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Expression and effects of cardiotrophin-1 (CT-1) in human airway smooth muscle cells

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1 Cellular hypertrophy and/or a reduced rate of apoptosis could increase airway smooth muscle mass. As cardiotrophin-1 (CT-1) induces hypertrophy and inhibits apoptosis in cardiomyocytes, we tested for the expression and effects of CT-1 in human bronchial smooth muscle cells (HBSMC).

2 CT-1 was detected in abundance in normal adult human lung and was expressed in both fetal and adult HBSMC.

3 Following serum deprivation, CT-1 was released by reintroduction of serum and by TGF-β2/IL-4 in fetal but not adult cells. TGF-β2/IL-4 triggered the release of CT-1 in serum-fed adult cells. Hypoxia and strain had no effect on the release of CT-1.

4 CT-1 reduced the apoptosis induced both by serum deprivation and by Fas antibody/TNF-α treatment in adult cells, with greater efficacy than other members of the IL-6 superfamily. The MAPK/ERK kinase inhibitor PD98059 (1–10 μM) reduced the effect of CT-1. Fetal cells were more resistant to apoptosis.

5 CT-1 (10 ng ml^{-1}) induced a significant increase in cell size as judged by protein/DNA ratios and flow cytometry. No effects on smooth muscle α-actin or vimentin proteins were noted, although CT-1 qualitatively alters the cytostructural distribution of SM22, an actin filament-associated protein, and increased SM22 protein abundance. No effect on proliferation or migration was evident.

6 These data suggest CT-1 expression primarily in fetal and synthetic HBSMC phenotypes. By reducing the rates of apoptosis and inducing hypertrophy, CT-1 may contribute to increased smooth muscle mass in airway disease.

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Keywords: Asthma; flow cytometry; smooth muscle phenotype; cardiotrophin-1; IL-6; apoptosis; hypertrophy

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CT-1, cardiotrophin-1; ERK, extracellular signal-regulated kinase; HBSMC, human bronchial smooth muscle cells; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; nmMHC, nonmuscle myosin heavy chain; OSM, oncostatin M; TNF-α, tumor necrosis factor

Introduction

Severe asthma is accompanied by an increase in smooth muscle mass. The amount of airway smooth muscle is greater with increased duration of asthma and may be an important determinant of the severity of airway responsiveness (Bai *et al*., 2000); yet, the mechanisms responsible for the increased mass are unclear. The increased smooth muscle mass appears to be due to an increase in both myocyte size and number (Hossain & Heard, 1970; Ebina *et al*., 1993), but *in vivo* rates of proliferation are likely very low (Benayoun *et al*., 2003). Cardiotrophin-1 (CT-1), a member of the IL-6 superfamily, was initially defined as a factor that has the capacity to induce cardiac myocyte hypertrophy (Habecker *et al*., 1995; Pennica *et al*., 1996). Further study showed that CT-1 caused hypertrophy of differentiated cardiac muscle cells, and inhibited the onset of cardiac myocyte apoptosis following serum deprivation or cytokine stimulation, an effect mediated in part *via* a mitogen-activated protein kinase (MAPK)-dependent pathway (Sheng *et al*., 1997). CT-1 has been grouped within the interleukin (IL)-6 family, based on structural homology and utilization of the gp130 signal transducer (Habecker *et al*., 1995). The activation of gp130 is reported to be critical in myocardial survival and hypertrophy (Wollert & Chien, 1997; Kunisada *et al*., 1998). Hirota *et al*. (1999) found that loss of gp130 leads to massive cardiac myocyte apoptosis following the biomechanical stress of pressure overload. Recent reports show that CT-1 expression is increased in cardiomyopathy, as part of a panel of fetal genes induced by the heart in an attempt to compensate for dysfunction (Zolk *et al*., 2002). Whether CT-1 has the same effects in airways as it does in the heart is unexplored.

We postulate that CT-1 is released within the airway wall during chronic asthma and contributes to airway wall thickening by inhibition of airway smooth muscle apoptosis and by stimulation of smooth muscle hypertrophy. To begin to test these hypotheses, we have determined if CT-1 is expressed in human fetal and adult smooth muscle cells, as well as in *ex vivo* human lung, tested the potential stimuli for extracellular release shown to be relevant in cardiac myocytes

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(hypoxia, cytokines, mechanical strain) (Pan *et al*., 1999; Brar *et al*., 2001; Lahiri *et al*., 2001), determined if proliferative and chemotaxis effects occurred, and determined whether CT-1 diminishes airway smooth muscle apoptosis induced by serum deprivation and/or the TNF-α/Fas death pathway (Sheng *et al*., 1996; Mukhina *et al*., 2000). Finally, we determined if the MAPK/ERK pathway is involved in antiapoptotic effects and, given that CT-1 has been reported to induce cytostructural remodeling in cardiac myocytes (Cheng *et al*., 1997; Kunisada *et al*., 1998), tested whether CT-1 affects smooth muscle protein abundance and cytostructure.

