Bcl-2 Sustains Increased Mucous and Epithelial Cell Numbers in Metaplastic Airway Epithelium

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Bcl-2, an inhibitor of apoptosis, is expressed in LPS-induced metaplastic goblet cells of rat airways. The present study investigated expression of Bcl-2 in airway mucous cells of persons with cystic fibrosis and tested in rats and mice whether its expression is responsible for sustaining metaplastic mucous cells. A significantly higher percentage of mucous cells expressed Bcl-2 in humans with cystic fibrosis compared with control subjects with no disease or subjects with other diseases. In LPS-instilled F344/N rats, the percentage of Bcl-2–positive mucous cells was decreased to background levels before the resolution of goblet cell metaplasia. Furthermore, intraperitoneal injection of rats with antisense oligonucleotides significantly reduced Bcl-2 expression and goblet cell metaplasia in nasal and pulmonary airway epithelia in rats. In contrast, sustained expression of Bcl-2 in transgenic mice by a metallothionein promoter caused increased LPS-induced goblet cell metaplasia over 8 days compared with wild-type mice. These studies demonstrate that Bcl-2 expression sustains goblet cell metaplasia in various species, that epithelial cell numbers are directly linked to the regulation of the numbers of goblet cells, and that downregulating Bcl-2 expression reduces goblet cell metaplasia.

Keywords: apoptosis; antisense oligonucleotides; cystic fibrosis; inflammation; LPS

One of the main reasons for the morbidity of patients with chronic pulmonary diseases, such as cystic fibrosis (CF) and asthma, is increased mucous secretions causing airway obstruction. Reduction of mucous secretions in CF is of great interest, because intervention strategies by gene therapy approaches to replace the CFTR gene or by reducing inflammation have largely been unsuccessful.

Inflammatory responses recruit nonciliated columnar epithelial cells into the cell cycle in large numbers (1) and thereby increase the number of cells per millimeter of basal lamina (BL) (2). This increase is accompanied by the appearance of mucus-producing cells in areas normally devoid of these cells and is called goblet cell metaplasia (GCM) (3). GCM allows the epithelium to produce larger amounts of mucus, because both preexisting and proliferating cells synthesize mucus (2). The generation of metaplastic mucous cells and the mechanisms by which inflammatory mediators induce the synthesis of mucus and the differentiation of surface epithelial cells into mucus-producing cells have been extensively studied. In contrast, mediators involved in reducing the numbers of metaplastic mucous cells after inflammatory responses subside are not well understood.

In an attempt to understand mechanisms that reduce numbers of metaplastic mucous cells during the resolution of inflammatory responses, we discovered that metaplastic mucous cells express regulators of apoptosis from the Bcl-2 family of proteins (4–6). Exposure of rats to LPS or allergen causes airway epithelial cells to proliferate, and nonproliferating and proliferating cells differentiate into mucous cells to establish GCM (2). Approximately 20 to 30% of the developing metaplastic mucous cells express Bcl-2 (5). Furthermore, exposure of rats to ozone induces GCM in nasal epithelia and Bcl-2 is expressed in epithelia where GCM occurs (4).

The Bcl-2 family of cytoplasmic proteins can register diverse forms of intracellular damage, gauge whether other cells have provided a positive or negative death stimulus, and determine the progression or inhibition of the suicide program (7). Bcl-2, an inhibitor of apoptosis, is a member of a large group of regulatory proteins that prevent or induce apoptosis. The activation of proapoptotic signals leads to inactivation of Bcl-2 by Bcl-2 homology region-3 domain-only family members and triggers Bax or Bak to permeabilize mitochondria to release cytochrome c, which initiates the activation of caspases and DNases responsible for the appearance of the apoptotic morphology (8–10). This morphology includes DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly of the cell (10).

The present study tested the hypothesis that disruption of mechanisms to resolve GCM could account for sustained GCM in CF where mucous hypersecretion reduces airflow. Our findings suggest that Bcl-2 plays a central role in the resolution of GCM and may be a useful target for reducing mucus-secreting cells in chronic diseases. Some results from these studies have been previously reported in the form of an abstract (11).

METHODS

See the online supplement for additional details regarding laboratory animals, *in situ* hybridization, microdissection of airway epithelia, polymerase chain reaction, and Western blot analyses.

Tissues from Human Subjects

Paraffin-embedded lung tissues with small airways were obtained under the auspices of the University of North Carolina and the Lovelace Respiratory Research Institute institutional review boards' approved protocols. Tissue sections (5-μm thick) originated from 14 subjects without lung diseases, 8 subjects with CF, and 8 subjects with diseases such as interstitial pulmonary fibrosis, pulmonary hypertension, and α1-antitrypsin (Table 1). All histology sections represent sections of airways and lungs excised during transplantation for end-stage lung disease or excess portions of donors.

Laboratory Animals

Male F344/N rats (8–10 weeks old) were purchased from Frederick Cancer Research, Frederick, MD. Bcl-2 transgenic mice on a C57Bl/6 background

TABLE 1. DEMOGRAPHICS OF AIRWAY TISSUE DONORS

	Normal (*n = 14*)	CF (*n = 8*)	Other (*n = 8*)
Mean age, yr	43	27	46
Sex	9 M, 5 F	7 M, 1 F	5 M, 3 F
Diagnosis		5 M, 1 F DF/DF	2 M IPF
		1 M DF/G551D	1 M, 1F PHTN
		1 F DF/621 + 1G > T	1 M α1-AT
			1 M COPD, 2 F non-CF bronchiectasis

Definition of abbreviations: α1-AT = α1-antitrypsin; CF = cystic fibrosis; COPD = chronic pulmonary obstructive disease; DF = deletion of phenylalanine; F = female; G > T = transversion mutations; IPF = interstitial pulmonary fibrosis; M = male; PHTN = pulmonary hypertension.

