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Regulation of pulmonary and systemic bacterial lipopolysaccharide responses in transgenic mice expressing human elafin

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The control of lung inflammation is of paramount importance in a variety of acute pathologies, such as pneumonia, the acute respiratory distress syndrome, and sepsis. It is becoming increasingly apparent that local innate immune responses in the lung are negatively influenced by systemic inflammation. This is thought to be due to a local deficit in cytokine responses by alveolar macrophages and neutrophils following systemic bacterial infection and the development of a septic response. Recently, using an adenovirus-based strategy which overexpresses the human elastase inhibitor elafin locally in the lung, we showed that elafin is able to prime lung innate immune responses. In this study, we generated a novel transgenic mouse strain expressing human elafin and studied its response to bacterial lipopolysaccharide (LPS) when the LPS was administered locally in the lungs and systemically. When LPS was delivered to the lungs, we found that mice expressing elafin had lower serum-to-bronchoalveolar lavage ratios of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), macrophage inflammatory protein 2, and monocyte chemoattractant protein 1, than wild-type mice. There was a concomitant increase in inflammatory cell influx, showing that there was potential priming of innate responses in the lungs. When LPS was given systemically, the mice expressing elafin had reduced levels of serum TNF-α compared to the levels in wild-type mice. These results indicate that elafin may have a dual function, promoting up-regulation of local lung innate immunity while simultaneously down-regulating potentially unwanted systemic inflammatory responses in the circulation.

The regulation of inflammatory cytokines and cell influx of neutrophils and macrophages is important in a variety of lung and systemic pathologies, such as acute respiratory distress syndrome, pneumonia, and sepsis (11, 22, 25, 44). Recent studies have highlighted the importance of cytokine-chemokine gradients between the alveolar space and the blood compartments in influencing the outcome of lung and systemic inflammations (3, 43). Such studies have shown that in rats concomitant lung administration and systemic administration of bacterial lipopolysaccharide (LPS) resulted in a reduction in the inflammatory cell influx in the alveolar space compared to the influx in animals treated only via the pulmonary route because of a reduced lung-blood chemotactic gradient. In related studies, it was shown that endotoxemic rats and mice with experimentally induced bacterial pneumonia have a poor outcome, possibly because of a lack of pulmonary neutrophilic migration and clearance of organisms (8, 28, 46).

Of interest in this context are low-molecular-weight mucosal elastase inhibitors, such as secretory leukocyte protease inhibitor (SLPI) and elafin/elastase-specific inhibitor (34, 35). These agents have been shown to be induced by early wave cytokines, such as interleukin-1 and tumor necrosis factor (TNF) (32), and to have antimicrobial properties (17, 36, 37, 47). Their concentrations are very low in blood, and they are not expressed in the liver (1, 20, 27, 29). Recently, it has been shown that a transient overexpression approach in which adenovirus is used as a gene vector is efficient for delivering human elafin (driven by the powerful mouse cytomegalovirus [MCMV] promoter) to mouse lung tissues (37, 38). In addition, it was found that in mice there was an increase in inflammatory cell migration to airways in response to intratracheally instilled bacterial LPS (38), suggesting that this inhibitor may be important as a chemoattractant and in the priming of innate responses in lung cells.

Because lung-targeted adenovirus protocols give rise to lung compartmentalization of transgene expression (49) and hence only local elafin expression in the previously described model (37), we decided to create transgenic mice expressing human elafin more ubiquitously in order to have coexistent local expression and systemic expression of elafin. To do this, a mouse cytomegalovirus [MCMV] promoter to mouse lung expression and systemic expression of elafin. To do this, a mouse transgenic line expressing human elafin cDNA under control of the MCMV promoter was generated, and its characteristics were assessed in two self-limiting models of inflammation, first by using intratracheally instilled bacterial LPS (akin to the method used in the adenovirus study mentioned above) and second by using a systemic LPS administration protocol.

MATERIALS AND METHODS

Generation of transgenic mice expressing human elafin cDNA under control of the MCMV promoter. A 6.3-kb fragment containing the elafin cDNA fragment under control of the MCMV promoter was isolated from the PDK6 plasmid (33) and used for microinjection. This elafin cDNA codes for the full-length elafin...
molecule, which contains transglutamination sites thought to be important for the binding of the molecule to the interstitium (27, 29, 33).