Methods

Cells and reagents

Adult (males aged 18, 37 and 21) and fetal (21-week fetus) human primary bronchial smooth muscle cells (HBSMC) were obtained from Clonetics (La Jolla, CA, U.S.A.), or were isolated by us as previously described (Halayko *et al*., 1996). CT-1, IL-6, oncostatin M (OSM), IL-13, TGF-β2, INF-γ and IL-4 were from R&D Systems (Minneapolis, MN, U.S.A.), TNF-α was from CalBiochem (La Jolla, CA, U.S.A.), anti-Human Fas monoclonal antibody (CH-11) was from Immunotech (Marseille, France), Apo-BrdU™ kit was from Pharmingen (Mississauga, Ontario, Canada). PDGF AB was obtained from Sigma (Louis, U.S.A.). [^{3}H] thymidine was from Amersham Pharmacia Biotech. Matched antihuman antibody pairs used for CT-1 and IL-6 ELISA were obtained from R&D Systems. Primary antibodies used for Western blot and immunocytochemistry included monoclonal antibodies for sm-α-actin (clone 1A4) and vimentin (clone V9) (Sigma-Aldrich, St Louis, U.S.A.), polyclonal antibodies for SM22 (generously provided by J. Solway, University of Chicago), and for nm-MHC (generously provided by R. Adelstein, National Institutes of Health, Bethesda, MD, U.S.A.).

Human lung homogenates

Methods were as previously described (Knight *et al*., 1999). In summary, segments of macroscopically normal lung taken from lung resected from lifelong nonsmokers ($n=6$) or heavy smokers with mild COPD ($n=6$) were either immediately homogenized and incubated with lysis buffer containing protease inhibitors to measure basal total CT-1 levels (per μg total protein), or were minced with fine scissors and cultured in DMEM supplemented with L-glutamine, streptomycin, penicillin and gentamicin for 24 h. Aliquots of supernatant were assayed for CT-1 using an ELISA from R&D (Minneapolis, MN, U.S.A.).

HBSM cell culture and treatment

For experiments on CT-1 production and release, cells were grown to $\sim$80% confluence in 24-well plates in SmGM containing 5% FBS (Clonetics cells), or DMEM medium containing 10% FBS and 100 μM nonessential amino-acid solution (our primary isolates), plus 10 ng ml^{-1} EGF, 2 ng ml^{-1} FGF, 5 μg ml^{-1} insulin, 50 μg ml^{-1} gentamicin and 50 ng ml^{-1} amphotericin. They were then washed once with PBS and incubated for 48 h in serum-free SmBM or F12 medium containing insulin (10 μg ml^{-1}), transferrin (5.5 μg ml^{-1}) and selenium-A (6.7 ng ml^{-1}). Serum (5% FBS) or cytokines were then added for 48 h as follows: IL-4 (20 ng ml^{-1}) $\pm$ TGF-β2 (0.01–10 ng ml^{-1}); TNF-α (1 ng ml^{-1}) and INF-γ (5 ng ml^{-1}); IL-6 (1 ng ml^{-1}), IL-13 (10 ng ml^{-1}), IL-1 (0.1–1 ng ml^{-1}), or vehicle. At the end of the incubation, an aliquot of culture medium was taken and frozen at -70°C for subsequent measurement of CT-1 by ELISA. The cell monolayers were then washed and incubated with lysis buffer containing protease inhibitors, and frozen for CT-1 quantification as above.

Mechanical strain as a stimulus for CT-1 release

To determine the effect of mechanical strain on CT-1 and IL-6 release, HBSMC were plated on collagen type I-coated silastic membranes in six-well culture plates, and subjected to strain using a commercially available apparatus (Flexercell) programmed to apply 30% maximum deformation of the membrane for 2 s with 2 s relaxation. At intervals, the culture medium was collected and frozen until analyzed; after 120 h of stretch, the cells remaining after removal of medium were lysed with lysis buffer in the presence of protease inhibitors, and frozen until analyzed. CT-1 and IL-6 synthesis and release were measured by ELISA.

Hypoxia protocol

To determine if CT-1 is released in response to hypoxia, adult HBSMC were grown to near confluence in six-well plates, washed with PBS once and then incubated in serum-free SmBM medium. After 24 h incubation, the media was replaced with fresh serum-free SmBM pretreated with a gas mixture of 95% N_2 and 5% CO_2 for 15 min. The plates were then placed in a controlled atmosphere chamber, which was flushed with a gas mixture of 95% N_2 and 5% CO_2 at a flow rate of 4 l min^{-1} for 15 min. The chamber was then placed in a 37°C incubator on a rocking platform set at 12 cycles min^{-1}, and cells were exposed for 2, 6, 17 and 24 h, respectively. The hypoxia-exposed cells were reoxygenated with a gas mixture of 95% O_2 and 5% CO_2 for either 24 or 48 h. The supernatants were collected and frozen at -70°C for CT-1 analysis.

Apoptosis assays

HBSMC at passages 5–8 were used for studies of apoptosis. Three protocols were employed. (1) cells at $\sim$80% confluence grown in 25 cm^2 flasks (for flow cytometry), or 24-well culture plates (for ELISA), were washed with PBS once and then incubated in serum-free SmBM medium with or without CT-1 (0.1–10 ng ml^{-1}) for 3 days; (2) cells at $\sim$80% confluence were washed with PBS once and then incubated in serum-free SmBM with or without CT-1 (1 ng ml^{-1}) or combinations of CT-1 and PD98059 (0.1–10 μM) for 3 days. PD98059 was added to the culture 30 min prior to the addition of CT-1; (3) cells at $\sim$80% confluence were treated with or without CT-1 (0.1–10 ng ml^{-1}) or 10 ng ml^{-1} TNF-α for 8–48 h in SmGM medium. After treatment with the cytokines, the cells were washed with PBS once and then incubated in serum-free SmBM medium in the presence or absence of 200 ng ml^{-1} CH-11 anti-human Fas monoclonal antibody (IgM) for 24 h. At

the end of the incubation with the cytokines and the antibody, the cells were collected for the identification of apoptosis.