with the Bcl-2 transgene under the control of the mouse metallothionein promoter (provided by Dr. Nelson Fausto, University of Washington, Seattle, WA) were bred at the Lovelace Respiratory Research Institute. Instillation for rats and mice was as described (12, 13). All experiments were approved by the Institutional Animal Care and Use Committee and were performed at the Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

Rat Nasal Explant Cultures and Treatment with Antisense Oligonucleotides

Nasal explants were treated with 100 μg/ml endotoxin in medium (n = 4/group) for 24 hours and maintained in culture in endotoxin-free medium for 2 additional days. They were then fixed in zinc formalin and processed for histologic analyses as described (14). They were treated with 30 μM antisense oligonucleotides (ASODNs) in 40 μl culture medium and 3 μl lipofectamine (Life Technologies, San Diego, CA). Table 2 shows the sequences of the ASODNs and control ODNs, which were 2-O-methoxy ethyl thioate derivatives (15).

TABLE 2. SEQUENCES OF THE ANTISENSE OLIGONUCLEOTIDES AND THEIR POSITION WITHIN THE Bcl-2 mRNA

ASODN No.	mRNA Region	Base Position	Sequence
1011 60	5′ UTR	1	CAGCTTTTTATTTCATGAGG
1011 61		39	TGGCATGAGATGCAGGAAAT
1011 62		75	AGTTATGACGAACACTTGAT
1011 63		110	ATGTGCTTTGCATTCTTGGA
1011 64		181	ACTTCGCGTCCCGGCTCCCG
1011 65		205	AAAGAAGCTGCAGGTACCAA
1011 66	Coding R	221	CGCCATCCTTCCGGGGAAAG
1011 67		241	TACCCTGTTCTCCCGGCTTG
1011 68		255	TCTCCCGGTTATCATACCCT
1011 69		265	TTCATCACGATCTCCCGGTT
1011 70		270	TGTACTTCATCACGATCTCC
1011 71		295	CCCCTCTGTGACAGCTTATA
1011 72		301	TCGTAGCCCCTCTGTGACAG
1011 73		321	CTTCATCTCCAGTATCCCAC
1011 74		370	TGGAAGGAGAAGATGCCAGG
1011 75		390	GCGTTCGGTTGCTCTCAGGC
1011 76		421	GTCCTGGCAGCCGTGTCTCG
1011 77		541	AAGTCGCGACGGTAGCGACG
1011 78		671	CCCACCGAACTCAAAGAAGG
1011 79		675	TGACCCCACCGAACTCAAAG
1011 80		781	TTATCCTGGATCCAGGTGTG
1011 81		850	AGCCAGGAGAAATCAAACAG
1011 82		911	GTATGCACCCAGAGTGATGC
1011 83		971	CTTTAGTGAACCTTTTCATA
1011 84	3′ UTR	1021	ACAACTTTGTTTCATGGTCC

Definition of abbreviations: ASODN = antisense oligonucleotide; UTR = untranslated region.

Injection of Rats with ASODN and LPS Instillations

Rats were injected intraperitoneally with 0.5 μl saline, control ODN, or ASODNs two times a day at 5 mg/kg in saline for 10 consecutive days. On Day 7 or 8 of injection, rats were intratracheally instilled with l mg LPS in 0.5 ml saline or intranasally instilled with 100 μl/naris of 1 mg/ml LPS and killed 72 or 48 hours later, respectively.

Histochemical Staining, Quantification, *In Situ* Hybridization, Real-Time Polymerase Chain Reaction, and Western Blot Analysis

Tissue blocks that corresponded to the same nasal anatomic site examined in the *in vitro* experiments described previously and intrapulmonary airways of the left lung lobes were obtained as described (16). Staining and quantification of mucous cell numbers and intraepithelial stored mucosubstances were performed as described (17–19). Bcl-2 was detected by immunohistochemistry using two different Bcl-2 antibodies (BD-Pharmingen, San Diego, CA, or Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 or 1:50, respectively, as described (12). *In situ* hybridizations, real-time polymerase chain reaction, and Western blot analyses were performed as described previously (20–22). *See* the online supplement for a detailed method description and regarding RNA isolation and polymerase chain reaction.

Statistical Analysis of Data

Data were expressed as the mean group value ± SE of the mean. Differences among groups were examined by analysis of variance and *t* tests using SAS software (Cary, NC) and by application of Bonferonni correction for multiple comparisons. Group means were considered significantly different when p values were 0.05 or less.