Transgenic mice were generated by a standard protocol (18) by injecting linear DNA (5 μg/μl) into the male pronuclei of fertilized ova derived from C57BL/6 × CBA F1 mice. In ovo at the musculature stage, DNA was immediately performed in two serial instillations of 250 and 200 μl of phosphate-buffered saline (PBS). Recovered BAL fluid (BALF) (typically 350 to 450 μl) was centrifuged at 2,000 × g for 5 min. Supernatants were frozen at −20°C until they were used, while each cell pellet was resuspended in 100 μl of PBS. Cell counting (with a hemacytometer) and cytoospinnings (300 rpm for 5 min at room temperature) were performed. After cytoospinning preparations were stained with DiffQuick (Dade Diagnostika, GmbH, Germany) for analysis of differential cell counts.

(i) Intraperitoneal instillation of LPS. Female transgenic mice expressing elafin and WT mice (ages, 8 to 10 weeks) each received one intraperitoneal dose of 120 μg of LPS (as described above) in 100 μl of PBS. Control mice were injected with PBS alone. Serum samples were collected after 1.5 h and 24 h by tail bleeding.

Thioglycollate challenge and culture of peritoneal macrophages. Female transgenic mice expressing elafin and WT mice (ages, 8 to 10 weeks) were injected intraperitoneally with 1 ml of 4% thioglycollate broth to induce peritonitis. The mice were sacrificed after 12 h, and peritoneal lavage performed with 5 ml of cold PBS. The cells were pooled and washed with PBS. A total of 5 × 10^6 cells were then cultured in 48-well plates in 1 ml of RPMI 1640 (Gibco BRL) medium supplemented with 25 mM Hepes, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum. After overnight incubation, nonadherent cells were removed by washing with serum-free medium, and all of the adherent cells were macrophages. LPS was then added at different concentrations (0 to 5 ng/ml), and the cells were cultured for 48 h in serum-containing medium. Each supernatant was removed and stored at −20°C until it was used for enzyme-linked immunosorbent assay (ELISA) analysis.

ELISA. ELISA analyses to detect murine TNF alpha (TNF-α), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2) were performed by using commercially available ELISA kits (R & D Systems, Abingdon, Oxon, United Kingdom) in accordance with the manufacturer’s instructions. Human elafin was measured by using an ELISA available in-house (37). Culture supernatants from peritoneal macrophages were diluted 1:20 before ELISA analysis of cytokines. Serum samples (collected either 1.5 or 24 h after treatment) and BALFs were either not diluted or diluted fivefold in PBS.

Statistical analysis. Normally distributed data were analyzed by the Student unpaired t test. When data were not normally distributed, nonparametric tests (Kruskal-Wallis and Mann-Whitney tests) were employed. Data were considered to be statistically significantly different when the P value was <0.05.

RESULTS

Genotype analysis of transgenic mice expressing elafin cDNA. The genotypes of mice were determined in order to determine whether elafin cDNA was present. Tail biopsy DNA was prepared and digested with HindIII (generation of a 562-bp fragment showed that the transgene was intact), as described in Materials and Methods. Heterozygous F1 mice were selected by Southern analysis and PCR. Male and female mice heterozygous for the elafin cDNA were crossed to generate homozygous F2 animals (Fig. 1). Homozygous (+/+) mice expressing elafin cDNA (referred to as elafin mice below) were crossed to establish the mouse line expressing elafin.

Northern blot and RT-PCR analyses of elafin mice. Figure 2 shows the tissue distribution of human elafin in elafin mice as determined by Northern blot analysis (Fig. 2A) and RT-PCR (Fig. 2B and C).