Apoptosis was initially detected by a flow cytometry protocol; however, as an ELISA for DNA fragmentation gave similar results, this was used in later experiments.

Flow cytometry method Free-floating and attached cells from 25 cm^2 flasks were trypsinized and collected. Cells were fixed in 1% buffered formaldehyde for 30 min, washed in PBS, and permeabilized with ice-cold 70% ethanol. HBSM apoptosis was identified by an APO-BrdU™ kit using the standard protocol provided by the manufacturer (Pharmingen, Mississauga, Ontario, Canada) (Rucker-Martin *et al*., 1999; Nagahara *et al*., 2000). Dual-wavelength flow cytometric analysis was performed on a FACS analyzer (EPICS XL-MCL, Coulter Miami, FL, U.S.A.) equipped with a 488 nm argon ion laser. In pilot experiments, when apoptosis was indicated by flow analysis, results were confirmed by inspection of nuclei using a fluorescent microscope after staining of DNA in cells with Hoechst 33258.

ELISA method for DNA fragments Histone-associated DNA fragments were detected as described by the manufacturer (Roche, Indianapolis, IN, U.S.A.), using a colorimetric ELISA assay.

Cell size

As a measure of a potential hypertrophy effect of CT-1, cell size was estimated by flow cytometry, based on the parameters of forward scatter (FS) and side scatter (SS) (Mandy *et al*., 1995), and by protein/DNA ratios. HBSMC were grown in growth medium with 1 ng ml^{-1} CT-1 or with 10 ng ml^{-1} CT-1 or vehicle for 5 days, then cultured in serum-free medium under the same three conditions for 7 days. DNeasy Tissue Kit (QIAGEN, Ontario, Canada) was used for DNA purification. BCA Assay Kit (Sigma-Aldrich, St Louis, U.S.A.) was used for protein quantification.

Proliferation and migration experiments

HBSMC at passage 5 were used for studies of proliferation. The effect of CT-1 on HBSM cell proliferation was estimated by measuring [^{3}H]thymidine incorporation as previously described (Billington *et al*., 1999). Migration in response to CT-1 was determined using a micro-Boyden chamber as previously described (Mukhina *et al*., 2000; Irani *et al*., 2002).

SDS–PAGE, Western blotting and immunocytochemistry for contractile proteins

Total protein lysates were obtained from serum-fed cultured HBSMC at 80% confluence, and from cultures that were also serum-deprived for 96 h in media with or without CT-1, 1 or 10 ng ml^{-1}. Proteins (40 μg $lane^{-1}$) were resolved on either 5% or 10% SDS–PAGE gels and processed for Western blot analysis, as described previously (Halayko *et al*., 1996). For immunocytochemistry, cultured HBSMC were cultured on 25 mm coverslips in 12-well dishes, grown and treated with CT-1 as described for Western Blot analysis.

Statistical analysis

Each data point in individual experiments was calculated from two to five determinations. All values are reported as mean ± s.e.m. from four to six separate experiments. Analyses of variance or paired *t*-tests were used to determine significance. *P*-values <0.05 were considered significant.

Results

Quantification of CT-1 in human lung and isolated smooth muscle cells, and stimuli for release

Human lung homogenates At baseline, lung homogenates from six nonsmoking patients contained 76.3 ± 9.3 pg CT-1 μg^{-1} total protein, not significantly different from samples from six heavy-smoking patients (85 ± 17 pg CT-1 μg^{-1} protein). Supernatants from lung homogenates contained 5200 ± 220 pg CT-1 ml^{-1} after 24 h incubation.

Fetal cells Serum-deprived HBSMC treated with combinations of TGF-β2 between 0.1 and 10 ng ml^{-1} and IL-4 (20 ng ml^{-1}), or with re-introduction of 5% serum, released substantial quantities of CT-1: 174.8 ± 14.9 pg ml^{-1} (maximal release at 1 ng ml^{-1} TGF-β2) and 890 ± 68 pg ml^{-1}, respectively (Figure 1, panel (a)). A time course analysis using 1 ng ml^{-1} TGF-β2 and 20 ng ml^{-1} IL-4 showed CT-1 release at points beyond 6 h, maximal at 48 h (Figure 1, panel (b)).

Adult cells At baseline in serum-deprived adult HBSMC, CT-1 levels in culture media were below the threshold of detection of our assay (32 pg ml^{-1}). Cell lysates contained 651 ± 65 pg ml^{-1} CT-1 after 48 h treatment with TNF-α

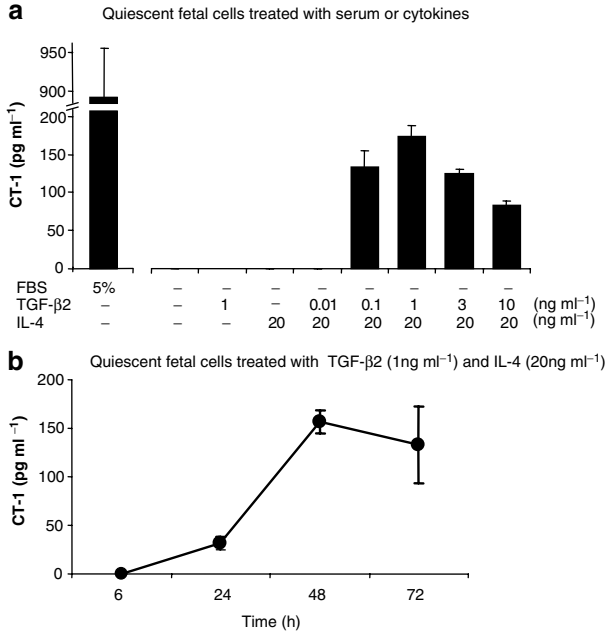

Figure 1 Panel (a) Effect of FBS (5%); or TGF-β2 ± IL-4 on CT-1 release from growth arrested fetal smooth muscle cells. Panel (b) Time course of effect of optimal concentrations of TGF-β2 in combination with IL-4 in causing release of CT-1. The mean data ± s.e.m. from four experiments are shown.