RESULTS

Bcl-2 Expression in Mucous Cells from Subjects with CF

We have previously reported that Bcl-2 is expressed in metaplastic mucous cells of rats exposed to ozone, cigarette smoke (4), or LPS (5). To further study the relevance of Bcl-2 expression in human chronic disease, we analyzed lung tissues that were obtained from individuals with no disease and from individuals who have CF, pulmonary hypertension, or bronchiectasis. Lungs from patients with CF were chosen because infections with gram-negative bacteria are common in these patients and may resemble the response we see in the rat lung after instillation of LPS (5). Although an average of 1 to 2% of mucous cells were Bcl-2–positive in airways from normal subjects or from subjects with other lung diseases, such as pulmonary hypertension and bronchiectasis, an average of 8% of mucous cells in lung epithelia of patients with CF showed immunostaining for Bcl-2 (Figures 1A and 1B). We noticed heterogeneity in Bcl-2 positivity of mucous cells in some tissue sections from subjects with CF. In some airways, up to 40% of goblet cells expressed Bcl-2, whereas in adjacent airways, only 1 to 2% of goblet cells were Bcl-2–positive. The identity of Bcl-2 was further verified by *in situ* hybridization, which showed a strong signal in epithelia where Bcl-2 was detected by
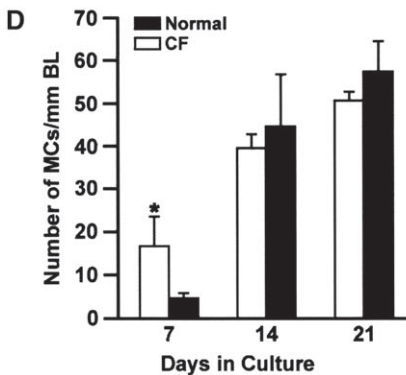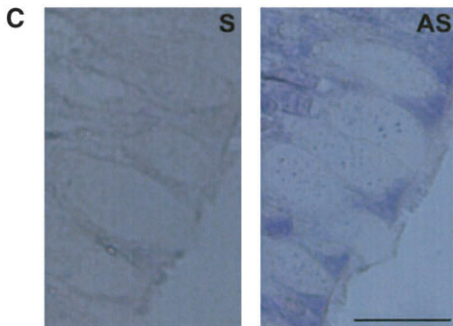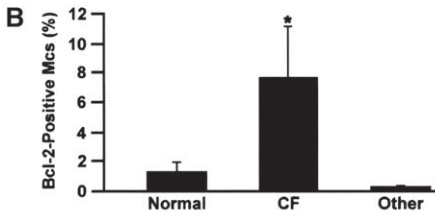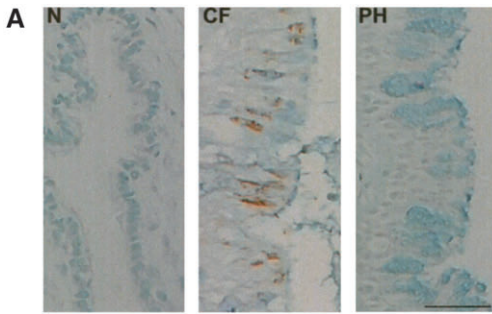
Figure 1. Increased percentages of mucous cells are Bcl-2–positive in airways of patients with cystic fibrosis (CF) compared with airways from subjects who have other lung diseases than CF or control subjects with no disease. Tissue sections were immunostained for Bcl-2 as described in METHODS. Nuclei and intraepithelial mucosubstances stain blue, and Bcl-2 immunostaining was detected with diaminobenzidine and shows brown. (*A*) The percentage of Bcl-2-positive mucous cells (MCs) was quantified by enumerating the number of Alcian blue (AB-)–positive MCs and the MCs that had immunostained with the Bcl-2 antibody (BD-Pharmingen). *Bar* represents 20 μm. N = normal; PH = pulmonary hypertension. (*B*) At least 250 AB-positive cells were counted to determine the percentage of positive Bcl-2 MCs. Values are group means ± SEM; n = 7 to 10 subjects/group. *Significantly different from control subjects with no disease, p < 0.05. (*C*) *In situ* hybridization of tissue sections from a subject with CF with antisense (AS) and sense (S) cRNA probes to Bcl-2. *Bar* represents 10 μm. (*D*) Increase in MCs per millimeter of basal lamina (BL) in well differentiated cultures of primary airway epithelial cells from three subjects without lung disease and three subjects with CF. *Significantly different from control subjects with no disease, p < 0.05.

immunohistochemistry, whereas the sense control probe showed no signal (Figure 1C).

To test whether CFTR deficiency is directly responsible for Bcl-2 expression, primary airway epithelial cells from three subjects with no lung disease and three subjects with CF were maintained in culture on an air–liquid interface and allowed to differentiate into columnar cells. Cells from control subjects and subjects with CF showed a time-dependent increase in goblet cells per millimeter of BL over 7, 14, and 21 days (Figure 1D). Significant differences in goblet mucous cells/millimeter of basal lamina were observed in the cultures obtained from three normal subjects and three subjects with CF at 7 days, when cells had just reached confluence. No differences were found at 14 and 21 days, when cells had formed a differentiated columnar epithe-

lium. This finding shows that the change in the number of mucous cells/millimeter of BL from 7 to 14 days was greater for normal subjects compared with those with CF. No differences were observed in the percentage of Bcl-2–positive cells in control and CF samples (data not shown).

Resolution of LPS-induced GCM in Rats

The number of cells that contain mucosubstances increased from an average of 15 to an average of 76 cells/mm BL from 2 to 4 days and decreased to approximately 43 cells/mm BL at 8 days after instillation. The mucous cell numbers decreased to background levels at 12 days to the numbers found in rats instilled with saline (8–20 cells/mm BL) throughout the 16 days after instillation (Figure 2A). The morphology of airway epithelial
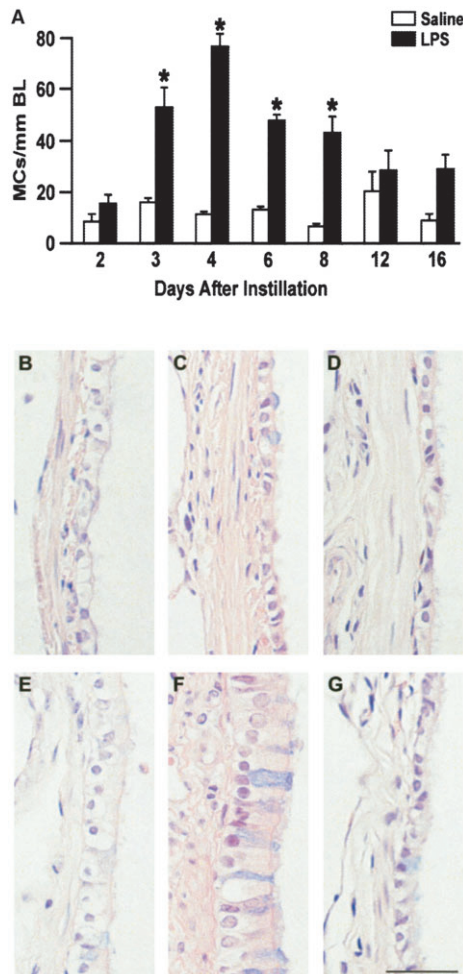
Figure 2. (*A*) The number of MCs per millimeter of BL in saline- and LPS-instilled rats. The number of AB/periodic acid-Schiff (PAS)–positive epithelial cells were enumerated and normalized to millimeter length of BL. Values are group means ± SEM; n = 5 rats/group. *Significantly different from saline-instilled control animals, p < 0.05. (*B–G*) Photomicrographs of airway epithelia from rats instilled with saline (*B–D*) or LPS (*E–G*). Tissue sections were stained with hematoxylin–eosin and AB; photomicrographs are representative for 2 (*B* and *E*), 3 (*C* and *F*), and 12 (*D* and *G*) days after instillation. *Bar* represents 20 μm.

