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Keratinocyte growth factor protects against Clara cell injury induced by naphthalene

A.Ö. Yildirim*,¶, M. Veith*,¶, T. Rausch*, B. Müller*, P. Kilb*, L.S. Van Winkle# and H. Fehrenbach*

ABSTRACT: Airway epithelial cells are exposed to environmental toxicants that result in airway injury. Naphthalene (NA) causes site-selective damage to Clara cells in mouse distal airways. N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF) protects against acute lung injury. The present study investigated whether or not ΔN23-KGF protects against NA-induced acute Clara cell damage by measuring airway responses specifically and in order to identify underlying molecular mechanisms.

Mice were treated with ΔN23-KGF or PBS 33 h prior to injection of 200 mg·kg body weight^{-1} NA. Lung function was analysed by head-out body plethysmography. Distal airways isolated by microdissection were assessed for cell permeability using ethidium homodimer-1. Immunohistochemistry of Clara cell-specific protein in conjunction with a physical dissector was used to quantify Clara cell numbers. RNA was isolated from frozen airways in order to analyse gene expression using quantitative RT-PCR.

ΔN23-KGF prevented NA-induced airflow limitation and Clara cell permeability, and resulted in twice as many Clara cells compared with PBS pre-treatment. ΔN23-KGF-pre-treated mice exhibited increased expression of proliferating cell nuclear antigen mRNA. Cytochrome P_{450} isoform 2F2, which converts NA into its toxic metabolite, was reduced by ~50%.

The present results demonstrate that pre-treatment with N-terminally truncated recombinant human keratinocyte growth factor protects against naphthalene-induced injury. This suggests that N-terminally truncated recombinant human keratinocyte growth factor exerts its beneficial effect through a decrease in the expression of cytochrome P_{450} isoform 2F2.

KEYWORDS: Airway epithelial injury, cytochrome P_{450}, keratinocyte growth factor, naphthalene, secretoglobin

Airway injury, repair and remodelling are implicated in the pathogenesis of diverse lung diseases, such as asthma, chronic obstructive pulmonary disease and lung cancer [1]. Among the many agents that cause lung injury, hazardous air pollutants have chronic adverse effects on lung function [2], and are likely to contribute significantly to morbidity and mortality in humans [3]. The airway epithelium is composed of a few major cell types, ciliated epithelial cells, Clara cells, neuroepithelial cells, basal cells, mucous cells and serous cells [4]. It provides a selectively permeable barrier between the internal tissues and the potentially hazardous agents entering the body *via* the airways. In particular, the distal airways are exposed at high levels to various environmental oxidants, which may lead to epithelial injury in this specific region [5].

Naphthalene (NA), a harmful environmental toxin, can be found in ambient air, ground water and cigarette smoke and is produced by combustion [6]. NA is used as feedstock in various chemical industrial processes and was suggested to pose a problem in the workplaces of various industries [7]. NA, which has been found to be the most abundant polycyclic aromatic hydrocarbon in sidestream cigarette smoke [8], selectively injures nonciliated Clara cells of the conducting airways. The toxicity of NA requires metabolic activation, which is catalysed by cytochrome P_{450} monooxygenases [9]. In addition to their general secretory role [10], mouse Clara cells are the primary cellular site of cytochrome P_{450} monooxygenase isoform 2F2 (CYP2F2) [11], an enzyme with both high catalytic activity for NA and great abundance in the mouse airway [12].

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AFFILIATIONS
*Clinical Research Group ''Chronic Airway Diseases'', Medical Faculty, Philipps University of Marburg, Marburg, Germany, and
#Dept of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine and Center for Health and the Environment, University of California, Davis, CA, USA.
¶Both authors contributed equally to this study.

CORRESPONDENCE
A.Ö. Yildirim, Clinical Research Group ''Chronic Airway Diseases'', Medical Faculty, Philipps University Marburg, Hans-Meerwein Str.1, 35043 Marburg, Germany.
Fax: 49 64212866095
E-mail: ayildiri@med.uni-marburg.de

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STATEMENT OF INTEREST
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Therefore, murine Clara cells are more susceptible to NA-induced cytotoxic injury than other types of airway epithelial cell [13].

Keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF) 7, has been identified as an important paracrine mediator of proliferation, migration and differentiation of alveolar epithelial type II cells [14]. KGF binds specifically to the KGF receptor, a splice variant of FGF receptor 2 (isoform IIIb), which is expressed by epithelial cells [15]. In animal studies, KGF reduces alveolar injury and mortality in rat and/or mouse models of acute lung injury to the gas exchange area [14]. Knowledge is much more limited with regard to the role of KGF in acute airway epithelial injury and repair. KGF was demonstrated to be important for the detoxification of reactive oxygen species [16], and to enhance proliferation in airway cells [17]. Administration of KGF was shown to protect against radiation-induced increases in airway epithelial barrier permeability [18], exert protective effects against oxidant injury [19] and limit allergen-induced alterations in airway epithelial integrity [20]. Furthermore, KGF accelerated epithelial wound closure in bronchial epithelial cells cultured *in vitro* [21], and studies in the heterotopic tracheal transplant model revealed that KGF enhanced airway epithelial repair involving both local resident progenitor epithelial cells and the mobilisation and engraftment of circulating epithelial progenitor cells [22].

Despite the important function of KGF in the lung, the role of KGF in the airways is not well understood. Microarray analyses of human primary bronchial epithelial cells incubated with KGF *in vitro* revealed a decrease in the expres-sion of CYP2F1 mRNA [23]. Human CYP2F1 exhibits 80% homology with mouse CYP2F2 and is also expressed in the respiratory tract [24]. Therefore, it was hypothesised that pre-treatment of lungs with N-terminally truncated recombinant human KGF (ΔN23-KGF; palifermin), which is characterised by enhanced protein stability [25], protects against NA-induced acute Clara cell damage of the murine distal airways. In order to address this question, four experimental approaches were used: 1) distal airways from mice pre-treated with ΔN23-KGF or PBS were isolated by microdissection [26] and analysed for cell membrane integrity, cell proliferation and Clara cell number following NA-induced injury; 2) serial noninvasive lung function analysis was performed in unanaesthetised mice by means of head-out body plethysmography [27] in order to assess the functional relevance of NA-induced acute airway injury and the potential protective effect of ΔN23-KGF at the organ level; 3) the effect of KGF treatment on mRNA expression of CYP2F2, which catalyses the conversion of NA into its cytotoxic 1*R*,2*S*-oxide [12], was analysed in micro-dissected distal airways, as well as in Clara cell-enriched pulmonary epithelial cells isolated from mouse lungs; and 4) mice were therapeutically treated with ΔN23-KGF at different time-points following the injection of NA in order to test the hypothesis that the protective effect of KGF treatment was dependent upon the presence of Clara cells.

