Antiapoptotic effect of found in inflammatory zone (FIZZ)1 on mouse lung fibroblasts

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Abstract
Myofibroblasts play an essential role in the abnormal deposition of extracellular matrix in pulmonary fibrosis. The presence or prolonged survival of these cells may be a key factor in the pathogenesis of progressive pulmonary fibrosis. Found in inflammatory zone (FIZZ)1 can induce myofibroblast differentiation and has an antiapoptotic effect on embryonic lung explant cultures. In this study, we investigated whether FIZZ1 also has an antiapoptotic effect on mouse lung fibroblasts (MLFs). Cells were treated with FIZZ1 for 24 h and then apoptosis was induced by TNFα in the presence of cycloheximide (CHX). FIZZ1 exhibited an antiapoptotic effect in MLFs, as assessed by flow cytometric analysis and TUNEL staining. Moreover, the cell number was higher in the FIZZ1–treated group relative to the non–treated control group after treatment with TNFα and CHX. FIZZ1 treatment also inhibited the apoptotic agent–induced activities of caspase-3 and caspase-8. Examination of potential signalling pathways revealed that FIZZ1 induced rapid phosphorylation of ERK-1/2, while PD98059, a MEK/ERK inhibitor, markedly induced activation of caspase-3. This antiapoptotic effect of FIZZ1 was associated with induction of myofibroblast differentiation in response to FIZZ1 stimulation. Taken together, these findings suggest that FIZZ1 is involved in pulmonary fibrosis through both induction of myofibroblast differentiation and increased or prolonged survival of myofibroblasts. This effect of FIZZ1 was mediated by inhibition of caspase-3 and -8, with involvement of the ERK pathway.

Keywords: FIZZ1; myofibroblast; apoptosis; caspase-3; caspase-8; ERK

Introduction
FIZZ1 (found in inflammatory zone 1), also known as RELM-α (resistin–like molecule α), was originally discovered in lung allergic inflammation [1], but its role is unclear. The FIZZ/RELM family has four members, FIZZ1/RELM-α, FIZZ2/RELM-β, FIZZ3/resistin, and RELM-γ, characterized by their conserved 10-cysteine residue motif, with two members, resistin and FIZZ2, having an additional cysteine residue [1]. FIZZ1 is a 9.4 kDa secreted protein and is expressed in white adipose tissue, spleen, lung, heart and mammary glands [1–3]. FIZZ1 inhibits adipocyte differentiation and nerve growth factor–mediated dorsal root ganglion survival. In normal lung, FIZZ1 expression is essentially undetectable, but in allergic airway inflammation it is highly expressed by macrophages, bronchial epithelium and type II alveolar epithelial cells (AECs) [1]. FIZZ1 is also highly induced in bleomycin–induced lung fibrosis and can induce myofibroblast differentiation in lung fibroblast culture [4]. The Th-2 cytokines, IL-4 and IL-13, induce AEC FIZZ1 expression via STAT6 and JAK-1 [5]. STAT6, or IL-4 and/or IL-13 deficiency abolish lung FIZZ1 expression and fibrosis in the bleomycin model. FIZZ1 has the capacity to promote lung cell proliferation, angiogenesis and inflammation [6,7]. Its expression is an indicator of alternative activation of macrophages [8,9]. All of these properties indicate a role for FIZZ1 in the regulation of cell growth/proliferation and differentiation.