Figure 3. (*A*) Expression of Bcl-2 at various time points after LPS instillation. The percentage of Bcl-2–positive MCs in rats instilled with saline or 1000 μg LPS from 2 to 16 days after instillation. The tissue sections were immunostained for Bcl-2 and counterstained with AB to identify denoting the mucus-producing cells. At least 400 MCs were counted in each rat to determine the percentage of Bcl-2–positive MCs. Similar immunostaining was observed with two different antibodies (BD-Pharmingen and Santa Cruz) to Bcl-2. *Significantly different from saline-instilled control animals, p < 0.05. (*B–D*) Representative photomicrographs show that Bcl-2 is expressed in mucus-producing cells following LPS instillation. Tissue sections from the left lung at generation 5 from rats at 2 (*B*), 3 (*C*), and 12 (*D*) days after instillation were immunostained for Bcl-2 and AB to identify mucus-containing cells. Bcl-2 was detected with diaminobenzidine and shows brown. *Bar* represents 20 μm.

cells remained cuboidal in saline-instilled rats throughout the 16 days after instillation (Figures 2B–2D). In LPS-instilled rats, the thickness of the epithelium was already increased at 2 days, reached maximum (∼ 21 ± 1.4 μm) at 3 to 4 days, and returned to the original thickness (9.2 ± 0.001 μm) at 12 to 16 days after instillation (Figures 2E–2G).

Accompanying this GCM, a significant increase in Bcl-2–positive mucous cells (20–30% in LPS rats compared with 3–7% in saline rats) was found at 2, 3, 4, and 6 days after instillation in LPS-instilled rats (Figure 3A). No differences between saline- and LPS-instilled rats were evident 8 to 16 days after instillation. Although the granular staining of Bcl-2 is visible in Alcian blue–positive cells at 2 to 6 days, very few cells were immunopositive at 12 days after instillation (Figures 3B–3D).

Bcl-2 Sustains LPS-induced GCM in Nasal Epithelia

The direct role of Bcl-2 in sustaining metaplastic mucous cells in airway epithelia was investigated by downregulating its ex-
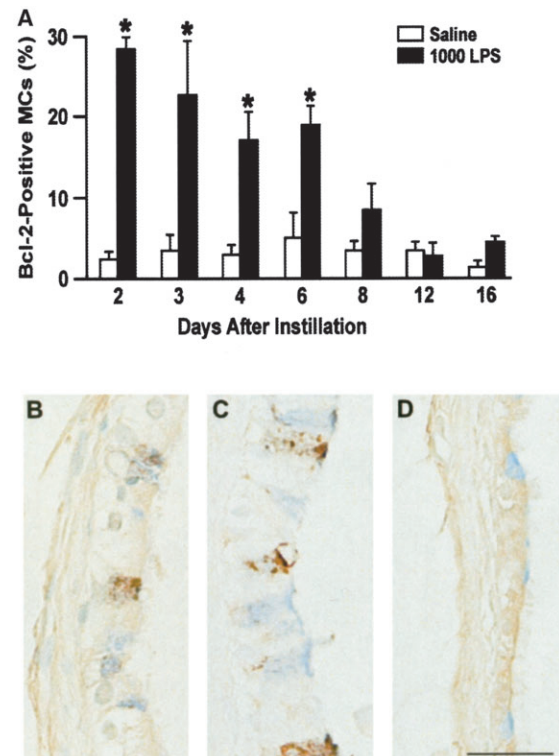
pression with ASODNs in cultured nasal explants. We screened 25 ASODNs for inhibition of GCM and Bcl-2 mRNA expression in the organ culture system where GCM can be induced by treating the distal midseptum from the rat nose with LPS (14). This screening identified four ASODNs that significantly reduced LPS-induced mucous cells in nasal explant cultures (Figure 4A). Two of four ASODNs (64 and 83) showed a significant reduction of GCM in repeat experiments and were therefore selected for testing their efficacy by treating nasal explant cultures at 3-, 30-, 60-, or 90-μM concentrations. ASODN 83 effectively reduced GCM, even at 3-μM concentrations, whereas ASODN 64 was most effective at the 90-μM concentration (Figure 4B). *In situ* hybridization for Bcl-2 mRNA showed a marked reduction of the mRNA in nasal explants treated with ASODN 64 (Figure 4C). Similar results were observed for ASODN 83 (data not shown).