MATERIALS AND METHODS

Animals

Pathogen-free male C57BL/6 mice (Harlan Winkelmann, Hanover, Germany) weighing 25–29 g and aged 8–10 weeks were used in all of the experiments. The animals were allowed free access to food and water. All of the animals were euthanised by an over-dose of sodium pentobarbital (100 mg·kg body weight^{-1} intraperitoneally) followed by exsanguination. All animal procedures were performed according to the guidelines of good animal experimental practice of the Philipps University of Marburg (Marburg, Germany) and approved by the local authorities for animal experiments.

Application of growth factor

Mice were anaesthetised by short-term inhalation of isoflurane (Abbot, Wiesbaden, Germany). Each mouse received a single bolus of 10 mg·kg body weight^{-1} ΔN23-KGF (Amgen, Thousand Oaks, CA, USA) by oropharyngeal aspiration or an equivalent volume of PBS (80 μL). This dose was found to induce the maximal proliferative response of alveolar epithelial type II cells in mice [28]. Treatment was performed 33 h prior to intraperitoneal injection of NA or corn oil.

Application of NA

NA was purchased from Fisher (Aschaffenburg, Germany) and dissolved in corn oil. C57BL/6 mice were injected with 200 mg·kg body weight^{-1} NA or corn oil alone as vehicular control and were sacrificed 12 h later. This time-point was chosen because experiments studying the kinetics of NA-induced acute airway injury revealed that, after 12 h, Clara cells were already damaged but exfoliation was still incomplete, whereas complete exfoliation was observed 24 h after injection of NA (see online supplementary material). Distal airways were microdissected and processed for histological analysis. In order to evaluate the protective effect of ΔN23-KGF, mice were pre-treated with ΔN23-KGF or PBS. Mice were sacrificed 12 h after NA treatment and distal airways were isolated by microdissection. In order to exclude effects of circadian rhythm, all applications were performed between 08:00 and 10:00 h.

In order to evaluate whether the effect of ΔN23-KGF is primarily related to Clara cells, mice were treated with 200 mg·kg body weight^{-1} NA 2 or 24 h prior to oropharyngeal aspiration of 10 mg·kg body weight^{-1} ΔN23-KGF. Animals were sacrificed 5 days later.

Tissue preparation and microdissection

Procedures for the microdissection of defined airway generations have been described in detail previously [26]. Briefly, after sacrifice by cervical dislocation, the trachea was exposed and cannulated. The lungs were removed from the thorax and inflated with 1% low melting-point agarose (Sigma-Aldrich, Munich, Germany) dissolved in 2× Waymouth's medium (Sigma-Aldrich) and incubated in Ham's F-12 nutrient mixture (Sigma-Aldrich) for 30 min on ice in order to allow the agarose to solidify. Distal airways were isolated from the left lung of each mouse.

Assessment of cell permeability

For cell permeability studies, microdissected airways were incubated with ethidium homodimer (EH)-1 (Sigma-Aldrich) in Ham's F-12 nutrient mixture for 20 min at 37°C in order to label the nuclei of any cells with permeable membranes, as described previously [29]. Airways were washed three times with F-12 medium at 37°C to remove unincorporated EH-1 and

fixed with 330 mOsm Karnovsky's fixative. Airways were stored in fixative in the dark until embedment into glycol methacrylate (Technovit 7100; Heraeus Kulzer, Wehrheim, Germany). Embedded airways were cut into 1-μm thick sections. A water soluble nonfluorescing mounting medium, Fluoromount-G™ (Southern Biotechnology, Birmingham, AL, USA), was used.

For imaging, an epifluorescent microscope (Olympus, Tokyo, Japan) equipped with a wide-band ultraviolet (UV) fluorescence excitation–emission filter set was used. As there is a phase shift in the wide-band UV excitation–emission filter set, the tissue autofluoresces green and EH-1-positive nuclei pinkish-white.

Airway fixation

For quantitative morphological analysis, at least four microdissected distal airways from each mouse (n=3) were fixed in 1% phosphate-buffered formalin, orientated such that the resulting sections were normal to the longitudinal axis of the airways, and embedded in paraffin. For RNA isolation and subsequent gene expression analyses, three microdissected airways from each mouse (n=3) were snap frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry

As described previously [17], cross-sections of airways 2 μm in thickness were deparaffinised in xylene and rehydrated in ethanol and PBS. Endogenous peroxidase activity was inactivated using 1% hydrogen peroxide in methanol (Roth, Karlsruhe, Germany; pH 7.2) for 30 min. Antigen retrieval was performed by microwave treatment in 3% citrate buffer (Roth; pH 6.0). After washing in PBS, the sections were incubated in PBS containing 1% bovine serum albumin (Serva, Heidelberg, Germany) for 30 min followed by incubation with a polyclonal rabbit antibody (courtesy of J. Klug, Justus Liebig University, Marburg, Germany) directed against Clara cell-specific 10-kDa protein (CC10) diluted 1:3,000 in the same solution for 1 h at 37°C. Sections were then incubated with an anti-rabbit secondary antibody diluted 1:10 for 30 min at room temperature. This was visualised using 3,3′-diaminobenzidine as chromogen according to the ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) and following the manufacturer's instructions. In order to identify ciliated cells and cell proliferation, immunostaining was performed using mouse monoclonal antibodies directed against β-Tubulin-IV (Bio Genex, San Ramon, CA, USA; 1:150 dilution) and against proliferating cell nuclear antigen (PCNA; Dako Cytomation, Hamburg, Germany; 1:600 dilution), respectively. Antibodies were detected using a mouse-on-mouse immunodetection kit (Vector MOM kit; Vector Laboratories). All sections were counterstained with haematoxylin.

Quantitative–morphological analysis

The number of Clara cells was determined by design-based stereology using a physical dissector [30]. Clara cell numbers were normalised to the area of the airway epithelial basal lamina. In addition, the arithmetic mean thickness of Clara cells was estimated by point and intersection counting. Four to six paraffin-embedded airways were studied per mouse. From each airway, two serial cross-sections were cut using a motorised microtome with a block-cooling device (H 355 S; Microm, Walldorf, Germany), which was calibrated for block advance. Sections 2 μm in thickness were stained for CC10 and analysed by means of a computer-assisted Olympus BX 51 light microscope, which was equipped with a CAST-Grid System (Olympus, Ballerup, Denmark).

The arithmetic mean thickness of Clara cells ($\bar{\tau}_{CC}$) was calculated using the following equation:

$$\bar{\tau}_{CC} = 2P_{CC} \cdot l_c / \pi I_{BL}, \quad (1)$$

where P_{CC} is the sum of points that hit the Clara cells, l_c is the length per point on a cycloid grid and I_{BL} is the sum of intersections of cycloid test lines with the epithelial basal lamina.