During active fibrosis, de novo genesis of the myofibroblasts is a key common feature and it is thought that, due to their importance as a major cellular source of extracellular matrix and fibrogenic cytokines, their persistence or survival may contribute to progressive fibrosis instead of resolution and complete healing. Thus, understanding the basis for their survival may provide further insight into their role in the pathogenesis of progressive pulmonary fibrosis. Germane to this issue is the previous observation that, in addition to myofibroblast differentiation, FIZZ1 has antiapoptotic effects on embryonic lung explants and causes increased lung cell density [10], consistent with its mitogenic effect on pulmonary vascular smooth muscle cells [6]. However, the effect of FIZZ1 on lung fibroblast or myofibroblast survival/apoptosis and proliferation is unknown.
In this study we hypothesized that FIZZ1 may have antiapoptotic or proliferative effects on lung fibroblasts, which can impact on the survival or persistence of the myofibroblast in the context of pulmonary fibrosis. We examined the antiapoptotic role of FIZZ1 in cultured mouse lung fibroblasts (MLFs), using biochemical assays, flow cytometric analysis and TUNEL staining, and surveyed potential associated signalling pathways. The results showed that FIZZ1 had protective effects against apoptosis in MLFs, which were mediated by the ERK signalling pathway.

Materials and methods

Cell culture

Six-week old female CBA/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out in accordance with the guidelines of the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan for the care and use of laboratory animals and with the Committee’s approval. Lung fibroblasts were isolated as before [11] and cultured in DMEM, supplemented with 10% plasma-derived serum (PDS; Cocalico Biologicals Inc., Reamstown, PA), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, 1% ITS (insulin, transferrin, selenium; Sigma, St. Louis, MO), 5 ng/ml PDGF (R&D Systems, Minneapolis, MN) and 10 ng/ml EGF (R&D Systems). The cells were used at passages 3–5.

Induction and detection of apoptosis

Cells (5 × 10^5) were plated onto 60 mm plates and when subconfluent they were serum deprived in DMEM with 0.5% PDS for 24 h. Apoptosis was induced by treatment with 5 ng/ml TNFα (R&D Systems) and 500 ng/ml cycloheximide (CHX) for 4 h, or 200 ng/ml anti-Fas monoclonal antibody (BD, San Diego, CA) and 5 µg/ml CHX for 14–16 h. Since FIZZ1 induces myofibroblast differentiation after 24 h treatment in lung fibroblast culture [4,5], the antiapoptotic effect of FIZZ1 was studied by pretreating cells with 25 ng/ml murine FIZZ1 (Leinco Technologies, St. Louis, MO) for 24 h before TNFα/CHX treatment. Apoptosis was evaluated using annexin V-propidium iodide (PI) staining (TACS™, Annexin V-FITC; R&D Systems). Briefly, cells were collected after trypsinization, washed with ice-cold PBS and resuspended in 100 µl Annexin V incubation reagent (10 µl 10× binding buffer, 10 µl propidium iodide, 1 µl Annexin-V-FITC, 79 µl distilled water) at a concentration of 3–5 × 10^5 cells/100 µl, and incubated for 15 min at room temperature (RT) in the dark. Samples were washed with binding buffer and analysed by FACS Caliber (Becton-Dickinson). Apoptotic cells were identified as an annexin-V-positive/PI negative population.

Additionally, TUNEL assay was performed for in situ detection of apoptotic cells, using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon, CA, USA). Induction of apoptosis and FIZZ1 treatment were identical to the method used in FACS analysis of apoptosis. This was followed by determination of apoptotic cells, in accordance with the manufacturer’s protocol, and fluorescence microscopic analysis by counting the number of positive cells in randomly chosen, non-contiguous, high-power (∗×40 objective) fields until a minimum of 400 total cells were counted. Cell number was counted after 48 h starvation with or without FIZZ1 treatment, using a Coulter counter.

Cell transfection

A dominant negative Akt construct was used to evaluate the role of Akt in response to FIZZ1 treatment. The construct, Akt–K179A, had a point mutation with a substitution of alanine for lysine at position 179 (gift from Dr Victor Thannickal, University of Michigan). The empty vector PCIS2 was used as negative control. Transfection of these constructs by Nucleofector technology was performed according to the optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). Briefly, the cells were gently resuspended in 100 µl primary mammalian fibroblast nucleofector solution (Amaxa Biosystem), mixed with cDNA and pulsed with the program U-23. Immediately afterwards, the cells were transferred into prewarmed fresh medium in six-well plates. The cells were used 24 h after transfection to evaluate the involvement of Akt in the antiapoptotic effect of FIZZ1. A control vector, pmaxGFP (Amaza Biosystem), was used to gauge transfection efficiency.