We further tested whether LPS-induced GCM in distal nasal midsepta can be reduced by injection of rats with ASODNs to the Bcl-2 mRNA. Rats were injected intraperitoneally with saline, control ODN, or ASODN 64 twice per day at 5 mg/kg for 7 days before LPS instillation and 2 days after instillation. The amount
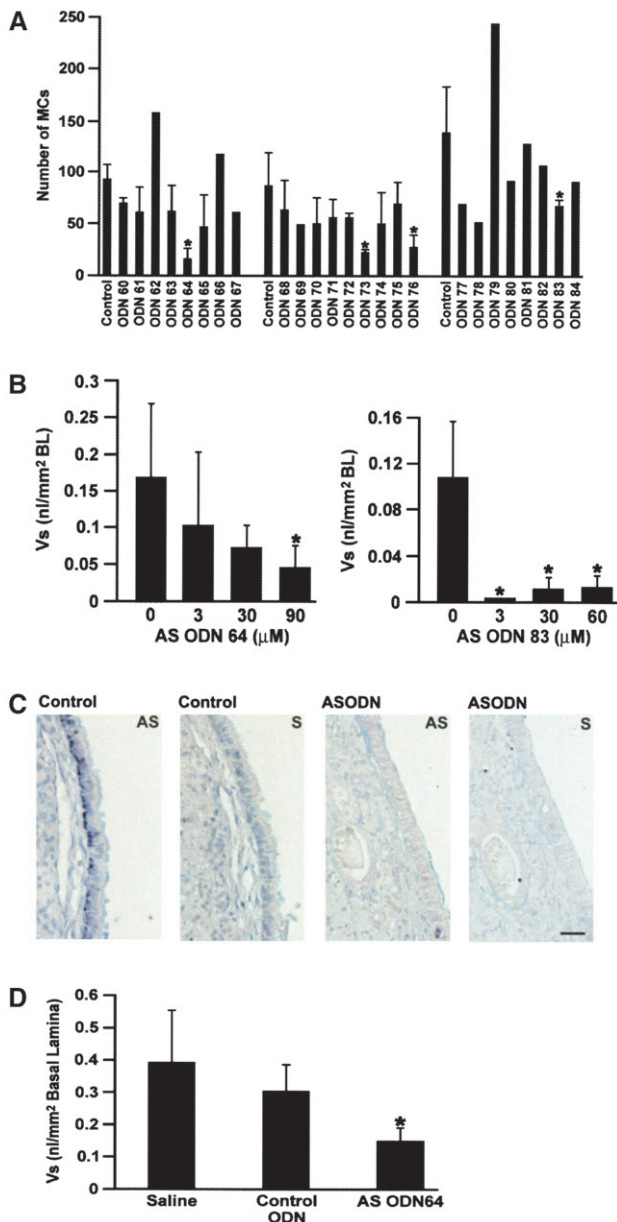
Figure 4. (*A*) Four of 25 antisense oligonucleotides (ASODNs) significantly reduced the number of MCs in explant cultures. Organ cultures from rat distal nasal midsepta were placed in culture on transwell membranes in an air–liquid interface and treated with 100 μg/ml LPS for 24 hours and treated with 30 μM ASODNs to Bcl-2 mRNA throughout the 3-day culture period or were left untreated as control. Explant cultures were fixed 2 days later in zinc formalin, histochemically stained with AB/PAS to detect acidic and neutral intraepithelial mucosubstances stored in the epithelial cells, and analyzed for the number of MCs. The 25 ASODNs were tested in three separate experiments (8–9 ASODNs with an untreated group as control in each experiment). *Error bars* represent group mean values ± SEM (n = 4 explant cultures/experimental group). *Significantly different from untreated control explant cultures, $p < 0.05$. (*B*) A dose-dependent effect of ASODNs 64 and 83 on stored mucous substances in the nasal explant cultures. Nasal explants were placed in culture on transwell membranes, exposed to 100 μg LPS for 24 hours, and treated with the respective ASODNs at 3-, 30-, 60-, or 90-μM concentrations throughout the culture period. Tissues were processed 2 days after LPS treatment, and volume density (Vs) of stored mucosubstances in the surface epithelium was determined by morphometry. ASODN 83 shows a significant reduction at 3-μM concentration. ASODN 64 shows a significant reduction only at 90-μM concentration. *Bars* represent group mean values ± SEM (n = 4 explant cultures/group). *Significantly different from untreated controls, $p < 0.05$. (*C*) *In situ* hybridization of control ODN- or ASODN 64–treated nasal septa with antisense probe to Bcl-2 (AS) or with the sense cRNA probe as control (S). *Bar* represents 15 μm. (*D*) ASODN 64 reduces the LPS-induced goblet cell metaplasia (GCM) in the distal nasal septum in *in vivo* rats. Rats were injected intraperitoneally with saline control ODN or ASODN 64 mornings and evenings with 5 mg/kg for a total of 10 mg/kg/day for 9 consecutive days. On Day 7, all rats received an intranasal instillation (100 μl/naris) of a 1-mg LPS solution in saline. Two days after LPS instillation, rats were killed, and GCM in the epithelium of the distal nasal septum was quantified by morphometry after histochemical stain with AB/PAS. *Bars* represent group mean values ± SEM (n = 6 rats/experimental group). *Significantly different from distal septal epithelium of rats injected with saline or control ODN, $p < 0.05$.

of stored mucosubstances in respiratory epithelia of distal nasal septa was significantly reduced in rats injected with ASODN 64 compared with those injected with control ODN or saline as control (Figure 4D).