The number of Clara cells (n_{CC}) was calculated, using Equation 2, from their numerical density (n_{CC} per volume of epithelium (V_{EPI}; $nV_{CC,EPI}$)), which was determined using Equation 3 and normalised to the surface area of the basal lamina (S_{BL}) in the same reference volume, estimated using Equation 4:

$$nS_{CC,BL} = nV_{CC,EPI} / SV_{BL,EPI} \quad (2)$$

$$nV_{CC,EPI} = 0.5ct / P_{EPI} \cdot A_{fr} \cdot H \quad (3)$$

$$SV_{BL,EPI} = \pi I_{BL} / 2l_c \cdot P_{EPI} \quad (4)$$

where $nS_{CC,BL}$ is the n_{CC} per S_{BL}, $SV_{BL,EPI}$ the S_{BL} per V_{EPI}, ct the number of nuclear profiles of CC10 immunoreactive cells in the look-up section of the dissector, which are not seen in the reference section, P_{EPI} the sum of points hitting airway epithelium, A_{fr} the area of the unbiased counting frame and H the height of the dissector (*i.e.* the calibrated mean block advance of 2 μm). Since counting was performed in both directions (double dissector), ct was multiplied by 0.5 in Equation 2.

RNA isolation and real-time RT-PCR

Total RNA was isolated from microdissected airways using the SV Total RNA Isolation System (Promega, Mannheim, Germany), and cDNA was synthesised using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed using the QuantiTect® SYBR® Green Master Mix (Abgene, Hamburg, Germany) and BioRad ICycler (BioRad Laboratories, Munich, Germany). QuantiTect® SYBR® Green Master Mix (10 μL), 1 μL of each primer at a concentration of 50 pmol·μL^{-1} and 20 μL water were added to 3 μL cDNA, standard or water (negative control). Primer sequences were generated from the respective mRNA sequences obtained from the European Molecular Biology Laboratory gene bank (Heidelberg, Germany; table E1 of online supplementary material). Primers were synthesised by MWG Biotech (Ebersberg, Germany). Change in cycle threshold analysis was used to determine expression in comparison to reduced glyceraldehye-3-phosphate dehydrogenase (GAPDH). In additional, because NA caused Clara cell exfoliation and the CYP2F2 isozyme is predominantly expressed in Clara cells, CYP2F2 mRNA expression was determined relative to CC10 mRNA expression.

Effects of ΔN23-KGF on Clara cells cultured in vitro

Mouse Clara cells were isolated from C57BL/6 mice using the protocol described by ELIZUR *et al.* [31], from which a Clara cell

purity of 70% was obtained. Briefly, the lung was perfused free of blood with PBS *via* the right ventricle, 1 mL 1% low melting-point agarose in PBS was instilled through the trachea followed by 0.5 mL 0.25% bovine pancreatic trypsin (Sigma-Aldrich). Following incubation on ice, the lungs were excised and incubated for 10 min at 37°C. The tissues were diced in medium (Dulbecco's modified Eagle medium containing 250 $\mu g \cdot mL^{-1}$ DNase and 2× antibiotic/antimycotic) filtered sequentially through 100- and 40-μm filters. The final pellet was resuspended in 10 mL medium. For experiments, cells were placed into 35-mm cell culture plates. Cells received fresh medium supplemented with either ΔN23-KGF (50 $ng \cdot mL^{-1}$) or an equivalent volume of PBS as control. After 1 and 4 h of *in vitro* culture at 37°C, the RNA Lysis buffer (SV Total RNA Isolation System) covering the cells was collected and the cells harvested for RNA isolation and subsequent real-time RT-PCR analysis as described above.

Noninvasive measurement of lung function

Immediately prior to sacrifice, mid-expiratory airflow (EF_{50}; measured in $mL \cdot s^{-1}$), a parameter suitable for monitoring broncho-obstruction [27], was measured using head-out body plethysmography as described previously [32]. Briefly, the system consisted of a glass body to which four head-out body plethysmograph chambers were attached (Forschungswerkstätten, Hanover Medical School, Hanover, Germany). A calibrated pneumo-tachograph (PTM 378/1.2; Hugo Sachs Electronics, March-Hugstetten, Germany) and a differential pressure transducer (8T-2; Gaeltec, Dunvegan, UK) coupled to an amplifier (Gould Universal Amplifier; Gould, Dietzenbach, Germany) were attached to the top port of each plethysmograph chamber. The amplified analogue signal from the pressure transducer was digitised *via* an analogue-to-digital converter (DAS-16; Keithley, Germering, Germany) at a sampling frequency of 2,000 s^{-1}. For airflow measurements, one mouse was placed into each chamber with the head protruding through a flexible neck collar so that the chamber was sealed in an airtight fashion against the main glass body. Airflow was measured during a 15-min period after the animals had resumed normal breathing. EF_{50} measured following treatment with ΔN23-KGF and/or NA were expressed as percentage changes from the mean baseline value of the respective control group.

Statistical analysis

Data are presented as mean±SD unless otherwise stated. One-way ANOVA was used to determine the significance of differences observed between groups if normality and equal variance corresponded to $p>0.1$. Otherwise, Kruskal–Wallis ANOVA on ranks was used. A p-value of <0.05 was considered significant.

RESULTS

Pre-treatment with ΔN23-KGF protects Clara cells against naphthalene-induced necrosis

Failure of cell membrane integrity was assessed using EH-1, a sensitive indicator of irreversible cell injury [29]. EH-1-positive cells, which were identified as Clara cells based on morphological characteristics, were abundant in the distal airways of mice 12 h after injection of 200 $mg \cdot kg$ body $weight^{-1}$ NA (fig. 1b), whereas EH-1-positive cells were completely absent from control mice (fig. 1a) or mice treated with ΔN23-KGF (fig. 1c). Pre-treatment of lungs with ΔN23-KGF prior to injection of NA markedly reduced the appearance of EH-1-positive cells in distal airway epithelium (fig. 1d).

ΔN23-KGF induces PCNA activation and airway epithelial proliferation

PCNA is a proliferation marker as its gene is expressed from early in the G_1 phase to the S phase of the cell cycle, with the protein confined to the nucleus. Epithelial cells showing nuclear staining for PCNA were rarely observed in untreated airways (fig. 2a). Immunoreactivity for PCNA was regularly observed in airway epithelial cells of mice 12 h after injection of NA (fig. 2b). However, PCNA-positive cells were abundant in the distal airways of lungs pre-treated with ΔN23-KGF (fig. 2c and d).

Quantitative real-time RT-PCR revealed that, in mice pre-treated with ΔN23-KGF 33 h prior to injection of NA, PCNA mRNA expression was significantly increased compared with mice injected with NA that had been pre-treated with PBS (fig. 2e).

