An essential role for C/EBPβ binding protein in bleomycin-induced pulmonary fibrosis

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Abstract

Pulmonary fibrosis is characterized by inflammation, genesis of myofibroblasts, and abnormal tissue repair. Despite extensive research, its pathogenesis remains incompletely understood. Previously, the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) was found to be a key regulator of myofibroblast differentiation in vitro, and to be involved in the acute phase and inflammatory responses. In an attempt to test the role of C/EBPβ in the development of pulmonary fibrosis, experiments using C/EBPβ null mice and their wild-type littermates were conducted. Our findings indicated that, compared to wild-type mice, animals deficient in C/EBPβ showed significantly reduced fibrotic lesions and collagen deposition in the lung upon endotracheal injection of bleomycin. Further studies on the mechanisms by which C/EBPβ regulates fibrosis indicated that knockout of C/EBPβ attenuates inflammatory cytokine expression in bleomycin-treated mice. The reduced α-smooth muscle actin gene expression in either isolated lung fibroblasts or lung tissue from bleomycin or saline-treated C/EBPβ deficient mice suggests that C/EBPβ regulates myofibroblast differentiation during fibrosis. Consistent with this finding, cells from C/EBPβ deficient mice exhibited higher proliferative rates than those from wild-type mice. These data suggest that C/EBPβ plays an essential role in pulmonary fibrosis and that this role appears to be multifactorial with respect to cytokine expression, cell differentiation, and proliferation.

Keywords: C/EBPβ; fibrosis; smooth muscle actin; bleomycin

Introduction

A key phenomenon considered to be important in the pathogenesis of pulmonary fibrosis is the de novo genesis of the myofibroblast, which presumably arises by differentiation from precursor cells, such as the adventitial fibroblast [1–4]. A common and useful marker for myofibroblast differentiation is the expression of α-smooth muscle actin (α-SMA) [1,2,5,6]. Hence, insight into the induction of myofibroblast differentiation is afforded by studies of regulation of induction of this gene. A recent study shows that CCAAT/enhancer binding protein β (C/EBPβ), a basic leucine zipper transcription factor, is a key regulator of α-SMA expression and myofibroblast differentiation in isolated lung fibroblasts, by binding to a C/EBPβ binding element identified in the α-SMA promoter [7]. Two major isoforms of C/EBPβ have been described and found to be expressed in certain cells [8–10]. The larger of these isoforms has been designated as liver-enriched activator protein (LAP), while the truncated form is known as liver-enriched inhibitor protein (LIP). The latter contains the DNA binding domain of LAP but lacks its activation domain [8–10]. LAP activates α-SMA gene expression, while LIP inhibits expression by competing for binding to the C/EBPβ binding consensus on the α-SMA promoter [7]. Thus the LIP isoform by virtue of its ability to bind DNA but lacking in the activation domain might be construed as behaving in a dominant negative manner. Despite this in vitro evidence of cellular regulation by C/EBPβ isoforms, their in vivo role in the pathogenesis of pulmonary fibrosis, and specifically its role in genesis of the myofibroblast, remains to be determined.

In this paper, the regulatory role of C/EBPβ in the development of pulmonary fibrosis was determined by comparing the responses of C/EBPβ null mice and wild-type mice with endotracheal bleomycin treatment. The results indicated that, upon endotracheal injection of bleomycin, C/EBPβ deficient mice showed significantly reduced pulmonary fibrosis and collagen deposition compared with their wild-type littermates. Further attempts to identify the mechanism for the attenuated fibrosis in C/EBPβ null mice indicated that C/EBPβ deficiency attenuated collagen gene transcription and inhibited the genesis of the...
myofibroblast, the major cell that produces collagen lung fibrotic lesions. Furthermore, lung expression of some cytokines was diminished in bleomycin-treated deficient mice relative to that in wild-type controls. In vitro studies of fibroblasts isolated from these mice revealed that C/EBPβ null fibroblasts grew faster than wild-type fibroblasts despite being deficient in myofibroblast differentiation. Taken together, these data suggest that C/EBPβ play an essential role in the induction of pulmonary fibrosis.