Western blot analysis

Whole cell lysates were prepared and analysed by western blotting for the protein species indicated, as previously described [4]. The following antibodies were used: anti-Akt, anti-phosphoAkt and anti-ERK1/ERK2 (R&D systems); anti-phosphoERK1/ERK2 (Thr 202/Tyr204) (Cell Signalling, Beverly MA); anti-α-smooth muscle actin and anti-caspase-3 (Sigma, St. Louis, MI); and anti-caspase-8 (BD, San Diego, CA). Blots were developed using corresponding HRP-conjugated secondary antibodies, followed by chemiluminescence detection (New England Biolabs, Beverly, MA).

Caspase-3 activity assay

The bioactivity of caspase-3 was measured with a Colorimetric Activity Assays Kit (Chemicon International, Temecula, CA). In brief, MLFs were resuspended in lysis buffer and centrifuged. The supernatants were incubated with caspase-3 substrate Ac-DEVD-pNA. The free pNA from Ac-DEVD-pNA was measured at 405 nm, using a microplate reader.
Results

Antiapoptotic effect of FIZZ1 on mouse lung fibroblasts

In preliminary observations, after serum and growth factor deprivation for 48 h, 5.5 ± 0.29% (mean ± SE, n = 4) of non-treated MLF underwent early apoptosis, as evaluated by annexin V–PI staining with analysis by flow cytometry. In cells treated with FIZZ1 (25 ng/ml), the number of apoptotic cells was significantly (p < 0.05) reduced to 3.7 ± 0.54% (mean ± SE, n = 4) of total cells. This suggested the antiapoptotic activity of FIZZ1 in lung fibroblasts, which correlated with its myofibroblast differentiation-inducing activity [4]. However, due to the relatively low level of apoptosis induced by serum deprivation, another approach is required to optimally study this effect. Optimal conditions for inducing apoptosis were observed when cells were treated for 4 h with 5 ng/ml TNFα and 500 ng/ml CHX, or for 14–16 h with 200 ng/ml anti-Fas and 5 µg/ml CHX. TNFα or Fas alone had no effect on apoptosis under these conditions. TNFα/CHX treatment caused a four-fold increase (to 22.5 ± 0.46%) in apoptotic cells by annexin V staining (Figure 1A, B). Pretreatment with 25 ng/ml FIZZ1 significantly (p < 0.05) diminished TNFα/CHX-induced apoptosis by >40%. Anti-Fas antibody/CHX treatment showed a greater proportion of apoptotic cells (26.1 ± 0.79%), which was also significantly (p < 0.05) reduced by 32 ± 4% by FIZZ1 pretreatment.

To confirm that FIZZ1 has an antiapoptotic effect, TUNEL staining was used as a complementary approach. The results confirmed that FIZZ1 had an antiapoptotic effect on lung fibroblasts (Figure 1D, E). FIZZ1 did not affect the basal or control level of apoptosis. Thus, using three different approaches to monitor apoptosis, FIZZ1 was found to have significant antiapoptotic activity.

![Figure 1. Effect of FIZZ1 on mouse lung fibroblast (MLF) apoptosis. Apoptosis in MLF was detected by FACS analysis after annexin V–propidium iodide (PI) staining (A, B) and by TUNEL assay (C, D). Cells were cultured in the absence or presence of FIZZ1 for 24 h and then treated with TNFα/CHX for 4 h. Representative FACS histogram (A) and micrograph from TUNEL assay (C) are shown. The experiments were repeated twice and the combined results from the respective assays are shown (B, D) as mean ± SE (n = 3). In (B, D), an asterisk indicates that the mean value for the TNFα + FIZZ1 group was significantly different (p < 0.05) from that for cells treated with TNFα/CHX (TNFα) only, as assessed by ANOVA](image-url)
Antiapoptotic effect of FIZZ1