Pre-treatment with ΔN23-KGF inhibits Clara cell loss

Clara cells were identified immunohistochemically *via* CC10, which is also known as CC16, uteroglobin or secretoglobin [33]. Clara cell numbers were analysed using design-based stereology with a physical dissector approach. Quantitative morphological analysis revealed that, 12 h after injection of NA, the number of CC10-positive Clara cells per square millimeter of basement membrane (BM; 5,047±829) was reduced to 50% of the Clara cell number characteristic of the distal airways of control mice (10,157±229 cells·mm BM^{-2}). Pre-treatment of lungs with ΔN23-KGF caused a slight increase in Clara cell number after 33 h (12,142±399 cells·mm BM^{-2}) and significantly restricted the loss of Clara cells 12 h after NA treatment (9,404±528 cells·mm BM^{-2}) to only 23% of that of lungs treated with ΔN23-KGF alone (fig. 3).

Consistent with the physical dissector counts of Clara cell numbers, real-time RT-PCR showed significantly reduced CC10 relative to GAPDH mRNA expression in the distal airways of NA-treated mice (4.4% of PBS control), whereas the CC10 mRNA level in airways of mice pre-treated with ΔN23-KGF prior to injection of NA was approximately four-fold higher (fig. 4). KGF has previously been shown to decrease CC10 mRNA expression levels in rat Clara cells [34]. Consequently, pre-treatment with ΔN23-KGF alone resulted in a reduction in CC10 mRNA expression compared with PBS-treated control mice despite the increase in proliferation and Clara cell numbers.

ΔN23-KGF downregulates cytochrome P_{450} 2F2 mRNA expression

CYP2F2 is important in the metabolism of NA since it converts NA into a cytotoxic epoxide intermediate. Administration of ΔN23-KGF with or without subsequent injection of NA resulted in significant downregulation of CYP2F2 mRNA expression (fig. 5). This was corroborated by real-time RT-PCR analyses of Clara cell-enriched lung epithelial cells isolated from mouse lungs. After 4 h of incubation of isolated Clara cells with ΔN23-KGF *in vitro*, the level of CYP2F2 mRNA

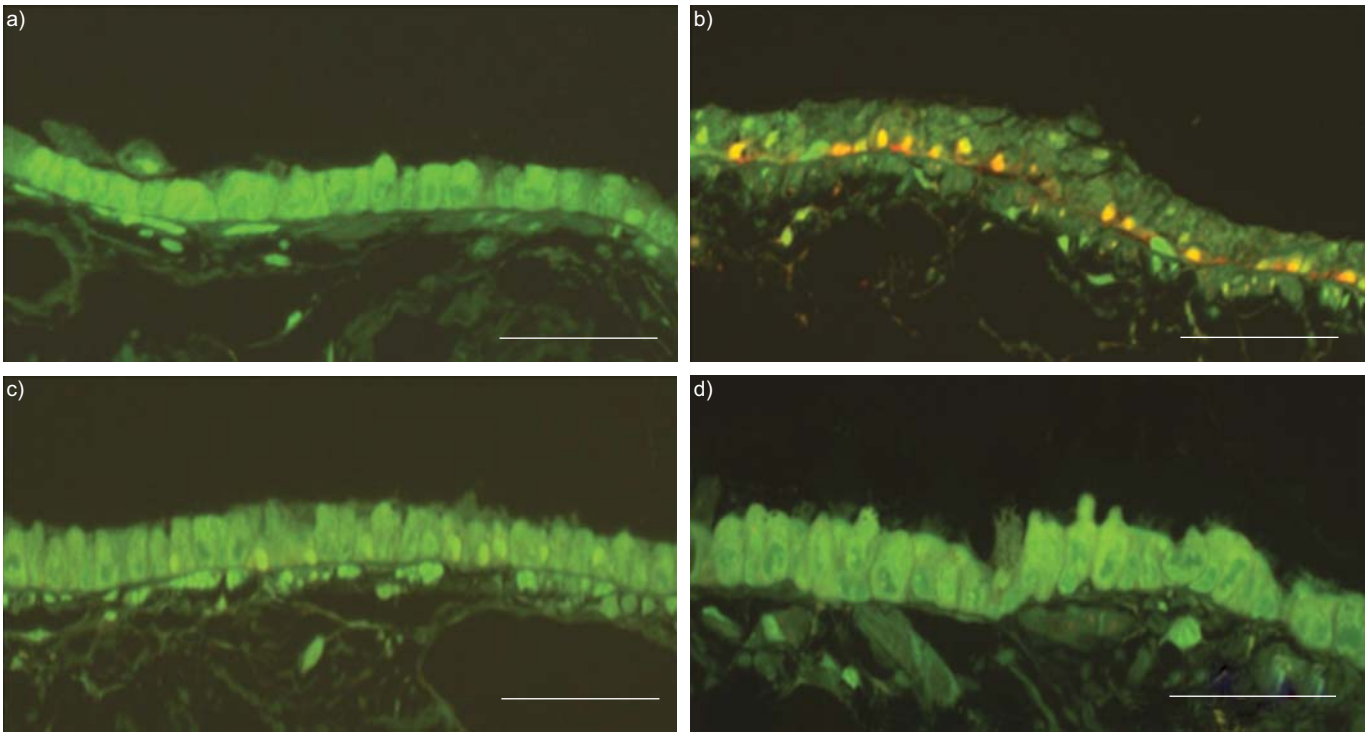

FIGURE 1. Ethidium homodimer-1 staining (red-orange) of the distal airways of mice. Mice were treated with a, c) corn oil and b, d) naphthalene (200 mg·kg body weight^{-1}) with (c and d) or without (a and b) N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; 10 mg·kg body weight^{-1}) pre-treatment. Pre-treatment with ΔN23-KGF protected Clara cells against naphthalene-induced necrosis. The images are representative of four distal airways per mouse (n=3 mice per group). Scale bar=50 μm.

expression was significantly decreased (fig. 6). Since NA resulted in a significant loss of Clara cells 12 h after injection, normalisation of CYP2F2 to GAPDH mRNA expression is problematic in that a reduction in CYP2F2 mRNA expression might simply reflect the loss of Clara cells. Therefore, CYP2F2 mRNA expression was instead normalised to CC10 mRNA expression. Since treatment with ΔN23-KGF alone reduces CC10 expression, this procedure is likely to result in underestimation rather than overestimation of the effect of ΔN23-KGF on CYP2F2.

Pre-treatment with ΔN23-KGF prevents naphthalene-induced impairment of lung function

In order to assess the functional relevance of NA-induced acute airway injury and of the effect of ΔN23-KGF, airflow was investigated by means of head-out body plethys-mography. At 33 h following administration, ΔN23-KGF or PBS alone had no significant effect on EF_{50} compared with EF_{50} recorded immediately prior to pre-treatment (0 h), 0.74 ± 0.18 mL·s^{-1} in PBS treated mice and 0.78 ± 0.43 mL·s^{-1} in mice treated with ΔN23-KGF. However, 12 h after injection of NA, there was a significant reduction in EF_{50} recorded from mice pre-treated with PBS, whereas EF_{50} did not change in mice pre-treated with ΔN23-KGF (fig. 7).