(1 ng ml^{-1})/INF-γ (5 ng ml^{-1}), whereas media contained only 70 ± 30 pg ml^{-1} CT-1 and control cell lysates at this time point expressed higher quantities of CT-1 (1203 ± 68 pg ml^{-1}, $P<0.05$). TGF-β2 (1 ng ml^{-1})/IL-4 (20 ng ml^{-1})-treated cells released trace amounts of CT-1 (circa 30 pg ml^{-1}) in the supernatant, whereas cell lysates contained 1359 ± 8 pg ml^{-1} CT-1, significantly more ($P<0.05$) than control cell lysates that contained 842 ± 34 pg ml^{-1} CT-1. In contrast, in HBSMC in which serum was not withdrawn, TGF-β2 (1 ng ml^{-1})/IL-4 (20 ng ml^{-1}) stimulated the release of 228 ± 22 pg ml^{-1} CT-1 *versus* undetectable amounts in controls; cell lysates under these conditions contained 1314 ± 54 *versus* 1591 ± 207 pg ml^{-1} CT-1, P not significant. IL-1, IL-6 and IL-13 did not induce the release of CT-1. Cyclical strain of adult cells for 24–120 h did not augment the release CT-1, whereas IL-6 levels increased significantly in both control and strained cells. Adult cells subjected to hypoxia showed no significant release of CT-1 (data not shown).

Effects of CT-1 on apoptosis in cultured smooth muscle cells

Fetal cells were very resistant to the apoptosis induced by either serum deprivation or anti-Fas 200 ng ml^{-1}/TNF-α 10 ng ml^{-1} (data not shown).

The effect of CT-1 on spontaneous apoptosis in serum-deprived adult HBSMC is shown in Figure 2a. A representative plot from flow cytometry demonstrates that 17.9% smooth muscle cells were apoptotic after 3 days serum deprivation *versus* only 9.5% of CT-1-treated cells. Overall, 1 ng ml^{-1} CT-1 inhibited HBSMC apoptosis by 40.2 ± 8.9% after 3 days of serum deprivation (16.8 ± 3.7% in vehicle-treated cells *versus* 10.6 ± 3.7% CT-1 treated cells, $n=3$). This inhibition occurred in a concentration-dependent manner, as demonstrated by the DNA fragmentation assay, with the optimal concentration of CT-1 being 1 ng ml^{-1} (Figure 2b).

TNF-α (10 ng ml^{-1}) alone was ineffective in inducing HBSMC apoptosis (data not shown). Anti-Fas alone (200 ng ml^{-1}) induced a weak apoptotic effect (11.3 ± 4.4% vehicle-treated cells *versus* 17.6 ± 4.5% anti-Fas-treated cells, $n=4$, $P<0.05$). As expected, TNF-α plus anti-Fas had a potent effect on HBSM apoptosis, the degree of apoptosis being eight fold > control (11.3 ± 4.4% vehicle-treated cells *versus* 87.8 ± 3.8% anti-Fas plus TNF-α-treated cells, $n=4$, $P<0.05$) (Figure 3a and b). The effect of CT-1 on the inhibition of anti-Fas/TNF-α-induced HBSMC apoptosis is also shown in Figure 3a and b. A marked inhibition of the anti-Fas and TNF-α-induced HBSMC apoptosis by CT-1 was observed at 48 h, with CT-1 dose-dependently inhibiting the anti-Fas/TNF-α-induced HBSMC apoptosis by 50.0 ± 23.9% using flow cytometry, and 58.7 ± 14.2% using the DNA fragmentation ELISA, with a maximum effect at 10 ng ml^{-1} (Figures 3b and 5). Pretreatment of the cells with CT-1 for 8 h or less did not result in changes in the degree of apoptosis (Figure 3b), suggesting that the effects of CT-1 on anti-Fas/TNF-α-induced HBSMC apoptosis occur in both a time- and