Material and methods

Animal treatment
C/EBPβ deficient mice (C57BL/6 background) were bred from breeding pairs, which were generous gifts of Dr PF Johnson (National Cancer Institute, National Institutes of Health, Bethesda, MD) and generated as previously described [7]. Bleomycin-induced pulmonary fibrosis was induced as previously described [11]. The control group received the same volume of sterile phosphate-buffered saline only (saline treated).

Hydroxyproline assay

As a measure of fibrosis, total lung hydroxyproline content was determined by colorimetric assay after acid hydrolysis, as described previously [12].

Fibroblast proliferation assay

Mouse lung fibroblasts isolated from either bleomycin-treated C/EBPβ−/− or C/EBPβ+/+ mice were seeded (2 × 10^4 cells/well) in six-well dishes in complete DMEM medium as described above, and were allowed to adhere for 24 h. The cells were then washed and cultured in fresh media supplemented with 10% plasma-derived serum and growth factors as indicated above. At the indicated time points, the cells were briefly trypsinized for cell counting using a Cell and proteins by western blotting as before [7]. The anti-α-SMA and anti-procollagen I antibodies were from Sigma (St Louis, MO, USA). Equal loading was confirmed by blotting the membrane with anti-β-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

mRNA analysis

Determination of mRNA for the indicated genes was undertaken using real time polymerase chain reaction (PCR) as before [11]. With this method, a CT value reflects the cycle number at which DNA amplification is detected. The amount of target, normalized to endogenous reference and relative to a calibrator, is given by 2−ΔΔCT [14]. Total RNA was extracted from either murine lung tissue or fibroblasts as indicated. For each assay, 200 ng of total RNA was used as template and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as internal control to normalize the amount of input RNA. One-step real time reverse transcriptase (RT)-PCR (48 °C for 30 min, 95 °C for 10 s, followed by 50 cycles of 95 °C for 10 s, 60°C for 1 min) was undertaken with Taqman One Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using a GeneAmp 5700 Sequence Detection System (PE/ABI). Predeveloped primers and probes for transforming growth factor β1 (TGFβ1), interleukin (IL)-1β, tumour necrosis factor α (TNFα), monocyte chemotactic peptide 1 (MCP1), and GAPDH were purchased from Applied Biosystems (PE/ABI, Foster City, CA, USA). Primers and MGB probes (6-FAM conjugated) for α-SMA, procollagen I and C/EBPβ were designed with software Primer Express 2.0 (Applied Biosystems) and synthesized by Applied Biosystems. The primer sequences were as follows: mouse α-SMA: forward primer, 5′-TCCTTGAAAGAGCTCAGACT-3′; reverse primer, 5′-AAGCGTTTCATCATGTG-3′; and probe, 6-FAMCTGACGGGCAGGTGAMGBNFQ; mouse procollagen type I, alpha II: forward primer, 5′-CAACCGTGCTTCTCAGACATC-3′; reverse primer, 5′-TGCCCGTCTCTCATCCA-3′; and probe, 6-FAMACCACTACTGCAAGACMGDFQ; mouse C/EBPβ: forward primer, 5′-AGCTGAGCAGAGATGAGT-3′; reverse primer, 5′-GCTGCTGCTG-3′; and probe, 6-FAMCGCAACAATCGMGBNFQ.
Effects of C/EBPβ deficiency on lung histopathology

To further confirm such a role for this factor, mice deficient in C/EBPβ and their wild-type littermates were injected into the endotrachea with bleomycin and analysed for development of fibrosis 21 days later. C/EBPβ null mice have no detectable phenotype with reference to lung morphology and appear to develop normally, although female null mice have defective ovarian development and thus are infertile [15]. Routine H&E staining confirmed no detectable histopathological abnormality in lungs of control saline-treated C/EBPβ null mice, which were indistinguishable from the lungs of wild-type mice (Figure 2). When treated with bleomycin, the wild-type mice showed the expected extensive dense lung fibrosis characterized by increased interstitial wall thickness, inflammatory cell infiltration, increased number of fibroblasts, and interstitial collagen deposition. In contrast, although fibrotic lesions were observed, the fibrotic changes were markedly decreased in extent and severity for C/EBPβ null mice with the same treatment. The fibrotic lesions were less widespread and more narrowly confined to central peribronchial and perivascular areas. Cellularity was much decreased compared to wild-type lungs. Thus the histopathology suggested that C/EBPβ deficiency was associated with a reduction in bleomycin-induced pulmonary fibrosis.