Therapeutic effect of ΔN23-KGF after naphthalene-induced airway epithelial injury depends on the presence of Clara cells

In order to test the hypothesis that the protective effect of ΔN23-KGF is primarily related to Clara cells, treatment with ΔN23-KGF was performed 2 h after injection of NA, when Clara cells are already injured but still present, or 24 h after injection, a time-point at which Clara cells are almost completely lost from the distal airway epithelium (fig. E1 of online supplementary material). In mice treated with ΔN23-KGF 2 h after injection of NA, Clara cell numbers were significantly higher than in mice injected with NA and only slightly and insignificantly lower than in control mice injected with corn oil (fig. 8). In contrast, in mice receiving ΔN23-KGF 24 h after injection of NA, Clara cell numbers were similar to those found in mice injected with NA and significantly lower than in control mice injected with corn oil.

DISCUSSION

KGF is a potent mitogen for epithelial cells and has been demonstrated to protect the lung against acute injuries of the pulmonary parenchyma in various animal models [14]. However, its potential for protecting against airway epithelial injury has not been well characterised. Therefore, the aim of the present study was to investigate whether or not pre-treatment with ΔN23-KGF or palifermin, which results in enhanced KGF protein stability [25], protects the airway epithelium against NA-induced acute airway injury *in vivo*. Three experimental approaches were used in addressing this question. These approaches defined different aspects of the cell biology of the early injury process, the effect of treatment on pulmonary function and the effect of KGF treatment on the biology of the airway epithelium. It was found that ΔN23-KGF prevented the NA-induced loss of cell membrane integrity, increased airway epithelial cell proliferation and largely reduced the loss of Clara cells, as well as the impairment of

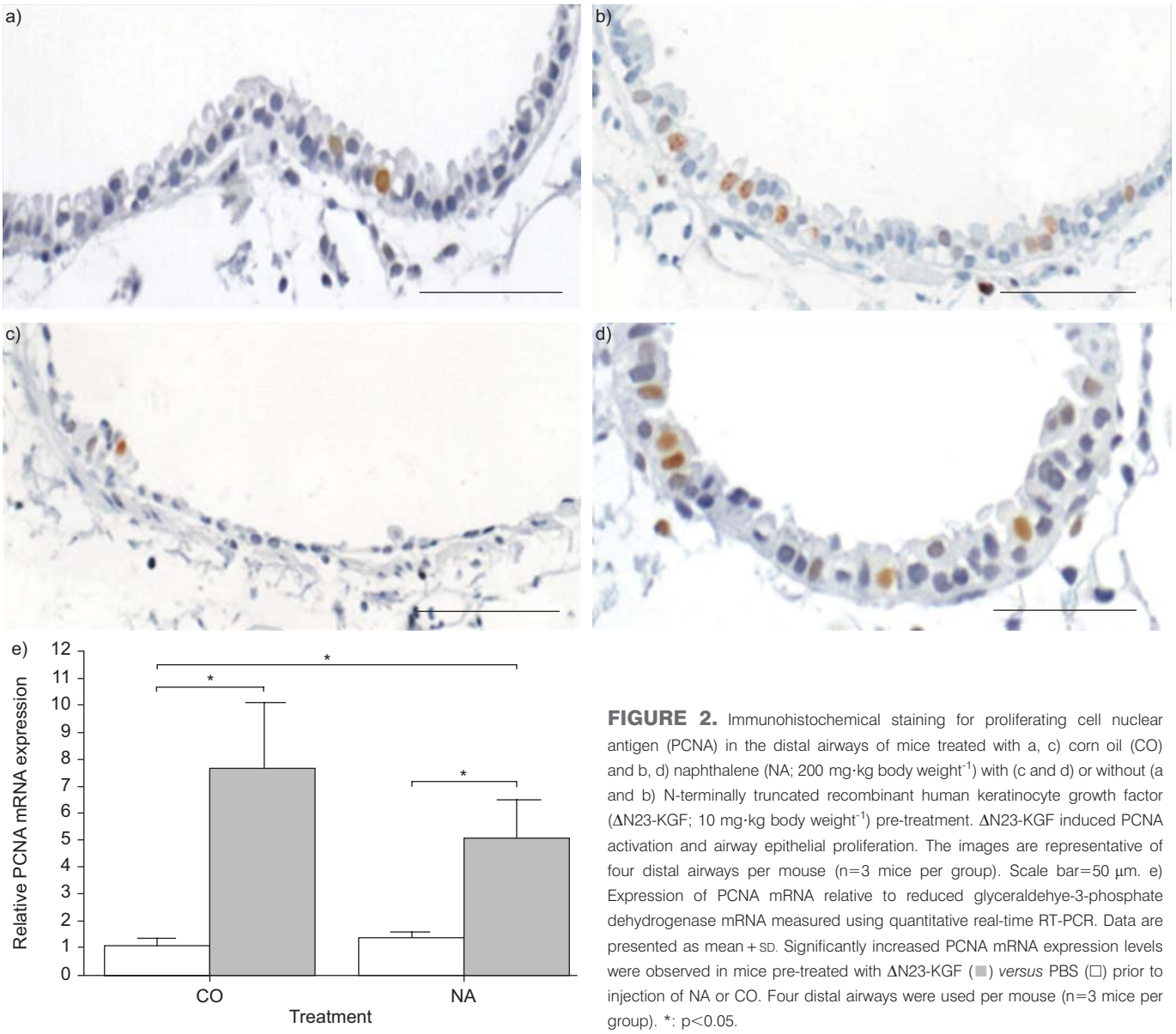

FIGURE 2. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in the distal airways of mice treated with a, c) corn oil (CO) and b, d) naphthalene (NA; 200 mg·kg body weight^{-1}) with (c and d) or without (a and b) N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; 10 mg·kg body weight^{-1}) pre-treatment. ΔN23-KGF induced PCNA activation and airway epithelial proliferation. The images are representative of four distal airways per mouse (n=3 mice per group). Scale bar=50 μm. e) Expression of PCNA mRNA relative to reduced glyceraldehye-3-phosphate dehydrogenase mRNA measured using quantitative real-time RT-PCR. Data are presented as mean + SD. Significantly increased PCNA mRNA expression levels were observed in mice pre-treated with ΔN23-KGF (■) *versus* PBS (□) prior to injection of NA or CO. Four distal airways were used per mouse (n=3 mice per group). *: $p<0.05$.

lung function due to NA-induced acute airway injury. The decrease in CYP2F2 mRNA expression in microdissected distal airways, as well as in isolated Clara cells, indicates that ΔN23-KGF exerted its protective effects, at least in part, through downregulation of this NA-metabolising xenobiotic enzyme. The failure of ΔN23-KGF to ameliorate NA-induced injury when applied 24 h after injection of NA, *i.e.* at a time-point at which Clara cells are almost completely lost from the distal airway epithelium, demonstrates that the protective effect of ΔN23-KGF is primarily related to Clara cells.