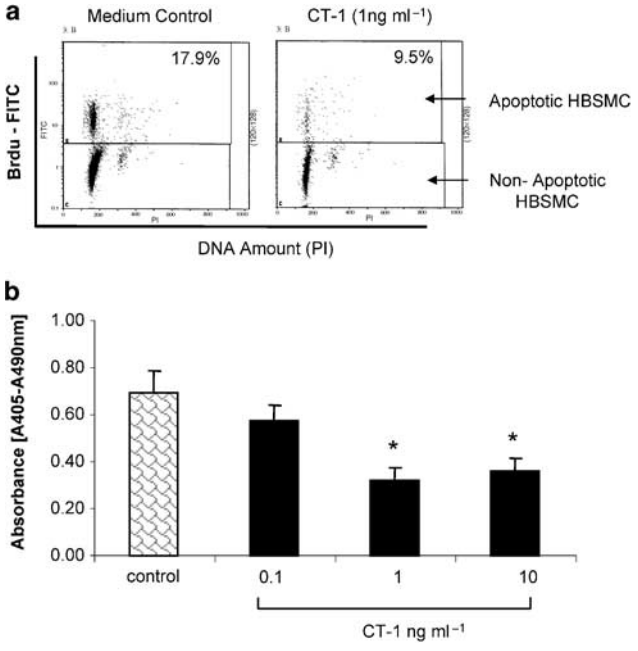

Figure 2 CT-1 inhibits apoptosis in serum-deprived HBSMC. (a) HBSMC were cultured in serum-free medium with or without 1 ng ml^{-1} CT-1 for 3 days. Binding of BrdU to apoptotic nuclei was detected by FITC fluorescence with flow cytometry, as described in Methods. Percentages of apoptotic cells are indicated on the graph (upper box). Data are representative of three separate experiments. (b) HBSMC were cultured in serum-free medium with or without CT-1 (0.1–10 ng ml^{-1}) for 3 days. Quantification of histone-associated DNA fragmentation was performed by ELISA as described in Methods. The mean data $\pm$ s.e.m. from four independent experiments are shown. *$P<0.05$.

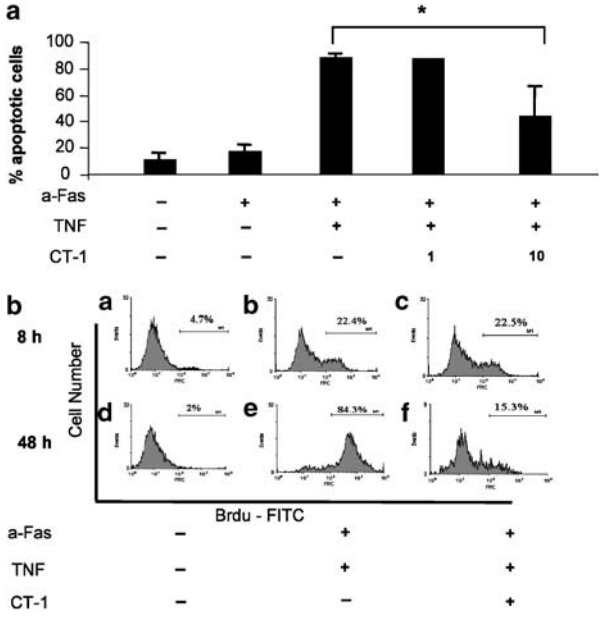

Figure 3 CT-1 inhibits anti-Fas/TNF-α-induced HBSMC apoptosis. (a) HBSMC were incubated with or without 1 or 10 ng ml^{-1} of CT-1 and/or 10 ng ml^{-1} TNF-α for 48 h. Cells were then washed with PBS and cultured in serum-free medium with or without CH-11 primary anti-Fas IgM antibody (200 ng ml^{-1}) for 24 h. The percent of apoptotic cells were detected by flow cytometry, as described in Methods. Results are the mean $\pm$ s.e.m. $n=4$. *$P<0.05$. (b) Experimental protocol is the same as in (a). Representative histograms indicate the time course of HBSMC apoptosis. Cells were incubated with or without CT-1 10 ng ml^{-1}/TNF-α 10 ng ml^{-1} for 8 h (a–c) and 48 h (d–f) before culturing with anti-Fas (200 ng ml^{-1}). Percentages of apoptotic cells are indicated on the graph. The data are representative of four separate experiments.

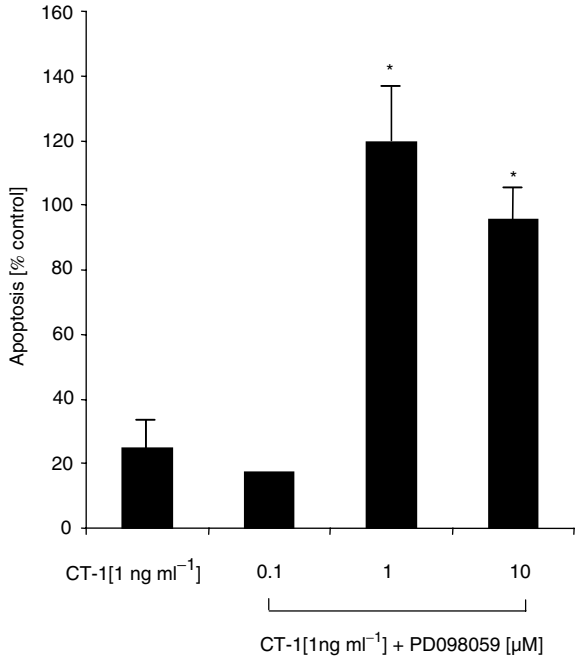

Figure 4 Effect of the MAPK/ERK kinase inhibitor PD98059 on the antiapoptotic effect of CT-1 serum-deprived HBSMC ($n=3$). *$P<0.05$ compared to control.

dose-dependent manner. Figure 4 shows the effect of PD98059, demonstrating a dose-dependent inhibition of the effects of CT-1 ($n=3$). To exclude the possibility of a nonspecific cytotoxic effect of PD98059, we tested whether it was capable of inducing cell death in the presence of 5% FBS. Serum-induced survival was not inhibited by 10 μM PD89059 (data not shown). Using the analysis of propidium iodide uptake by flow cytometry, there was no increase in HBSM cells in the S phase of the cell cycle in CT-1-treated cells after 4 days, indicating no evidence of cell proliferation in serum-free conditions.