Effects on lung collagen

To confirm and quantify the histological changes observed, lung collagen deposition was determined by the hydroxyproline assay. Whole lung hydroxyproline content was determined at the same time point, namely 21 days after saline or bleomycin injection. Figure 3 shows that bleomycin treatment caused a 60% increase (over saline-treated controls) in the lung hydroxyproline content of wild-type mice, which was significantly reduced to a 35% increase in C/EBPβ deficient mice. This >40% reduction in fibrosis as measured biochemically was consistent with the histopathology shown in Figure 1.

To further confirm the effect of C/EBPβ on collagen deposition, lung homogenate protein extracts from bleomycin or saline-treated C/EBPβ null mice were injected into the endotrachea with bleomycin relative to that in control saline-treated wild-type mice (Figure 2). When treated with bleomycin, the wild-type mice showed an increase in C/EBPβ expression. Total RNA extracted from lungs of either bleomycin- ('Bleo') or saline- ('Saline') treated C57BL/6 mice (n = 5) was analysed for C/EBPβ mRNA by real time PCR. The results were expressed as $2^{-\Delta\Delta CT}$, with GAPDH mRNA as the endogenous control and the values from the saline-treated controls used as reference. Data are shown as mean ± SE with n = 5. The difference between bleomycin-treated and saline-treated lungs was statistically significant ($p < 0.05$)
Figure 2. Reduced fibrosis in C/EBPβ null mice. Representative H&E stained lung tissue sections from saline- or bleomycin-treated wild-type and C/EBPβ null mice are shown. (A) C/EBPβ null, saline-treated; (B) wild-type, saline-treated; (C) C/EBPβ null, bleomycin-treated; (D) wild-type, bleomycin-treated. All were photographed at ×40 magnification.

Figure 3. Effects of bleomycin treatment on lung hydroxyproline. Whole lung homogenates from the indicated strain of mice treated with saline or bleomycin were acid hydrolysed and analysed for hydroxyproline content as described in 'Material and methods'. The results were expressed as a percentage of the respective saline control values. Mean ± SE (n = 5) values are shown, and the difference between the two strains of mice was statistically significant (p < 0.05).

Figure 4. Effects of bleomycin or saline treatment on lung type I collagen. Lung collagen I in homogenates from the indicated group of mice was analysed by western blotting. β-Tubulin was also detected using anti-β-tubulin antibody as a loading control.

and their wild-type littermates were analysed by western blotting for determination of type I collagen content. The results showed that lung type I collagen content was reduced in control saline-treated C/EBPβ deficient mice relative to that in wild-type mice. Bleomycin treatment caused an increase in lung type I collagen content in wild-type mice, which was blunted in C/EBPβ deficient mice to an extent that showed only minimal stimulation over the control saline-treated deficient mice. This reduction in basal and bleomycin-induced lung collagen I protein content was accompanied by significant reduction in lung α1(I) procollagen mRNA (Figures 4 and 5). A >50% reduction in basal mRNA levels was observed in the lungs of C/EBPβ deficient mice relative to wild-type mice. Moreover, while bleomycin caused a >2.5-fold increase in mRNA in wild-type mice, less than a 0.5-fold (or 50% increase) was noted for the mutant mice. Thus the biochemical evidence provided further support for deficiency in bleomycin-induced pulmonary fibrosis in C/EBPβ null mice.
Effects on lung cytokine expression

Lung cytokine expression is altered in pulmonary fibrosis, and some cytokines, such as TGFβ1, IL-1β, TNFα, and MCP-1, are found to be essential for pathogenesis in animal model studies [16–18]. To determine how C/EBPβ affects pulmonary fibrosis, assessment of cytokine expression in lungs of wild-type and C/EBPβ deficient mice was conducted by real time semi-quantitative PCR analysis. The results showed significantly blunted mRNA levels in the mutant mice (Figure 6A–D). Basal (in saline-treated controls) levels were not significantly different between mutant and wild-type lungs for IL-1β and TNFα mRNAs, but were significantly reduced for MCP-1 and TGFβ. However, bleomycin-induced levels in mutant mice were significantly reduced for all cytokine mRNA levels examined relative to those in wild-type controls, except for MCP-1, which despite its lower basal level was almost equally induced by bleomycin (both showed approximately a 12-fold increase in response to bleomycin treatment). Thus regulation of cytokine