The NA model of airway epithelial cell injury has been well established in the mouse [13, 35]. The high susceptibility to NA cytotoxicity has been revealed to be associated with the expression of CYP2F2 in Clara cells of the distal airway epithelium of the mouse [12]. Although the cytotoxic effect of NA had been extensively investigated in Swiss Webster [9, 36] and FVB mice [37–40], little was known about NA-induced injury to the distal airway epithelium of C57BL/6 mice [41, 42], this being particularly true for the early time course. In order to define the optimal time-point for the analysis of potential protective effects of ΔN23-KGF against NA-induced Clara cell injury, the kinetics of NA-induced effects on the distal airway epithelium were investigated first. The sequence of events in C57BL/6 mice following injection of 200 mg·kg body weight^{-1} NA was similar to that seen in Swiss Webster mice. At 3 h after injection of NA, Clara cells exhibited slight swelling and onset of vacuolisation, with exfoliation being almost complete by 24 h, which is in line with previous findings in C57BL/6 mice [41, 42]. After 12 h, most of the Clara cells were swollen and contained vacuoles, but only half of them were lost by exfoliation. Therefore, this time-point was chosen for revealing the potential protective effects of ΔN23-KGF on NA-induced Clara cell injury.

Previous studies have adapted EH-1 for use on microdissected airways and demonstrated it to be a reliable probe for cells

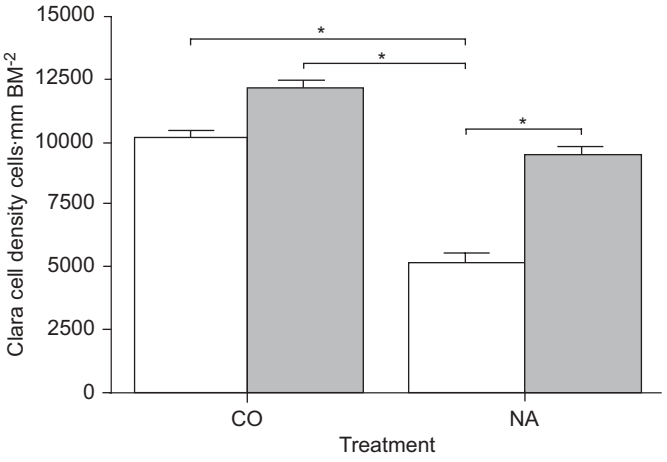

FIGURE 3. Design-based stereological estimation of the number of Clara cell-specific 10-kDa protein-positive Clara cells in distal airways obtained from mice pre-treated with PBS (□) or N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; ■) prior to injection of corn oil (CO) or naphthalene (NA). Data are presented as mean + SD. Pre-treatment with ΔN23-KGF inhibited Clara cell loss. Four distal airways were used per mouse (n=3 mice per group). BM: basement membrane. *: $p<0.05$.

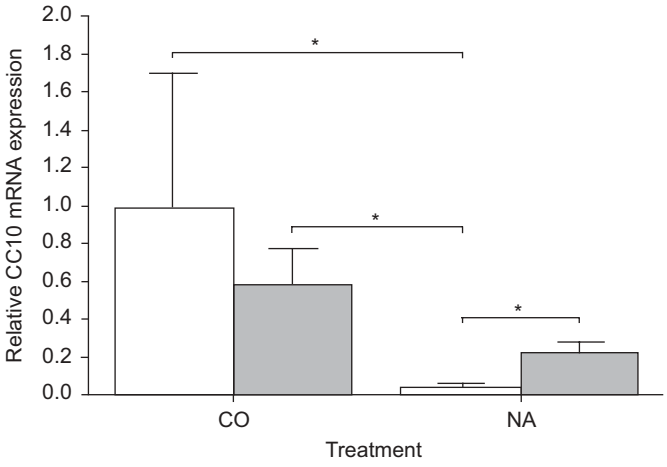

FIGURE 4. Quantitative real-time RT-PCR analysis using Clara cell-specific 10-kDa protein (CC10)-specific primer pairs. CC10 mRNA expression was determined relative to that of reduced glyceraldehye-3-phosphate dehydrogenase. Total RNA was isolated from mice pre-treated with PBS (□) or N-terminally truncated recombinant human keratinocyte growth factor (■) prior to injection of corn oil (CO) or naphthalene (NA). Data are presented as mean + SD. Three distal airways were used per mouse (n=3 mice per group). *: $p<0.05$.

undergoing necrosis [43]. Intraperitoneal injection of NA resulted in a marked increase in EH-1-permeable Clara cells by 6 h after injection in Swiss Webster mice [29], and 12 h after injection in C57BL/6 mice in the present study, indicating that NA-induced exfoliation of Clara cells was due to necrosis. Pre-treatment of C57BL/6 mice with ΔN23-KGF largely abolished the incorporation of EH-1 into Clara cell nuclei, which suggests that ΔN23-KGF protects Clara cells of the distal airways against NA-induced necrotic cell death. As a consequence, in mice pre-treated with ΔN23-KGF, only 23% of the Clara cells were lost 12 h after injection of NA, whereas 50% were lost in mice pre-treated with control vehicle, as demonstrated using design-based quantitative morphology [30]. Similarly, after injection of NA in mice pre-treated with PBS, CC10 mRNA expression was reduced to <5% of that of control mice, whereas, in mice pre-treated with ΔN23-KGF, expression of CC10 mRNA was 38% of that recorded in controls treated with ΔN23-KGF alone. The reduction in CC10 mRNA expression in ΔN23-KGF-treated animals is probably a consequence of the decrease in the number of CC10 transcripts per cell, demonstrated by *in situ* hybridisation studies [34], rather than a result of Clara cell exfoliation. The marked loss of Clara cells following injection of NA is likely to affect the barrier function of the airway epithelium and KGF can be suspected to maintain airway epithelial integrity, which is essential to, for example, lung fluid balance, host defence, and clearance or metabolism of inhaled agents [10]. This is in line with *in vitro* studies that have demonstrated KGF to protect monolayers of bronchial epithelial cells against loss of barrier function induced by hydrogen peroxide [19] or irradiation [18].