Bcl-2 Sustains GCM in Pulmonary Airways

The effect of downregulating Bcl-2 expression on LPS-induced GCM in pulmonary airways was examined by injecting rats intraperitoneally with ASODNs 83 and 64 every day for a total of 9 days starting 7 days before intratracheal instillation with 1 mg LPS. GCM was assessed at 3 days after LPS instillation in the airway epithelium at generation 5. The total cell number per millimeter of BL (Figure 5A), the number of mucous cells per millimeter of BL, and the amount of stored mucosubstances (Figure 5B) in the airways of rats instilled with LPS were significantly reduced by ASODN 64. Furthermore, the percentage of Bcl-2–positive mucous cells was reduced significantly by ASODN 64 (Figure 6A). ASODN 83 caused a minor, statistically nonsignificant reduction of total epithelial cell number and mu-

cous cells per millimeter of BL and of amount in intraepithelial stored mucosubstances. ASODN 84, which was not effective in organ culture experiments, showed similar numbers of total epithelial cells and mucous cells per millimeter of BL and similar percentages of Bcl-2–positive mucous compared with rats injected with control ODN (data not shown). No differences in inflammation were observed in lung tissues from control- or ASODN-treated rats. However, the epithelial thickness was reduced when rats were injected with ASODNs 83 and 64, but not with control ODN (Figure 5D).

The effect of treatment with control ODN or ASODN 83 and 64 on the levels of Bcl-2, Bcl-x_L, Bax, and Bak, the major regulators of this protein family, was tested. The percentage of Bcl-2–positive mucous cells was more efficiently reduced by ASODN 64 compared with control ODN or ASODN 83 (Figures 6A and 6B). The protein levels of Bcl-x_L, Bax, and Bak were tested in extracts prepared from the right lungs of three representative rats from each treatment group. Interestingly, not only Bcl-2 but also Bcl-x_L levels were reduced by treatment with ASODNs 83 and
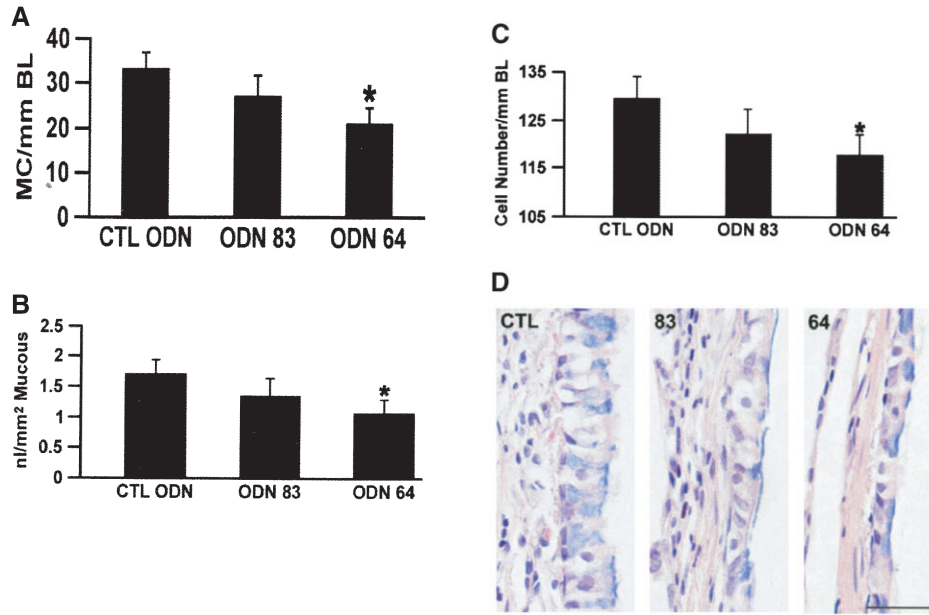
Figure 5. ASODN reduces LPS-induced GCM, the Vs, and the number of epithelial cells per millimeter of BL in pulmonary airways in *in vivo* rats. Rats were injected intraperitoneally with control (CTL) ODN, ASODN 83, or ASODN 64 twice a day for 10 consecutive days. On Day 7, all rats received an intratracheal instillation of 1 mg LPS in 500 µl. Three days after LPS instillation, rats were killed, and the numbers of MCs per millimeter of BL (*A*) and the amount of stored mucosubstances (*B*) in the epithelium of airway generation 5 were quantified by morphometry after histochemical stain with AB/PAS. Adjacent sections were stained with hamatoxylin–eosin followed by AB for quantification of the total numbers of cells per millimeter of BL (*C*). *Bars* represent group mean values ± SEM (n = 6 rats/experimental group). *Significantly different than distal septal epithelium of rats injected with control ODN, p < 0.05. (*D*) Photomicrographs of axial airways at generation 5 in the left lung lobe of rats injected with control ODN, ASODN 83, and ASODN 64. Tissue sections were stained with hematoxylin–eosin and counterstained with AB. *Bar* represents 30 µm.

64 compared with control ODN (Figure 6C). However, the levels of the proapoptotic proteins Bax and Bak were not affected by the ASODN treatments (Figure 6C).