This analysis showed that although most tissues tested were positive for the transgene (Fig. 2B), stomach, small intestine, skeletal muscle, and bone tissues were the tissues in which...
elafin was most abundant (Fig. 2A). Due to their lower level of elafin expression, blood and bone marrow samples were treated separately (Fig. 2C). Compared to the intensity of the elafin bands in the stomach sample (Fig. 2C, lane 5), the elafin bands in the blood and bone marrow samples were much less intense. In addition, PCR gave rise to at least two bands in the blood sample and eight bands in the bone marrow samples. The specificity of these products was therefore checked by Southern analysis (by using elafin cDNA as a probe), and only the 477-bp expected elafin product hybridized strongly, showing that both blood and bone marrow cells expressed the elafin cDNA transgene.

When tissues were analyzed for protein production (by ELISA), the elafin levels were not always reliably measured, which probably reflected the relative lack of sensitivity of the assay (data not shown).

**Inflammatory responses to intratracheally instilled bacterial LPS.** (i) Cytokines. In order to assess whether elafin mice responded differently to LPS than WT mice responded, both groups of mice were instilled intratracheally with 50 μg of LPS (or PBS). Because of the importance of TNF-α as an early mediator of inflammation, we first measured the TNF-α levels in BALF and serum. As shown in Table 1, as expected, both groups of LPS-treated mice had higher BALF TNF-α levels than their PBS-treated counterparts. It should be noted that the response to LPS was largely (but not totally) compartmentalized in the lungs in WT mice (there was a 22-fold increase in the TNF-α level in BALF and a 1.3-fold increase in serum after LPS treatment). There was also a trend (not statistically significant) toward increased levels of the cytokines in BALF from LPS-treated elafin mice compared to the levels in LPS-treated WT mice. When the systemic levels of TNF-α in serum were examined, the elafin mice had lower levels than the WT mice after PBS or LPS treatment, suggesting that elafin may have a stronger systemic anti-inflammatory activity than a local lung proinflammatory activity in these mice.

Since TNF-α is an early cytokine involved in initiation of the inflammatory response, we extended our study to two other cytokines, MCP-1 and MIP-2, which are chemokines for monocytes and neutrophils, respectively. Because of the potential dual activity of elafin in altering the TNF-α response (weakly proinflammatory in the lung and strongly anti-inflammatory systemically), we expressed the data as serum/BAL cytokine ratios (Fig. 3). In accordance with the TNF-α data, elafin mice

![FIG. 1. Construction and identification of the elafin cDNA transgene under control of the MCMV promoter. (A) Construct used to generate transgenic mice expressing human elafin cDNA under control of the MCMV promoter. A 1,400-bp fragment of the MCMV promoter (nucleotides -1336 to 36) drives expression of the elafin cDNA (538 bp). Restriction sites for HindIII, which were used to identify the presence of the transgene, are indicated. (B) Genomic DNA (10 μg) isolated from tail biopsies was digested with HindIII and resolved on a 1% agarose gel. The gel was transferred to a nylon membrane, and the position of the 562-bp human elafin cDNA transgene. The position of the 562-bp human elafin cDNA fragment is indicated by an arrow.

![FIG. 2. Expression of the human elafin cDNA transgene in female murine tissues. (A) Northern analysis of total RNA (20 μg) from lung (L), stomach (St), large intestine (Li), small intestine (Si), skin (S), skeletal muscle (SM), bone (B), ovary (O), uterus (U), bladder (Bl), heart (H), spleen (Sp), brain (Br), kidney (K), liver (Li), and thymus (Th) tissues. Ethidium bromide staining of gels prior to transfer (lower panel) was used to check equivalent sample loading. The positions of the 28S and 18S rRNA are indicated on the left. The positions of molecular weight markers are indicated on the left (lane M) (1.0-kb ladder; catalog number G 5711 for panel B; Promega). The abbreviations for the organs are the same as those described above; trachea (Tr) and wild-type liver RNA (WT) samples also were analyzed. (C) Total blood cells from two elafin mice were obtained by exsanguination and subsequently pooled. Bone marrow was obtained from the same mice, and samples were treated separately for RT-PCR. The specificity of the 477-bp elafin cDNA band (top panel) was verified by Southern blot analysis (bottom panel). Lane M, molecular weight markers (catalog number 15615-016; Promega); lane 1, negative control in which water was used in place of DNA; lane 2, total blood cell elafin cDNA; lanes 3 and 4, bone marrow elafin cDNA (independent samples); lane 5, stomach elafin cDNA.
exhibited the same decrease in serum/BALF MCP-1 and MIP-2 ratios as those WT mice. It should be noted that as shown in Table 1, although a trend was observed, the transgene did not have a significant effect on the absolute cytokine levels in the BALF.

