Mol. Genet* 2003;12:1945–1958. [PubMed: 12913066]
- Peake JL, Reynolds SD, Stripp BR, Stephens KE, Pinkerton KE. Alteration of pulmonary neuroendocrine cells during epithelial repair of naphthalene-induced airway injury. *Am. J. Pathol* 2000;156:279–286. [PubMed: 10623676]
- Plopper CG, Macklin J, Nishio SJ, Hyde DM, Buckpitt AR. Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III. Morphometric comparison of changes in the epithelial populations of terminal bronchioles and lobar bronchi in mice, hamsters, and rats after parenteral administration of naphthalene. *Lab Invest* 1992;67:553–565. [PubMed: 1434534]
- Sala A. B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis and cancer. *Eur. J. Cancer* 2005;41:2479–2484. [PubMed: 16198555]
- Schmeltz I, Tosk J, Hoffmann D. Formation and determination of naphthalenes in cigarette smoke. *Anal. Chem* 1976;48:645–650. [PubMed: 1259162]
- Setsuie R, Wada K. The functions of UCH-L1 and its relation to neurodegenerative diseases. *Neurochem. Int* 2007;51:105–111. [PubMed: 17586089]
- Sicinski P, Zacharek S, Kim C. Duality of p27Kip1 function in tumorigenesis. *Genes Dev* 2007;21:1703–1706. [PubMed: 17639075]

- Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, Palma J, Brody JS. Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc. Natl. Acad. Sci. U. S. A* 2004;101:10143–10148. [PubMed: 15210990]
- Stevens TP, McBride JT, Peake JL, Pinkerton KE, Stripp BR. Cell proliferation contributes to PNEC hyperplasia after acute airway injury. *Am. J. Physiol* 1997;272:L486–L493. [PubMed: 9124606]
- Sunday ME, Willett CG, Graham SA, Oreffo VI, Linnoila RI, Witschi H. Histochemical characterization of non-neuroendocrine tumors and neuroendocrine cell hyperplasia induced in hamster lung by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone with or without hyperoxia. *Am. J. Pathol* 1995;147:740–752. [PubMed: 7677185]
- Tomoda K, Kubota Y, Kato J. Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. *Nature* 1999;398:160–165. [PubMed: 10086358]
- Weng Y, Fang C, Turesky RJ, Behr M, Kaminsky LS, Ding X. Determination of the role of target tissue metabolism in lung carcinogenesis using conditional cytochrome p450 reductase-null mice. *Cancer Res* 2007;67:7825–7832. [PubMed: 17699788]
- Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* 1989;246:670–673. [PubMed: 2530630]
- Yang Y, Zhang Z, Mukherjee AB, Linnoila RI. Increased susceptibility of mice lacking Clara cell 10-kDa protein to lung tumorigenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a potent carcinogen in cigarette smoke. *J. Biol. Chem* 2004;279:29336–29340. [PubMed: 15148323]
- Yildirim AO, Veith M, Rausch T, Muller B, Kilb P, Van Winkle LS, Fehrenbach H. Keratinocyte growth factor protects against Clara cell injury induced by naphthalene. *Eur. Respir. J.* 2008

Figure 1. Pulmonary neuroendocrine cell hyperplasia following exposure to naphthalene and NNK

A Relative number of CGRP-containing neuroendocrine foci. A neuroendocrine focus is defined as solitary PNEC or NEB containing ≥ 3 PNECs. Foci per airway were quantified 1, 2, 3, 4 and 5 days after corn oil (white columns, control) or naphthalene (black columns) injection. On the right, neuroendocrine foci per airway following exposure to PBS (white column, control) or NNK (hatched column) for 10 weeks (Wk). Numbers in parentheses refer to the number of mice treated with naphthalene/NNK (n) or corn oil/PBS (c) in each group. Error bars denote standard deviation. A two-tailed t-test for comparing means was applied to assess the statistical significance of the difference in mean number of NE-foci in control and naphthalene treated animals for each day. (* $p < 0.05$; ** $p < 0.01$). **B** Size of CGRP- and PGP9.5-containing NEBs following NNK exposure. NEB-size was measured at 10 weeks following injection of PBS (white columns) or NNK (hatched columns). Numbers in parentheses refer to the number of NEBs measured in control/NNK-treated mice. Error bars denote standard error of the mean. Mann Whitney Rank Sum test was applied for comparing differences in median NEB-size between NNK and control treated animals and no significant difference was observed.

Figure 2. PGP9.5 is upregulated in non-neuroendocrine lung epithelium after naphthalene exposure

Immunohistochemical evaluation of mouse lung tissue following the exposure to naphthalene (N) and corn oil (O). Expression of CGRP exclusively in NEBs after 3 days of naphthalene (A) and corn oil (D) injection (arrowheads). Adjacent sections (B and E) reveal PGP9.5 immunoreactivity both in NEBs (arrowheads) and adjacent airway epithelial cells (arrows) only in the naphthalene-exposed lung (B). C Intense PGP9.5 expression 4 days after naphthalene injection in epithelial cells (arrows) and a NEB (arrowhead). F In a control lung 4 days after oil injection PGP9.5 immunoreactivity is limited to a NEB (arrowhead). Lu = airway lumen. Bar = 100µm.

Figure 3. PGP9.5-expression limited to neuroendocrine cells following NNK exposure
 Immunohistochemical evaluation of mouse lung tissue in NNK- and PBS (control)-exposed mice at 10 weeks. **A** CGRP staining was performed to identify PNECs and NEBs (arrowheads) 10 weeks after NNK exposure. **B** Adjacent, serial tissue sections were stained for PGP9.5, which revealed immunoreactivity in the same structures (arrowheads). **C** A CGRP-containing NEB in a control lung (arrowhead). **D** Two PGP9.5-containing NEBs in a PBS-exposed mouse. Lu= airway lumen.; Bar= 50 μ m.

Figure 4. PGP9.5 mRNA is upregulated after naphthalene exposure

Relative expression of PGP9.5 mRNA by quantitative RT-PCR 1–5 days after naphthalene injection (black columns) or 10 weeks after exposure to NNK (hatched column). PGP9.5 expression values were normalized to β -actin and the fold-change in expression between naphthalene and oil as well as NNK and PBS treated animals was calculated using the $2^{-\Delta\Delta C_t}$ -method (Livak and Schmittgen, 2001). A fold change of 1 represents no difference between naphthalene and oil treated samples (reference line). Numbers in parentheses refer to the number of mice treated with naphthalene/NNK (n) or corn oil/PBS (c) in each group. Error bars denote standard deviation. One-way ANOVA with Dunnett's multiple comparison test was applied to compare differences in mean fold change between day 1 and 5; 2 and 5; 3 and 5; 4 and 5 (using day 5 as reference control). Days 1 and 2 were found to be significantly different from day 5 as indicated by the vertical bars (*= $p < 0.001$).

Figure 5. p27^{Kip1} expression in the non-NE epithelium is weaker following naphthalene exposure Immunohistochemical evaluation of CGRP and p27^{Kip1} expression in adjacent mouse lung tissue sections following the exposure to naphthalene (N) and corn oil (O). CGRP staining was performed to identify PNECs and NEBs (arrowheads) 1 day after treatment with naphthalene (A) or corn oil (C). Adjacent sections (B and D) reveal nuclear p27^{Kip1} immunoreactivity mainly in NEBs (arrow-heads) after naphthalene exposure (B) while general p27^{Kip1} staining is observed in both NE and non-NE epithelium after exposure to corn oil (D). Lu = airway lumen.