Comparison with other members of the IL-6 family

Figure 5 shows the maximal effect of IL-6 (0.1, 1, 50 ng ml^{-1}), LIF (1–200 ng ml^{-1}), IL-11 (0.1, 10, 100 ng ml^{-1}) and OSM (0.02, 0.2, 2 ng ml^{-1}) in relation to CT-1. CT-1 (10 ng ml^{-1}) showed the greatest efficacy of the family members tested in the inhibition of anti-Fas/TNF-α-induced apoptosis.

Effects of CT-1 on cell size, proliferation and migration

CT-1 induced a significant increase in cell size in growth-arrested cells, as judged by both flow cytometry (data not shown) and protein/DNA ratio after 12 days total incubation. The protein/DNA ratio was 113±4.1% control after treatment with CT-1 1 ng ml^{-1} and 127±4.3% after treatment with CT-1 10 ng ml^{-1} ($P<0.05$ *versus* control).

Treatment of serum-deprived HBSM cells with CT-1 (1, 20 and 25 ng ml^{-1}) for 48 h did not induce DNA biosynthesis as compared with untreated controls. However, PDGF AB, a potent mitogen for human airway smooth muscle cells, significantly enhanced [^{3}H]thymidine incorporation into HBSM cells. PDGF, but not CT-1, significantly augmented smooth muscle migration over control values.

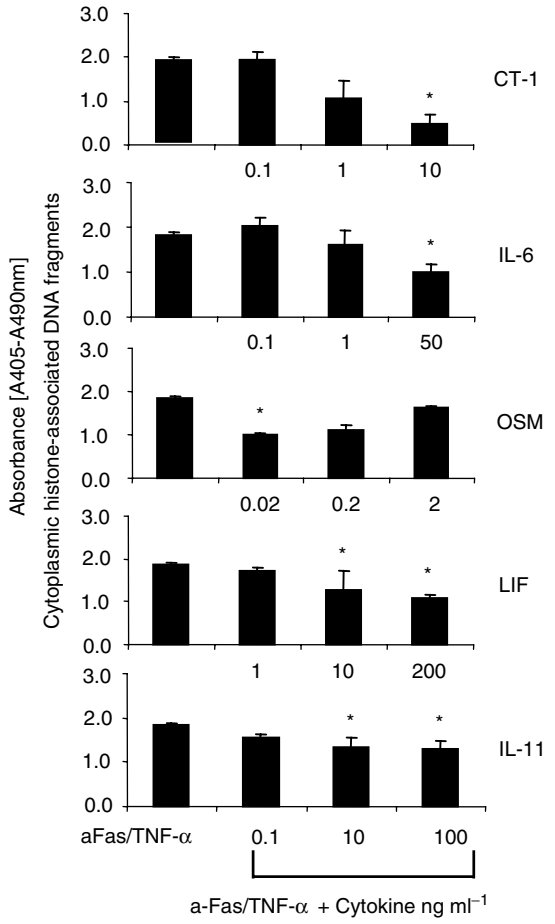

Figure 5 Effect of IL-6 family on anti-Fas/TNF-α-induced airway smooth muscle apoptosis. HBSMC were incubated with or without different concentrations of IL-6 family cytokines and TNF-α (10 ng ml^{-1}) for 48 h. Cells were then serum-starved and treated with CH-11 primary anti-Fas IgM antibody (200 ng ml^{-1}) for 24 h. Quantification of histone-associated DNA fragmentation was performed by ELISA, as described in Methods. The experiments were repeated three times with each condition done in quadruplicate. Data are expressed as mean±s.e.m. *$P<0.05$ compared to control.

Effects of CT-1 on phenotype and morphology of cultured airway smooth muscle cells

The effects of 96 h serum deprivation with and without CT-1 on protein abundance in primary human airway smooth muscle are summarized in Figure 6. Representative Western blots for sm-α-actin, SM22 and vimentin are shown in Figure 6. Densitometric analysis (Figure 6) revealed that serum deprivation induced a significant increase in sm-α-actin, with no difference between serum deprivation and serum deprivation/CT-1, but had no effect on vimentin abundance compared to serum-fed control cultures. SM22 increased after serum deprivation and CT-1 treatments, but this was only significant ($P<0.05$) compared to control for the CT-1 (1 ng ml^{-1}) condition. Typical examples of fluorescent immunocytochemical images are shown in Figure 7. Treatment of cultured HBSMC with CT-1 (10 ng ml^{-1}) for 96 h clearly induced the formation of filamentous arrays of SM22 oriented along the longitudinal axis of the cells. In contrast, the cytostructural distribution of sm-α-actin,vimentin or nmMHC appeared to be unaffected by treatment with CT-1.