Since the death signal of TNFα is mediated by caspase-8 activation, and caspase-3 is one of its downstream targets, the importance of these caspases to the antiapoptotic activity of FIZZ1 was examined. The TNFα-induced activation of these caspases was confirmed by examining the cleavage of procaspase-3 and -8 by western blotting. Cleavage of caspase-3 to its 17 kDa form was readily observed at 4 h after TNFα/CHX treatment by western blotting, while caspase-8 activation was detected as early as 30 min (Figure 2A). Pretreatment with FIZZ1 caused a reduction in TNFα/CHX activation of caspase-3 at 4 h, and caspase-8 at 1 and 4 h of TNFα treatment (Figure 2A, B). The results were confirmed using a colorimetric assay for caspase-3 (Figure 2C). Thus, inhibition of apoptosis by FIZZ1 was accompanied by reduction in activation of caspases-3 and caspase-8.

FIZZ1 effects on Akt and ERK

To further determine the intracellular mechanism involved in the antiapoptotic effect of FIZZ1, we analysed the effect of FIZZ1 on signalling pathways that are known to control cell survival, such as the PI-3 kinase–Akt and ERK pathways. Western blotting analysis for Akt and ERK and their respective phosphorylated species (pAkt and pERK, respectively) showed that FIZZ1 alone did not activate Akt, but rapidly activated ERK (Figure 3A).

In contrast to FIZZ1 treatment alone, combined treatment with TNFα/CHX caused a noticeable increase in pAkt at 10 and 60 min of treatment (Figure 3B), indicative of Akt activation under this condition. ERK activation was enhanced by the combined treatment after 10 min compared to FIZZ1 treatment alone, but also appeared diminished by 60 min. The levels of total ERK and Akt were not noticeably affected by any of the treatments. Pretreatment with

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**Figure 2.** FIZZ1 effect on TNFα/CHX-induced caspase-activation. Apoptosis of MLF was induced by 5 ng/ml TNFα/500 ng/ml CHX treatment for 4 h after cell culture in the absence or presence of FIZZ1 for 24 h. Caspase-3 and -8 activities were analysed by Western blot (A, B) and colorimetric assay (C, caspase-3 only), as described in Methods. Densitometric analysis of caspase-3 and -8 expression levels normalized to GAPDH at 4 h is shown in (B). Results are shown as percentages of the respective control (untreated) values. The results of colorimetric assay are expressed as mean ± SE of triplicate measurements. Asterisks in (B, C) indicate that the mean value for the TNFα + FIZZ1 group was significantly less (p < 0.05) than that for cells treated with TNFα/CHX (‘TNFα’) only, as assessed by ANOVA.
the MEK/ERK inhibitor PD98059 reduced the FIZZ1-induced ERK phosphorylation, but was not affected by the PI3 kinase inhibitor LY 294002 (Figure 3C). Thus, inhibition of TNFα-induced apoptosis by FIZZ1 was accompanied by activation of both Akt and ERK signalling, although FIZZ1 alone had no significant effect on Akt signalling.

The effects of these kinase inhibitors on apoptosis were then examined. Addition of the MEK inhibitor PD98059 alone caused enhanced activation of caspase-3 relative to that caused by TNFα/CHX alone, while LY294002 did not have a noticeable effect (Figure 4). Furthermore, PD98059 reversed the inhibitory effect of FIZZ1 on TNFα/CHX-induced activation of caspase-3 and -8. The PI3 kinase inhibitor did not have a detectable effect on the activation of caspase-8. Moreover, relative to cells transfected with the expression transfection vector only (PCIS2), transfection with a dominant negative Akt construct (Akt–K179A) did not significantly affect TNFα/CHX-induced caspase-8 activity, neither did it affect inhibition of this induced activity by FIZZ1 (Figure 5). Thus, only the ERK pathway appeared to be significantly involved in mediating the FIZZ1 signal. To further confirm the importance of ERK in mediating the FIZZ1 antiapoptotic effect, the effect of PD98059 on annexin V staining was evaluated. The results showed that the FIZZ1-induced reduction
Figure 4. Effects of kinase inhibition on caspase activity. The cells were treated as indicated and the protein extracts were then analysed by western blotting for caspase-3 and -8 (A). A representative blot is shown, which was repeated twice with comparable results. The results of quantification by densitometry and after normalization to GAPDH are shown in (B) as a percentage of the mean value of cells treated with TNFα/CHX only (i.e. without FIZZ1). Asterisks indicate significant difference ($p < 0.05$) from mean values of TNFα/CHX-treated cells. FIZZ1 suppression of TNFα/CHX-induced caspase-3 and -8 activation was abolished by the MEK inhibitor, PD98059, but not by LY294002.