(ii) Inflammatory cells. The finding that elafin mice had lower serum/BALF ratios of inflammatory cell cytokines suggested that the chemokine gradient generated may have resulted in an increase in the number of inflammatory cells in BALF from elafin mice compared to the number of inflammatory cells in BALF from WT mice. Figure 4 shows that as expected, LPS induced an influx of inflammatory cells in BALF from the two groups of mice compared to the number of cells after the PBS treatment. In WT mice the increase consisted mostly of neutrophils, whereas in elafin mice both the number of neutrophils and the number of macrophages were elevated. Indeed, when the LPS-treated groups were considered separately, BALF from elafin mice contained more inflammatory cells than BALF from WT mice contained.

Interestingly, although the levels of human elafin protein in sera and BALF were very low (consistently less than 50 pg/ml) in elafin mice and zero in WT mice, in elafin mice treated with PBS and LPS positive correlations were found between the levels of elafin in BALF and the levels of of TNF-α, MCP-1, and MIP-2 (Fig. 5).

When serum levels of cytokines and elafin were analyzed, only the relationship between TNF-α and elafin levels approached statistical significance (P = 0.081).

**Serum TNF-α levels after intraperitoneal LPS administration.** The in vivo experiments whose results are shown in Table 1 and Fig. 3 showed that intratracheal instillation of 50 μg of LPS did not lead to total lung compartmentalization of the LPS stimulus in WT mice since the TNF-α levels were increased in the serum of these mice. Since elafin mice had lower levels of circulating TNF-α (Table 1), we hypothesized that if elafin could down-regulate the systemic effect of LPS when LPS is administered intratracheally, it may dampen the responses when LPS is injected systemically. Since the level of TNF-α in serum is known to increase transiently after systemic LPS administration and TNF-α is considered a major initiator of the septic response (4--6, 13, 39, 42), we used previously established protocols to measure TNF-α immunoreactivity in serum 1.5 and 24 h after intraperitoneal LPS injection.

Figure 6 shows that as expected, the serum TNF-α levels in WT mice were greater 1.5 h after LPS administration and...
returned to the baseline value within 24 h. In sera from ela
fin mice, the TNF-/H9251 levels were signifi
cantly lower than those in WT mice at 1.5 h, suggesting that ela
fin mice would be less responsive to LPS, thereby explaining
the reduced systemic TNF-/H9251 expression in vivo (Fig. 6). To
further investigate this, we isolated monocyte-derived perito
neal macrophages 4 days after intraperitoneal thioglycolate
injection and stimulated the cells in vitro with different levels
of LPS. Figure 7 shows that peritoneal macrophages from
elafin mice produced less TNF-/H9251, MCP-1, and MIP-2 than
peritoneal macrophages from WT mice produced, and Fig. 8
shows that these cells produced elafin mRNA, as determined
by RT-PCR (the protein level was below the threshold of
detection) (data not shown).

Ex vivo stimulation of peritoneal macrophages with LPS. As
shown in Fig. 3, the serum/BAL cytokine ratios were lower in
elafin mice than in WT mice. In addition, we showed that bone
marrow cells and blood cells were positive for the transgene
(Fig. 2C). Given the reported anti-inflammatory properties of
the related molecule SLPI in monocytes and macrophages (10,
21, 23, 26), we hypothesized that monocytes derived from ela
fin mice would be less responsive to LPS, thereby explaining
the reduced systemic TNF-/H9251 expression in vivo (Fig. 6). To
further investigate this, we isolated monocyte-derived perito
neal macrophages 4 days after intraperitoneal thioglycolate
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detection) (data not shown).