Head-out body plethysmography was used to assess the effects of airborne chemicals on respiratory function [44]. The present study demonstrated for the first time that acute airway epithelial damage caused by NA was associated with significant airway dysfunction, indicating that the highly specific injury to Clara cells resulted in significant functional impairment at the organ level. Whether this is the result of changes in airway permeability or inflammation, a direct effect of NA on airway smooth muscle or an indirect effect due to the alterations in the airway epithelium remains to be elucidated. Pre-treatment of mice with ΔN23-KGF 33 h prior to NA-induced airway injury almost completely prevented the impairment of EF_{50}. In previous studies, comparing noninvasive measurement of EF_{50} with invasive measurement of pulmonary conductance (G_L) and dynamic compliance, very good agreements between EF_{50}, G_L and dynamic compliance were observed [45, 46]. During airway constriction, the main changes in the tidal flow signal, recorded by head-out body plethysmography, occur during the mid-expiratory phase. Therefore, the EF_{50} was defined as the tidal flow at the mid-point (50%) of expiratory tidal volume [46]. Consequently, EF_{50} is closely linked to tidal volume and expiration time. However, EF_{50} was clearly demonstrated to be independent of respiratory frequency [45]. Only a slight and insignificant decrease in EF_{50} was observed 33 h after instillation of ΔN23-KGF, whereas modest-but-significant changes in lung resistance and forced expiratory airflow were seen by invasive lung function analysis 3 days after treatment with ΔN23-KGF in rats [47]. These differences may relate to the fact that noninvasive assessment of lung function is less sensitive than invasive measurement of respiratory function parameters in anaesthetised and intubated animals [27], although the possibility that there may be quantitative differences between mice and rats in their response to ΔN23-KGF cannot be ruled out.

Clara cells are the preferred progenitor cell for regeneration of the distal airway epithelium following injury [9, 37, 38]. Transdifferentiation of ciliated cells has been suggested to significantly contribute to epithelial repair following NA-induced injury [40]. Recently, however, RAWLINS *et al.* [42] suggested that a small population of variant Clara cells, which

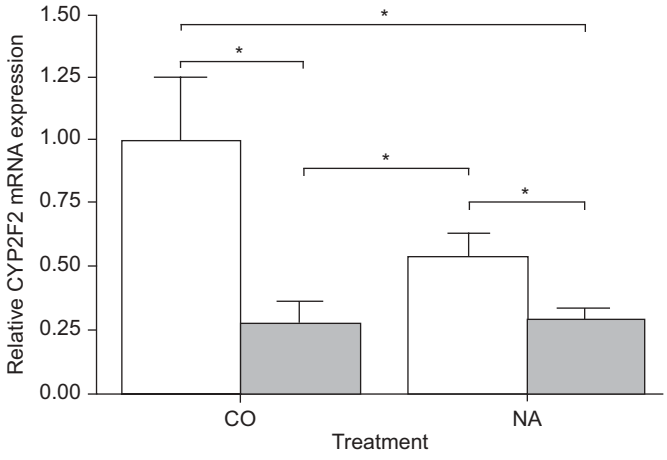

FIGURE 5. Quantitative real-time RT-PCR analysis using primer pairs specific for cytochrome P_{450} isozyme 2F2 (CYP2F2). CYP2F2 mRNA expression was determined relative to that of Clara cell-specific 10-kDa protein. Total RNA was isolated from mice pre-treated with PBS (□) or N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; ■) prior to injection of corn oil (CO) or naphthalene (NA). Data are presented as mean + SD. ΔN23-KGF downregulated CYP2F2 mRNA expression *in vivo*. Three distal airways were used per mouse (n=3 mice per group). *: $p<0.05$.

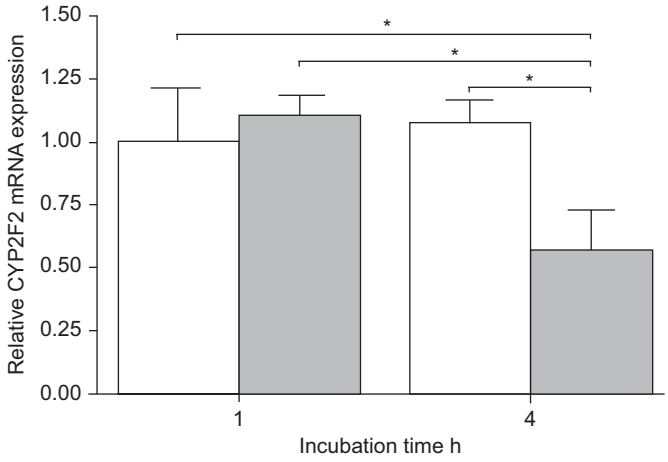

FIGURE 6. Quantitative real-time RT-PCR analysis using primer pairs specific for cytochrome P_{450} isozyme 2F2 (CYP2F2). CYP2F2 mRNA expression was determined relative to that of Clara cell-specific 10-kDa protein. Total RNA was obtained from Clara cell-enriched lung epithelial cells isolated from the lungs of C57BL/6 mice. Isolated cells were harvested after 1 and 4 h of incubation with PBS (□) or cell culture medium that contained 50 ng·mL^{-1} N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; ■). Data are presented as mean + SD. ΔN23-KGF downregulated CYP2F2 mRNA expression *in vitro*. n=3 mice per group. *: $p<0.05$.

survive after NA injury, are most probably the progenitors of Clara cells following NA injury. Their data suggest that ciliated cells do not transdifferentiate into Clara cells. Human recombinant KGF was shown to stimulate DNA synthesis and proliferation of human bronchial epithelial cells *in vitro* and of rat and mouse airway epithelium *in vivo*. It has previously been demonstrated that the KGF-induced stimulation of distal airway epithelial proliferation was restricted to Clara cells, and peaked between days 1 and 2 following intratracheal instillation of KGF into rat lungs *in vivo* [17]. In the present study, a similar strong induction of mouse Clara cell proliferation in response to exogenous ΔN23-KGF was observed, as evidenced by both PCNA immunohistochemistry and quantitative real-time RT-PCR for detection of PCNA mRNA expression. Consequently, Clara cell numbers were increased by ~20% in mice 33 h after treatment with ΔN23-KGF compared with control treated mice. At 12 h after NA treatment, no significant proliferation of airway epithelium was observed in C57BL/6 mice treated with NA alone. However, proliferation of distal airway epithelium was demonstrated at later time-points, and was shown to peak at day 2 after injection of NA into Swiss Webster mice [9, 37]. Notably, the induction of this endogenous repair process was recently suggested to be related to the upregulation of airway epithelial expression of KGF receptor at day 1 after NA-induced injury [48].