Sustained GCM in Bcl-2 Transgenic Mice

Transgenic mice with Bcl-2 under the control of the MT1 promoter, which is inducible by zinc water, were used to further establish the role of Bcl-2 expression in sustaining LPS-induced mucous cell metaplasia. We and others had previously shown that C56Bl/6 mice instilled with LPS display GCM (13, 23). Bcl-2 expression was 10-fold greater in transgenic compared with wild-type littermates 8 days after LPS instillation (Figure 7A), and goblet cells in transgenic but not wild-type mice showed Bcl-2 positivity (Figure 7B). Furthermore, GCM in wild-type mice was
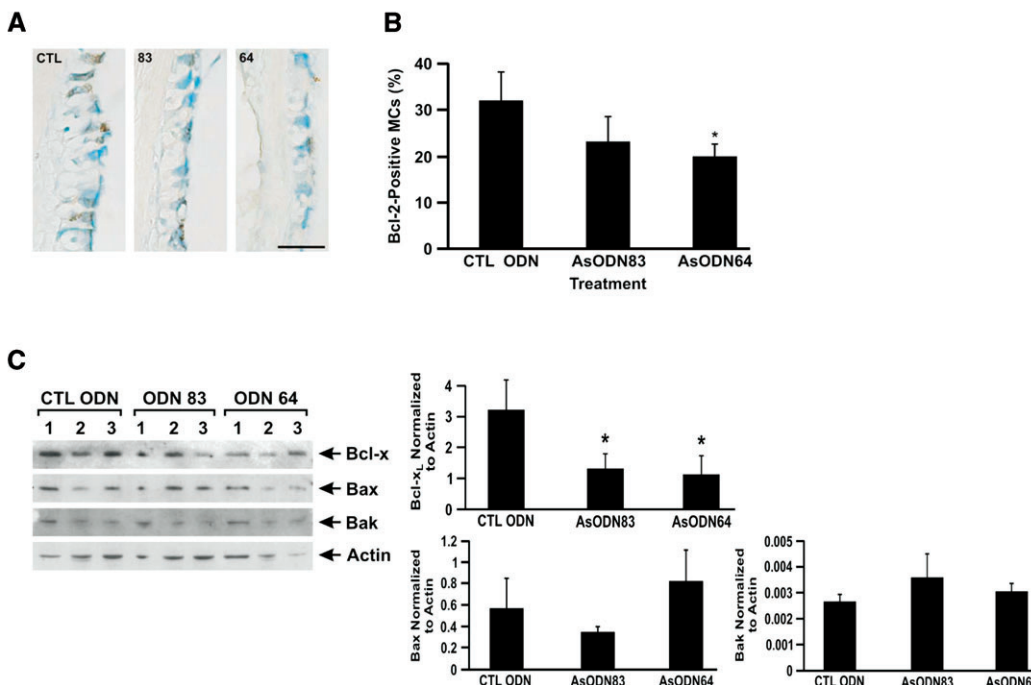

Figure 6. Bcl-2 protein levels are reduced in rats injected with ASODN 64 compared with control (CTL) ODN-injected animals. *Bar* represents 30 µm. (*A, B*) The percentage of Bcl-2–positive MCs was reduced in rats injected with ASODNs 83 and 64 compared with those injected with control ODN. *Bars* represent group mean values ± SEM (n = 6 rats/experimental group). *Significantly different from control ODN. (*C*) Densitometric quantification of Bcl-xL, Bax, and Bak after normalizing to actin in proteins extracted from three rat lungs each injected with control ODN and ASODNs 83 and 64.
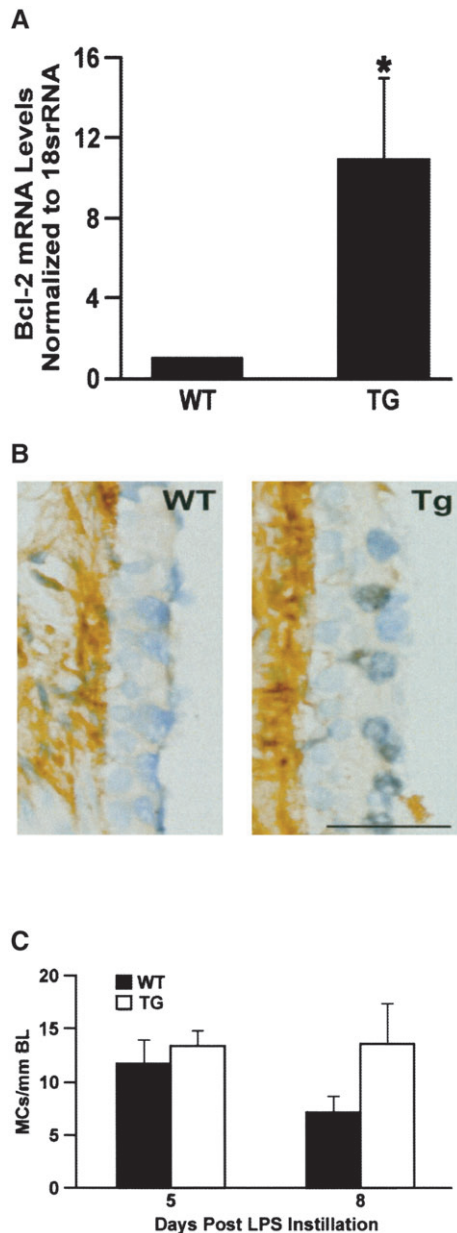
Figure 7. Sustained Bcl-2 expression in transgenic mice sustains LPS-induced GCM. Both wild-type (WT) and human Bcl-2 transgenic (TG) mice with the Bcl-2 transgene under the control of the mouse MT1 promoter on a C57Bl/6 background were maintained on 25 mM zinc sulfate drinking water to induce transgene expression. Mice were instilled with LPS on 3 consecutive days with 180 μg in 50 μL saline, a protocol previously shown to induce maximum GCM in C57Bl/6 mice. (*A*) Increased expression of Bcl-2 was detected 8 days after LPS instillation in lungs of TG mice that express a zinc-inducible Bcl-2 transgene in airway epithelia compared with WT mice when they were maintained on water with zinc sulfate. *Bars* represent group mean values ± SEM (n = 3/group). *Significantly different from WT, p < 0.05. (*B*) Representative photomicrographs of Bcl-2 immunostaining cells in WT and TG (Tg) mice at 8 days after LPS instillation. *Bar* represents 20 μm. (C) GCM was quantified in these mice over a period of 5 and 8 days after LPS instillation. GCM was similar in both WT and TG mice at 5 days after LPS instillation. However, at 8 days, GCM was decreased in WT but sustained in TG mice.

decreased at 8 days after instillation, but remained elevated in Bcl-2 transgenic mice (Figure 7C).

DISCUSSION

The present study shows that Bcl-2 is present in airway mucous cells of subjects with CF and that LPS-induced epithelial proliferation and GCM is transiently sustained by the expression of this antiapoptotic protein.