DISCUSSION

Human neutrophil elastase inhibitors, such as alpha-1 protei
nase inhibitor and SLPI, have recently been used in in vivo
models as purified or recombinant proteins to modulate lung
and systemic inflammation (24, 30, 31, 40). Workers in our
laboratory are particularly interested in elafin, a more specifi
cneutrophil elastase inhibitor which exhibits 40% homology
with SLPI. This 10-kDa molecule is part of a four-disulfide-
core protein family which has recently been named the trappin
family (35). In addition, it has been shown that in vitro and in
vivo elafin is a cationic molecule with activity against gram-
positive and gram-negative pathogens (36, 37) and can there
fore be considered a defensin-like molecule with a role in
innate immunity.

Recently, the use of adenovirus as a lung vector for transient
gene expression of potentially therapeutic transgenes under
control of the strong MCMV promoter allowed us to discover

FIG. 5. Correlation between BALF elafin and cytokine levels.
BALF from elafin mice instilled intratracheally with PBS or LPS (see the
legends to Fig. 3 and 4) were analyzed for elafin and cytokine
(TNF-/H9251, MCP-1, MIP-2) levels.

FIG. 6. Serum TNF-/H9251 levels are lower in elafin mice following intraperitoneal LPS injection. A TNF-/H9251 ELISA analysis was performed with
serum samples obtained from WT mice and elafin mice (EL) (ages, 8 to 10 weeks) either 1.5 or 24 h after intraperitoneal administration of 120
µg of LPS or PBS. The numbers of mice in the groups were as follows: WT mice treated with LPS, 10 mice; elafin mice treated with LPS, 10 mice;
WT mice treated with PBS, 5 mice; and elafin mice treated with PBS, 5 mice. All data are expressed as the mean ± standard deviation. An unpaired
two-tailed Student’s t test was used to establish statistical significance between groups. The levels of TNF-/H9251 in the serum of elafin mice 1.5 h after
LPS treatment were significantly less than the levels in LPS-treated WT mice (P = 0.02), as indicated by the asterisk. ND, not detected.
a new property for elafin, its chemotactic activity for lung inflammatory cells (38).

In order to determine thoroughly the role of this molecule in local and systemic inflammation, in the present study we used transgenic mice that were engineered to express human elafin constitutively under control of the same MCMV promoter.

Importantly, our elafin construct codes for the full-length protein, which contains transglutamination-cementoin sites (27, 29, 34, 35) thought to be important in binding of the molecule to the interstitium. Recently, Aiba-Masago et al. studied expression in transgenic mice of bacterial β-galactosidase driven by an MCMV promoter similar to the one used in our study (2). These authors found that the brain, stomach, kidney, and skeletal muscle were the major organs which expressed the transgene. Our study (Fig. 2) showed that the stomach, small intestine, skeletal muscle, and bone are major sites of expression. The reasons for the slight difference in the spectrum of organs in which the transgene is expressed between the two studies are unclear, but the difference could reflect differences in the read-outs (i.e., mRNA levels in our study and protein levels in the study of Aiba-Masago et al.) or differences in the chromosomal location of the transgenes. Alternatively, our human elafin cDNA construct has a 158-bp 3′ untranslated region which may contain positive or negative regulatory binding sites which could influence the MCMV-associated specificity of expression. Interestingly, when studying endogenous expression of human elafin RNA, Nara et al. found that in accordance with our results, stomach and small intestine tissues were positive as determined by an RNase

FIG. 7. Peritoneal macrophages from elafin mice are hyporesponsive to LPS. Peritoneal macrophages from elafin mice and WT mice were obtained as described in Materials and Methods. Cells (5 × 10⁵ cells) were then cultured in 48-well plates in 1 ml of RPMI 1640 containing 10% fetal bovine serum with or without different LPS concentrations (0 to 5 ng/ml). After 48 h, the medium was recovered, and the concentrations of TNF-α, MCP-1, and MIP-2 were determined by ELISA. An unpaired two-tailed Student’s t test was used to establish statistical significance between groups (n = 3). An asterisk indicates that a value is statistically significantly different (P < 0.05) than the value obtained with the equivalent LPS dose in WT mice.