In rats, Northern blot analysis of whole lung homogenate revealed that administration of KGF *in vivo* led to a significant decrease in CC10 mRNA expression on days 2 and 3 compared with vehicle-treated control lungs and then gradually recovered to normal values [34]. In the present study, treatment with ΔN23-KGF resulted in a slight, albeit insignificant, decrease in CC10 mRNA expression in the distal airways of C57BL/6 mice, which might relate to the earlier time-point examined in the present study. In rats, the decrease in CC10 mRNA levels appeared to be due to decreased expression per cell, as assessed by *in situ* hybridisation [34]. Correspondingly, CC10, assessed by quantitative immunohistochemistry, exhibited decreased immunoreactivity per cell in distal rat airway epithelium [17]. In response to intratracheal administration of exogenous KGF *in vivo*, a quantitative shift from Clara cells exhibiting normal strong staining for CC10 to them exhibiting weak immunoreactivity for CC10 was observed. The findings of these studies suggested that the reduction in CC10 mRNA and protein

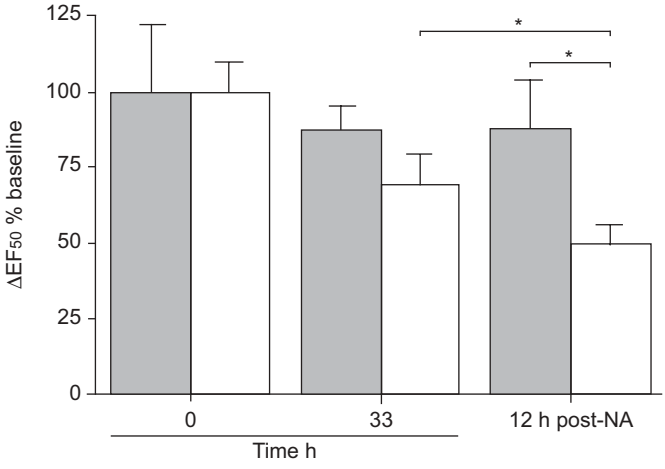

FIGURE 7. Noninvasive head-out body plethysmography of mice prior to (0 h; baseline) and 33 h after pre-treatment with N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; ■) or PBS (□), and 12 h after the injection of naphthalene (NA) following pre-treatment. Data are presented as mean + SD. Pre-treatment with ΔN23-KGF prevented NA-induced impairment of lung function. n=6 mice per group. ΔEF_{50}: change in mid-expiratory airflow. *: $p<0.05$.

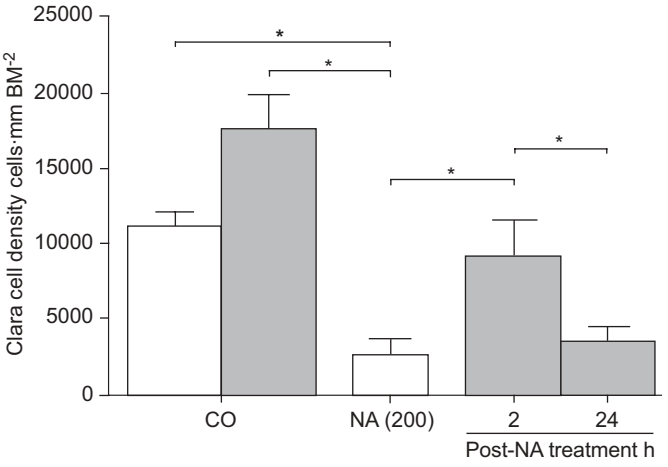

FIGURE 8. Design-based stereological estimation of the number of Clara cell-specific 10-kDa protein-positive Clara cells in distal airways obtained from mice treated with PBS (□) or N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF; ■) after injection of 200 mg·kg body weight^{-1} naphthalene (NA) or corn oil (CO). Airways were obtained by microdissection on day 5 after injection of CO or NA. The therapeutic effect of ΔN23-KGF following NA-induced airway epithelial injury depended upon the presence of Clara cells. Data are presented as mean + SD. Four distal airways were used per mouse (n=3 mice per group). BM: basement membrane. *: $p<0.05$.

expression was not the result of a decrease in the number of CC10-expressing Clara cells but reflected decreased expression per cell. This is supported by the present findings that Clara cell numbers were increased by 20%, whereas CC10 mRNA expression was concomitantly decreased down to ~60% in mice treated with ΔN23-KGF compared with vehicle controls.

The species-specific and region-selective cytotoxicity of NA has been directly linked to its metabolism to NA 1*R*,2*S*-oxide, which is catalysed by the xenobiotic enzyme CYP2F2 [12]. In adult mice, expression of CYP2F2 appears to be strictly limited to nonciliated airway epithelial cells [11]. Since both ΔN23-KGF and NA had significant effects on the number of Clara cells per distal airway, as shown above, the fact that a decrease in Clara cell numbers per airway could simulate a decrease in CYP2F2 mRNA expression relative to that of GAPDH had to be taken into account. Therefore, the expression of CYP2F2 mRNA was related to that of CC10, which is highly specific for Clara cells [49]. Despite the slight reduction in CC10 mRNA expression in lungs pre-treated with ΔN23-KGF, ΔN23-KGF reduced CYP2F2 mRNA expression to $\leqslant$25% of that of vehicle controls. Correspondingly, the present *in vitro* study of Clara cell-enriched lung epithelial cells isolated from C57BL/6 mice revealed a significant reduction in CYP2F2 mRNA expression in cells cultured for 4 h in the presence of ΔN23-KGF. Notably, a population of CC10-positive variant Clara cells, which are maintained within neuroepithelial bodies, was demonstrated to be resistant to NA-induced injury due to low CYP2F2 expression [39]. This subpopulation of Clara cells with low CYP2F2 expression was suggested to be critical to the renewal of proximal airway epithelium following NA-induced injury [50]. A similar NA-resistant Clara cell subpopulation was located to the bronchiolar–alveolar duct junction and exhibited a number of features characteristic of region-specific endogenous stem cells [39]. These data suggest that a reduction in the expression of CYP2F2 mediated by ΔN23-KGF pre-treatment is able to significantly limit the Clara cell cytotoxicity of NA.

The failure of ΔN23-KGF to ameliorate NA-induced injury when applied 24 h after NA-injection, *i.e.* at a time-point at which Clara cells are almost completely lost from the distal airway epithelium [41, 42], whereas Clara cell loss was significantly reduced by treatment with ΔN23-KGF 2 h after injection of NA, demonstrates that the protective effect of ΔN23-KGF is primarily related to Clara cells. This is in line with previous findings that the proliferative effect of KGF is restricted to Clara cells in rat distal airway epithelium [17].

As reviewed recently, NA is used as feedstock in various chemical industrial processes and is suggested to pose a problem in the workplaces of various industries [6, 7]. Hence it is conceivable that therapeutic application of ΔN23-KGF immediately following accidental occupational exposure to high levels of NA might help to limit the development of severe airway epithelial injury, although additional studies in humans are required before such an approach can be translated into clinics.

In summary, it has been demonstrated that pre-treatment of lungs with N-terminally truncated recombinant human keratinocyte growth factor significantly reduced the naphthalene-induced loss of Clara cells by necrosis and prevented the deterioration of respiratory function associated with airway injury. The present *in vivo* and *in vitro* findings suggest that the protective effect of N-terminally truncated recombinant human keratinocyte growth factor is mediated, at least in part, by marked downregulation of the expression of cytochrome P_{450} isoform 2F2, the xenobiotic enzyme responsible for the metabolism of naphthalene into its cytotoxic intermediate. The success of a therapeutic application of N-terminally truncated recombinant human keratinocyte growth factor depends upon the presence of Clara cells.

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