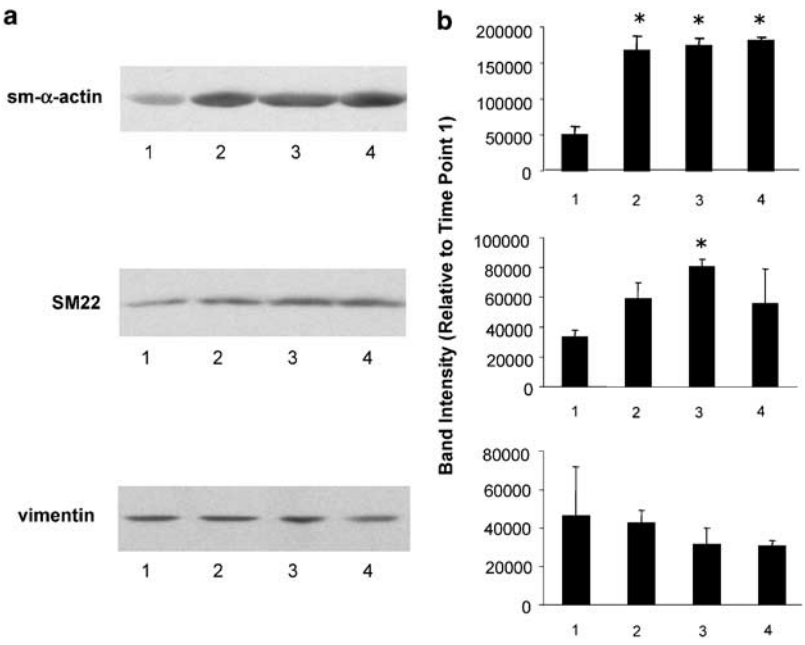

Figure 6 Effect of CT-1 on serum deprivation-induced changes in phenotype marker gene expression. Primary HBSMC were cultured to 80% confluence in growth medium (1), then grown in serum-free medium for 4 days (2), or in serum-free medium with 1 ng ml^{-1} CT-1 (3), or with 10 ng ml^{-1} CT-1 (4). Western blots (left panels) using antibodies for sm-α-actin, SM22 and vimentin were obtained for total protein lysates prepared, as described in Methods. Band intensity was quantified and compared by scanning densitometry (right panels). Data are representative of three individual experiments. Data are presented as mean ± s.d., $*P<0.05$.

Discussion

The findings in this study indicate that CT-1 is expressed in normal adult human lung, one lung source being the smooth muscle cells of fetal and synthetic phenotypes, although other cell sources such as fibroblasts are likely. In adult cells, CT-1 enhances airway smooth muscle survival under conditions of serum deprivation, markedly inhibits TNF-α/Fas-induced apoptosis, more than other members of the IL-6 family, induces cellular hypertrophy, qualitatively alters cytostructural distribution of SM22 and increases the abundance of this protein. No effect of CT-1 on proliferation or migration was evident. We postulate that the increase in smooth muscle mass in diseases such as asthma may be due in part to both induction of hypertrophy and a reduction in apoptosis *via* cytokines such as CT-1. CT-1 expression may be increased in airway inflammation, akin to diseased hearts, where a panel of fetal genes is induced during the development of heart failure (Zolk *et al*., 2002).

Our data demonstrated that CT-1 is synthesized in abundance in both fetal and adult airway smooth muscle cells, and there is *in vitro* CT-1 release in response to cytokines involved in the pathogenesis of asthma, consistent with our results obtained with the related cytokine LIF (Knight *et al*., 1999). Neither mechanical strain nor hypoxia was a major stimulus for release, dissimilar to cardiac myocytes. Furthermore, unexpectedly, we were unable to induce significant cellular proliferation or migration in response to CT-1. The conditions under which CT-1 release is triggered differed between adult and fetal cells. Growth-arrested fetal cells release CT-1 after treatment with TGF-β2 and IL-4, or the re-addition of serum, but adult cells do not. To respond to TGF-β2, adult cells require continuous serum conditions, that is, maintenance of a synthetic phenotype. It is likely that other simultaneous stimuli are required for maximal release of CT-1 from HBSMC, such as integrin receptor activation or activation by molecules released from inflammatory cells.

We demonstrated that CT-1-stimulated adult airway HBSMC were markedly resistant to anti-Fas/TNF-α-mediated apoptosis, as are untreated fetal cells that express and release CT-1 spontaneously in response to serum, unlike adult cells. Inhibition of airway smooth muscle apoptosis by CT-1 and related cytokines has not been previously documented. We adapted a flow cytometry assay employed for the detection of apoptosis in hematopoietic cells (Nagahara *et al*., 2000) and, in pilot studies, found that this method was more sensitive and robust than annexin-V staining in HBSMC (Jiang *et al*., 1999; Rucker-Martin *et al*., 1999; Nagahara *et al*., 2000). CT-1 promotes cardiac myocyte survival in serum deprivation conditions *via* the activation of an antiapoptotic signaling pathway that requires MAPKs (Sheng *et al*., 1997; Latchman, 2000). The effects of the specific MAPK/ERK kinase inhibitor PD98059 in our studies are in agreement with the results in cardiac myocytes. Recently, Hamann *et al*. (2000) demonstrated that Fas antigen is expressed in human airway smooth muscle cells, Fas crosslinking with CH-11 anti-Fas antibody induces apoptosis in cultured HHBSMC cells, and TNF-α synergistically augments Fas-mediated airway myocyte killing. TNF-α plus anti-Fas has a potent apoptotic effect on HBSMC apoptosis because TNF-α treatment increases surface Fas expression (Hamann *et al*., 2000). In agreement with these findings, the results of our study show that TNF-α alone is ineffective in inducing adult HBSMC apoptosis, and anti-Fas alone induces a weak apoptotic effect. We postulate that the suppression of anti-Fas/TNF-α-mediated apoptosis by CT-1 may be *via* downregulation of TNF-α-induced Fas antigen