Figure 5. Effects of bleomycin or saline treatment on lung type I collagen mRNA. C/EBPβ null mice (C/EBPβ−/−) and its wild-type littermates (C/EBPβ+/+) were treated with bleomycin or saline, respectively. Seven days after treatment, total RNA was extracted from the lung tissue and α1(I) procollagen mRNA was detected by real time PCR. Results were expressed as $2^{-\Delta\Delta CT}$, with GAPDH used as the endogenous control and the level in saline-treated wild-type mice used as reference. Data are shown as mean ± SE from triplicate samples

Figure 6. Effects of bleomycin or saline treatment on lung cytokine expression. Total RNA extracted from either bleomycin or saline-treated C/EBPβ null (C/EBPβ−/−) and wild-type mice (C/EBPβ+/+) was analysed for IL-1β (A); TNFα (B); MCP-1 (C); and TGFβ1 (D) mRNA by real time PCR. The results were expressed as $2^{-\Delta\Delta CT}$, with GAPDH used as the endogenous control and the values from the respective wild-type saline-treated controls were used as reference. Data are shown as the mean ± SE from triplicate samples
expression by C/EBPβ could partially account for the reduced fibrosis in C/EBPβ null mice relative to that in wild-type mice upon bleomycin treatment.

Effects on myofibroblast differentiation

De novo genesis of myofibroblasts in fibrosis is readily detectable by assessment of α-SMA expression [1,2,5,6]. In view of the important roles of the myofibroblast in pulmonary fibrosis [1–4], deficiency in its genesis would result in impaired fibrosis. To evaluate if this might be a contributory factor to the deficient fibrosis seen in C/EBPβ deficient mice, the expression of α-SMA in isolated lung fibroblasts was examined in saline or bleomycin-treated wild-type and mutant mice. Analysis of α-SMA by western blotting revealed comparable levels of expression in cells isolated from saline-treated wild-type and mutant mice (Figure 7A). However, while cells from bleomycin-treated wild-type mice had higher levels of α-SMA expression than those from their corresponding saline-treated controls, the cells from mutant mice were essentially unaffected by in vivo bleomycin treatment. This would suggest defective myofibroblast differentiation in cells from C/EBPβ deficient mice. Confirmation for such a defect was obtained by real time PCR analysis for α-SMA mRNA levels. Analysis using this approach showed an approximately 50% reduction in basal α-SMA mRNA levels in mutant mice compared to wild-type control mice (Figure 7B). This reduced basal mRNA level was minimally altered (<40% increase) in cells isolated from bleomycin-treated mutant mice, in contrast to the >2.5-fold stimulation (or >150% increase) noted in cells from bleomycin-treated wild-type mice. Analysis of the α-SMA gene expression in the lung tissue of C/EBPβ null mice and their wild-type littermates showed the same trend (data not shown). Thus C/EBPβ deficiency caused defective genesis of myofibroblasts in bleomycin-induced pulmonary fibrosis.

Effects on fibroblast proliferation

C/EBPβ is known to be a regulator of cell cycle as well as differentiation [19–22]. Thus, we examined the effect of C/EBPβ deficiency on lung fibroblast proliferation since one of the important phenomena related to pulmonary fibrosis is the increased cell number of fibroblasts in the lung, and the cell proliferation rate has an important effect on the numbers of fibroblasts. As is indicated, growth curve analysis showed that lung fibroblasts isolated from bleomycin-treated C/EBPβ deficient mice were more proliferative than cells from wild-type mice (Figure 8A). Doubling time at the linear phase was almost twice as long in the wild-type cells as in the C/EBPβ deficient cells. Furthermore, thymidine incorporation was also significantly higher in the mutant cells than in the wild-type cells (Figure 8B). This is consistent with the concept that less differentiated cells usually have a higher growth or proliferation rate, or alternatively that differentiated (especially terminally differentiated) cells have very low proliferative rates, or do not divide at all. Thus one possible explanation is that C/EBPβ null fibroblasts grew faster because of their failure to differentiate to myofibroblasts.