Discussion

This report demonstrates that FIZZ1 has antiapoptotic activity in primary cultured MLFs and that this effect is associated with activation of ERK and inhibition of caspase-3 and -8. Since FIZZ1 also promotes myofibroblast differentiation [4], this would contribute to the enhanced survival and persistence of the myofibroblast. In response to pulmonary injury with accompanying inflammation, myofibroblasts play a key role as a major cellular source of extracellular matrix deposition, a hallmark of pulmonary fibrosis. In normal wound healing, the de novo genesis of myofibroblasts peaks at the height of active fibrosis with elaboration of matrix components and subsequently gradually declines as the healing process is successfully completed. This decline in myofibroblast numbers is thought to be due to their apoptosis, although the trigger or inducer of apoptosis remains uncertain [12,13]. In contrast, these cells persist and can be found in various stages of human pulmonary fibrosis where the disease is progressive. Therefore, the altered regulation of the number of myofibroblasts may be a key factor in progressive pulmonary fibrosis [14–16].

Apoptosis is a physiological process leading to elimination of unwanted cells within living tissues [17]. Studies of wound healing suggest that myofibroblast...
disappearance occurs via apoptosis [12]. The mechanisms involved in physiological or pathological regulation of myofibrolast apoptosis are unknown, and growth factors, cytokines, extracellular matrix interaction or death receptor ligands appear to be potential modulators [13,18]. A role for cytokines as modulators of apoptosis susceptibility has also been proposed, but data on the effect of pro- or anti-fibrotic cytokines on fibroblast apoptosis are scanty. Transforming growth factor-β (TGFβ), one of the most potent fibrogenic cytokines, promotes myofibroblast survival by preventing the myofibroblast from undergoing apoptosis [19]. In contrast to the effect of TGFβ, interleukin-1β (via generation of NO) and the CD95-receptor–CD95-ligand system (Fas/FasL) are reported to induce apoptosis [20,21]. The present study revealed that, similar to TGFβ, FIZZ1 had an antiapoptotic effect on MLF, which was associated with myofibroblast differentiation [4]. The response that we observed is similar to a FIZZ1-induced inhibition of apoptosis noted in lung epithelial cells from lipopolysaccharide-induced apoptosis and in cultured embryonic lung [10,22]. This suggests that the antiapoptotic effect of FIZZ1 may be a generalized activity that is independent of its ability to induce myofibroblast differentiation. This possibility, however, cannot be ruled out from the data presented.

Although there is evidence that FIZZ1 has significant antiapoptotic activity in lung epithelial cells and fibroblasts, the mechanisms involved in the antiapoptotic effect of FIZZ1 are still unknown. The caspases are the ultimate effectors or executioners of programmed cell death. They are synthesized as inactive precursors that are usually converted to the active form by proteolytic cleavage, catalysed by other caspases. TNFα is known to induce apoptosis by modulating Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (FLIP), caspase-8, and surface Fas expression [21]. Caspase-8 activation is capable of inducing apoptosis mediated either directly through the activation of caspase-3, or by the activation of the Bid protein, which results in mitochondrial injury, the release of cytochrome c, and activation of caspase-9 and caspase-3. To characterize the mechanism of the antiapoptotic effect of FIZZ1 in MLF, the activation of caspase-8 and one of its downstream targets, caspase-3, was examined. FIZZ1 was found to be effective at inhibiting caspase-8 and -3 activation, correlating with its antiapoptotic activity in these cells. These observations suggested that FIZZ1 could prevent apoptosis of MLF by inhibiting the activation of these caspases, which could then contribute to the progression of pulmonary fibrosis by promoting myofibroblast survival and persistence.