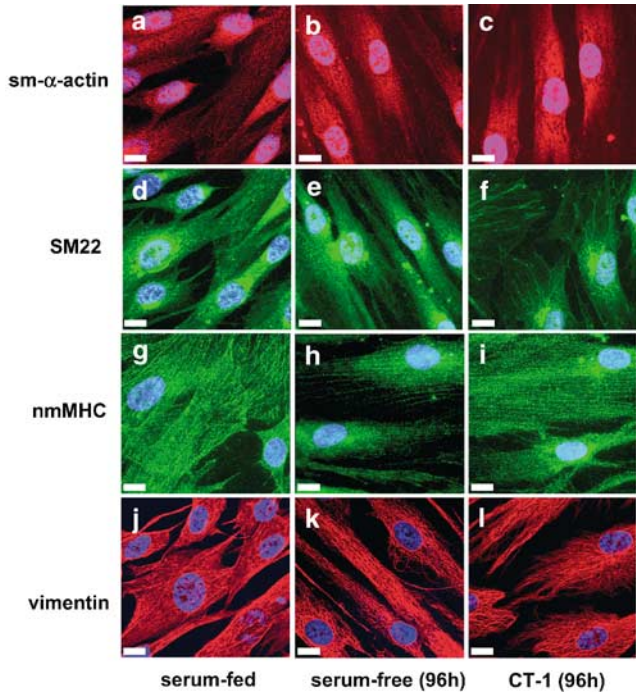

Figure 7 Fluorescent immunocytochemical analysis of CT-1-mediated effects on the cytostructural arrangement of smooth muscle marker proteins. HBSMC were grown to 80% confluence in growth medium (a, d, g, j), then cultured in serum-free medium for 4 days (b, e, h, k) or in serum-free medium with 10 ng ml^{-1} CT-1 (c, f, i, l). Cells were fixed and incubated with primary antibodies for either sm-α-actin (a–c), SM22 (d–f), nmMHC (g–i), or vimentin (j–l). Cy3-conjugated secondary antibodies (red) were used to label sm-α-actin and vimentin, whereas FITC-conjugated secondary antibodies (green) were used to label SM22 and nmMHC. Nuclei were stained in all cells using DNA-specific TOTO-3 dye (shown in blue). Images are representative of those from three individual experiments. Scale is 5 μm.

surface expression. Given that all receptor components of the IL-6 family are present in human airway smooth muscle (Knight *et al*., 1997; 1999; Lahiri *et al*., 2001), we tested the antiapoptotic effect of all members of this family, excepting cardiotrophin-like cytokine (NNT-1) and ciliary neurotrophic factor, and found CT-1 to show the greatest efficacy, although the effects of OSM require further research. The potency of CT-1 in inhibiting apoptosis in HBSM is similar to reports in cardiac cells (Sheng *et al*., 1997; Latchman, 2000), whereas the levels of release we have found are difficult to compare with human cardiomyocytes, as CT-1 assays used were insensitive compared to our assay (Ancey *et al*., 2002).

Although smooth muscle proliferation is thought to be involved in the development of the increased smooth muscle mass in asthma, and is readily induced *in vitro*, it has been difficult to detect proliferation *in vivo* in asthmatic airways (Benayoun *et al*., 2003; unpublished data). A decreased rate of apoptosis is expected to increase the total cell number and may also indirectly lead to increased muscle mass *via* hypertrophy by increasing the resident time of HBSMC in the airway, allowing other factors involved in hypertrophy to act. Longer CT-1 culture periods were necessary to demonstrate hypertrophy (12 days) than have been shown for cardiac myocytes (2 days). Our findings of decreased apoptosis by CT-1, yet no evidence of a proliferative effect, are not incompatible, as cell number will increase by CT-1 delaying or aborting apoptosis, even though no cells entered the synthetic phase, as demonstrated by lack of incorporation of BrdU or thymidine.

Our Western blot analyses revealed that though serum deprivation clearly mediated the accumulation of sm-α-actin, a marker for contractile state myocytes (Halayko *et al*., 1999), simultaneous addition of exogenous CT-1 did not affect this response. SM22, an actin-filament-associated protein normally expressed in high abundance in contractile myocytes (Hirota *et al*., 1999), increased in abundance with serum deprivation, but was only statistically different to control with the addition of 1 ng CT-1. Consistent with this finding, qualitative analysis of immunocytochemical data revealed that CT-1 did alter the cytostructural distribution of SM22, making distal, thick filamentous structures more readily apparent. CT-1 has also been reported to induce cytostructural remodeling in cardiac myocytes (Cheng *et al*., 1997; Kunisada *et al*., 1998). We also measured the abundance and distribution of both the intermediate filament protein vimentin and nmMHC isoforms (196 and 198 kDa) that are known to decrease in abundance as airway myocytes acquire a fully contractile state (Halayko *et al*., 1996). In addition, both vimentin and nmMHC have been reported to increase during hypertrophic growth of cardiac myocytes, and vascular and visceral smooth muscle cells in response to mechanical overload *in vivo* (Chiavegato *et al*., 1993; Patton *et al*., 1995). In our studies, serum withdrawal of cultured human airways myocytes induced a significant loss of nmMHC, and addition of CT-1 had no additional effect. Neither serum deprivation nor the addition of CT-1 to culture medium affected vimentin content, indicating that in our culture system vimentin expression is relatively stable.

In conclusion, CT-1 and related family members, by increasing the cell size and augmenting human airway smooth muscle survival pathways, may participate in the onset of increased airway smooth muscle mass in airway diseases.

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