Discussion

Two isoforms of C/EBPβ, LAP and LIP, have been described, which are translated from the same mRNA [8–10]. While both share the same C-terminal DNA binding domain, LAP has an intact N-terminal activation domain that is lacking in LIP [8–10]. Since the binding capability of LIP to the C/EBPβ binding consensus is four fold higher than that of LAP, the function of LIP is thought to repress the stimulatory effect of LAP on gene expression through competition for binding to the C/EBPβ binding consensus on the target gene promoter [8]. Previously we have shown that LAP activates, while LIP inhibits, α-SMA gene expression [7], which suggests that C/EBPβ may modulate pulmonary fibrosis by regulating myofibroblast differentiation.
C/EBPβ role in pulmonary fibrosis

Figure 8. Effects on lung fibroblast proliferation. Mouse lung fibroblasts isolated from either bleomycin-treated C/EBPβ null mice (C/EBPβ−/−) or wild-type littermates (C/EBPβ+/+) were counted at the indicated time point in culture (A) or using a scintillation counter for incorporated [3H]thymidine (B). The results were shown as mean ± SE from three independent experiments.

In support of such a possibility we first showed that bleomycin treatment significantly increased lung C/EBPβ expression. Furthermore, C/EBPβ deficiency in knockout mice caused significant blunting of fibrosis assessed by both histopathological and biochemical methods. This was accompanied by reduced α-SMA and collagen I gene expression in knockout mice, consistent with reduced myofibroblast differentiation. This is also consistent with previous reports that LAP is an activator for expression of several target genes [7,23]. Since the inhibitory effect of LIP depends on inhibition of the activating effects of LAP [8], reduced fibrosis in C/EBPβ deficient mice was expected. Indeed this was observed as the overall effect of C/EBPβ deficiency in the bleomycin model.

Pulmonary fibrosis is intricately controlled by a variety of cytokines [7,24–26], which are produced by many cell types [26]. Since myofibroblasts are key sources of some of these cytokines [27], including TGFβ1 [28], it is conceivable that the reduced myofibroblast differentiation in C/EBPβ deficient mice may contribute to the observed reduction in expression of this cytokine in lungs of deficient mice. However, direct effects of C/EBPβ deficiency on cytokine gene expression cannot be ruled out, and may represent an additional mechanism by which this transcription factor regulates pulmonary fibrosis.

As demonstrated in this paper, defective lung myofibroblast differentiation was found in bleomycin-treated C/EBPβ deficient mice, which was accompanied by significantly higher growth rate in lung fibroblasts isolated from these deficient mice. Nevertheless, despite the higher growth rate of C/EBPβ deficient fibroblast in vitro, we did not observe increased fibroblast numbers in C/EBPβ null mice after bleomycin treatment. Although this is somewhat paradoxical at first glance, the finding is entirely consistent with the notion that the more differentiated myofibroblasts would have a diminished ability to proliferate. This role for C/EBPβ in promoting differentiation whilst inhibiting cell proliferation has been previously observed and may be mediated by GADD45A [29]. Thus despite the potential higher proliferative capacity of the C/EBPβ deficient fibroblasts in vitro, their diminished ability to differentiate to myofibroblasts would have a greater overall inhibitory effect on fibrosis in light of the greater ability of myofibroblasts to elaborate extracellular matrix and cytokines [3,27,28].

Taken together, these findings demonstrate a multifactorial role for C/EBPβ in this model of pulmonary fibrosis. This is partly attributable to the diversity of target genes that are under the control of this complex transcription factor. Thus its deficiency is likely to have a multiplicity of effects. However, given the dismal outlook for treatment of chronic fibrotic diseases, especially using agents that target single molecules, it may be that these multiple effects by a single transcription factor may make it a better therapeutic target. Indeed there is evidence that this might be feasible in the case of C/EBPβ in studies of how aspirin or salicylates regulate cyclooxygenase-2 expression [30–32]. This approach relies on the importance of phosphorylation of this transcription factor in binding to its binding element, and thus targeting the kinase(s) responsible for this phosphorylation may be a feasible means by which C/EBPβ can be targeted in future studies.

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