Cell survival, death and apoptosis are also known to be regulated by certain mitogen-activated protein kinase (MAPK) pathways. MAPKs are cytoplasmic serine/threonine kinases that are activated in response to a various extracellular stimuli, including those that regulate cell proliferation, differentiation, cell survival, development and inflammation. ERK is a member of the MAPKs, and activation of the ERK pathway suppresses apoptosis induced by growth factor deprivation, matrix detachment signals and cytotoxic drugs [23–26]. Another major intracellular signalling pathway that is responsible for promoting cell survival is the PI3-kinase–Akt signalling pathway. Activation of PI3-kinase is thought to inhibit apoptosis by promoting translocation of Akt to the plasma membrane, where Akt can be phosphorylated at Thr308 and Ser473 by the kinases PDK-1 and PDK-2, respectively [27,28]. Phosphorylated Akt functions as a survival signal by inhibiting the activation of pro-apoptotic signals [29]. Neither the receptor nor the downstream signalling pathway for FIZZ1 has been identified with respect to its antiapoptotic activity. In rat pulmonary vascular smooth muscle cells, PI3-kinase and Akt are involved in FIZZ1 downstream signalling related to their proliferation. A recent study suggests that FIZZ1 induces vascular adhesion molecule-1 (VCAM-1) expression via the PI3K-Akt–NFκB signalling pathway in mouse endothelial and lung epithelial cells [30]. FIZZ2, another FIZZ family member, activates ERK and p38 in hepatocytes that is related to insulin resistance. [31]. However, there is so far no report that ERK participates in FIZZ1 downstream signalling. ERK1/2 was was activated upon treatment with FIZZ1, and this activation was suppressed by an inhibitor of this pathway, the MEK/ERK inhibitor PD98059. Furthermore, we demonstrated that FIZZ1-induced ERK activation was associated with inhibition of caspase-3 activation. This result is consistent with previous reports that activation of ERK is associated with inhibition of caspase-3 activation. ERK phosphorylates caspase-9 on Thr125 and it inhibits caspase-9 processing and caspase-3 activation [32,33].

In contrast to ERK, Akt was not activated by FIZZ1, except when combined with TNFa/CHX treatment. Thus, while the importance of ERK in FIZZ1-induced antiapoptotic activity was clearly suggested, the role of Akt was less clear and may involve more complex mechanisms associated with the additional response to TNFa/CHX treatment. Moreover, the importance of ERK was confirmed by the ability of PD98059 to abrogate the antiapoptotic effect of FIZZ1, including its effects on caspase activation, while the significance of Akt was not apparent, since LY294 002 failed to affect the FIZZ1-induced inhibition of caspase activation. Taken together, these data indicate that the ERK pathway is significantly involved in the antiapoptotic effect of FIZZ1. Whether this effect is dependent on the myofibroblast differentiation also promoted by FIZZ1 [4] remains to be determined. In any case, the data presented in this study suggest that FIZZ1 can contribute to the pathogenesis of progressive pulmonary fibrosis by induction of myofibroblast persistence via antiapoptotic effects.

Acknowledgements

We thank Dr Victor Thanmickal (Department of Internal Medicine, University of Michigan) for the generous gift of the
dominant negative Akt construct (Akt–K179A) and expression vector control (PCIS2). This work was supported by grants HL28737, HL31963, HL52285 and HL77297 from the National Institutes of Health, and an award to S.H.P. from the Sandler Program for Asthma Research.

References


8. Nair MG, Cochrane DW, Allen JE. Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and FIZZ1 that can be partly replicated in vitro. *Immunol Lett* 2003;85:173–180.


20. Antiapoptotic effect of FIZZ1