FIG. 8. Elafin mRNA production by peritoneal macrophages. Peritoneal macrophages from elafin mice were obtained and cultured as described in the legend to Fig. 7, except that the LPS doses were 0, 1, and 10 ng/ml. RT-PCR for the elafin and GAPDH transcripts was performed as described in Materials and Methods.
protection assay (27). Using Northern blot analysis, Pfundt et al. showed that the human tongue and pharynx (organs not assessed in the other studies mentioned here) are strong expressers of elafin, although these workers did not study the stomach, small intestine, skeletal muscle, and bone (29).

Although some organs expressed elafin more strongly than other organs, it was demonstrated by using RT-PCR that all the organs tested here were positive for expression of human elafin in the transgenic mice. The results of this study also confirmed the results of a previous study (33) showing that the human elafin cDNA probe does not cross-react with any murine message.

In the second part of our study we tested whether the elafin mice generated here were able to modulate the activity of intratracheally instilled LPS to the same extent that was observed in the adenovirus-elafin model (38).

Figure 4 shows that there was increased inflammatory cell influx (both neutrophils and macrophages) in BALF from elafin mice compared to that in WT mice following intratracheal LPS administration. A net increase in lung chemotactic activity is caused by chemokine gradients generated either by an increase in the levels of lung chemokines or a decrease in the levels of systemic chemokines (3, 28, 43, 46). We therefore measured serum and BALF chemokine levels and found that neutrophilic, monocytic cytokine, and chemokine (TNF-α, MIP-2, and MCP-1) ratios were lower in the elafin mice (Fig. 3). In addition, the cytokine levels were found to correlate with elafin concentrations (Fig. 5). These results are qualitatively similar to the results observed in our adenovirus study (38). Interestingly, although as indicated above there was a trend toward increased numbers of macrophages in BALF from mice given adenovirus elafin intratracheally, the number of neutrophils prominently increased in that study. In contrast, the numbers of both neutrophils and macrophages were elevated in elafin mice in the present study. The difference between the transient adenovirus and constitutive transgenic models could result from differential chemotactic activity due to a difference in lung elafin expression, which was much higher in the adenovirus study (nanogram levels, compared with the picogram concentrations in BALF) (Fig. 5). These results are qualitatively similar to the results observed in our adenovirus study (38). In addition, cells from elafin mice produced less TNF-α, MIP-2, and MCP-1 than cells from WT mice produced (Fig. 7). Interestingly, macrophages from elafin mice produced less TNF-α than macrophages from WT mice produced under basal (PBS) conditions, echoing data presented in Table 1, which show that the serum TNF-α levels were lower in the former mice.

In conclusion, these are the three main findings of the present study. (i) The elafin mice that we created expressed human elafin mRNA in a variety of organs (Fig. 1 and 2) and expressed protein levels in the lung and serum, as assessed by ELISA analysis. (ii) After intratracheal LPS administration, increases in numbers of inflammatory cells in BALF and decreases in the serum/BALF cytokine ratios were observed in the elafin mice compared to the values for WT mice (Table 1 and Fig. 3 and 4). In addition, the cytokine levels were correlated with the elafin concentrations in BALF (Fig. 5). (iii) Elafin mice were less responsive to LPS, as assessed in vivo, after intraperitoneal injection of LPS and measurement of circulating TNF-α (Fig. 6) and ex vivo (with peritoneal macrophages) (Fig. 7).

Because of the role of TNF-α in many of the pathophysiological events in sepsis (14, 15), inhibitors of the TNF-α cascade have been extensively studied (9, 10, 12, 16, 19, 21, 23, 24, 26, 48). We believe that elastase inhibitors, such as elafin, are particularly interesting because of their small size, which allows
better diffusion in tissues. Both in a previous adenovirus study (38) and in the present study a decrease in blood-BALF TNF-α–chemokine gradient in mice overexpressing elafin was found. We believe that the elafin mice should help in assessing which of the two strategies (i.e., local application or systemic application of elastase inhibitors) should be more beneficial in clinical applications. As pointed out above, because of possible differences in site expression, care should be taken to differentiate the physiological effects of an endogenous protein and the potential therapeutic effects of a transgene. We are currently pursuing this line of investigation in our laboratory and with our clinical colleagues.

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