Interestingly, Bcl-2 expression was significantly reduced (as shown by the percentage of Bcl-2–positive mucous cells and by Western blot analyses) but not completely suppressed by the ASODN treatment. This finding suggests that it is not crucial to completely inhibit Bcl-2 expression to cause the reduction of epithelial cell numbers and GCM and that proapoptotic signals initiate the proapoptotic cascade once Bcl-2 and Bcl-x_L are reduced to a certain threshold level. It is unclear why ASODN 64 was not as effective as ASODN 83 in downregulating GCM in organ culture but was more effective when injected in rats instilled with LPS. The presence of a large amount of inflammatory mediators in *in vivo* rats may result in differences in the stability of these ASODNs and contribute to this observed difference. Bcl-2 homology region-3–only subfamily members are known to induce apoptosis by association with antiapoptotic Bcl-2 family members or by stimulating other apoptosis-promoting family members (7, 9, 24, 25).

The reduction of Bcl-2 and Bcl-x_L, the major antiapoptotic proteins, may have allowed the proapoptotic proteins Bax and Bak to initiate the cell-death process. Although epithelial cells undergoing cell death are not presented in this study, terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL-)–positive cells have been detected at the early times after LPS instillation in mice (26). Furthermore, we have reported that the resolution of allergen-induced GCM is associated with the presence of TUNEL-positive cells and activated caspase 3 (27). Ongoing studies will characterize the cell death by which epithelial cells are removed from the epithelium.

The reason why Bcl-x_L levels were reduced in lungs when rats were injected with ASODNs to Bcl-2 is not clear. The homology of Bcl-2 and Bcl-x_L mRNAs in the regions where ASODNs 83 and 64 are localized is 50%. This level of homology may be sufficient to bind and downregulate Bcl-x_L mRNA because of similarity in the tertiary structures between these mRNAs. Consistent with our findings, a Bcl-2/Bcl-xL bispecific ASODN inhibits the expression of Bcl-2 and Bcl-xL in a representative breast carcinoma cell line and inhibits the growth and induces death in cell lines from lung, breast, colorectal, and prostate carcinomas *in vitro* (28).

The reduction of Bcl-2 expression with ASODNs decreased the total epithelial cell number and GCM, thereby directly linking Bcl-2 expression to GCM. Because the ASODNs were administered before LPS instillation, it is possible that the reduction of Bcl-2 expression affected the inflammation-induced epithelial cell proliferation. However, our previous studies demonstrate that approximately half of the Bcl-2–positive cells had undergone a cell cycle division whereas the other half had not incorporated BrdU and were therefore derived from preexisting epithelial cells (2). These studies suggest that Bcl-2 expression is not involved in regulating cell cycle in this system. Further support for the role of Bcl-2 in sustaining GCM was established by time-course experiments demonstrating that the reduction of Bcl-2 expression to background levels occurs before the resolution of GCM (5). In addition, the decrease in Bcl-2–positive mucous cells seen after injection of rats with bezafibrate was associated with significant reduction of LPS-induced mucous cells per millimeter of BL (12). These observations together with the known

antiapoptotic function of Bcl-2 (29, 30) confirm that Bcl-2 sustains the lifespan of mucous cells after inflammatory responses.

The reduction of LPS-induced GCM in distal septa as well as pulmonary airways of rats injected with ASODNs to the Bcl-2 mRNA suggests that the processes involved in the resolution of GCM in epithelia lining the nasal midsepta and the pulmonary airways are similar. Our previous studies have established that Bcl-2 is expressed in ozone- or LPS-induced GCM, both in nasal and pulmonary airways (4, 5). Because the distal region of the nasal septum and the main axial pulmonary airways are lined by a tall columnar respiratory epithelium with primarily serous-type (not mucous) secretory cells (31, 32), similar regulation of the resolution process of LPS-induced GCM in these epithelia is not surprising. These results suggest, therefore, that targeting Bcl-2 expression with ASODN may be useful to reduce GCM and airway obstruction in humans with bronchopneumonia or rhinitis.

In the rat, the proximal midseptum is lined by a mucociliary epithelium with endogenous mucous cells. Exposure to ozone does not change the numbers of mucous cells in this epithelium but induces GCM in epithelia that line the lateral wall and the naso- and maxilloturbinates (33). Interestingly, although the percentage of Bcl-2–positive cells remains low in the proximal midseptum, up to 50% of mucous cells express Bcl-2 where metaplastic changes occur (4). Together with our current finding that Bcl-2 sustains the life span of metaplastic mucous cells, we conclude that Bcl-2 is expressed only in epithelia where mucous cell numbers have to be regulated. In addition, the total number of epithelial cells per millimeter of BL that was reduced in rats injected with ASODN 64 was similar to the reduction in the number of mucous cells per millimeter of BL, suggesting that the epithelium removes primarily mucous cells to restore the original number of epithelial cells. Therefore, targeting Bcl-2 may primarily reduce metaplastic cells without affecting endogenous mucous cells, which makes this approach valuable and relevant for therapies in chronic diseases.

The presence of Bcl-2 in mucous cells from subjects with CF indicates that this protein may be a useful target for reducing mucous secretions. Our studies with cells from subjects with CF and control subjects show that cells from subjects with CF are capable of differentiating to mucous cells, even at the time when they reach confluence without the formation of a columnar epithelium. The fact that cells from subjects with CF do not have more mucus-producing epithelial cells compared with control subjects at confluence indicates a blunted capacity for *in vitro* differentiation of CF cells to a mucus-producing phenotype. Deficiency in CF is not directly responsible for Bcl-2 expression. Therefore, it is likely that the presence of bacterial infections in CF (34) may be causing sustained Bcl-2 expression. It is also possible that dysregulated expression of Bcl-2 or the presence of inflammatory mediators sustain activation of Bcl-2 promoter, causing sustained GCM and airway obstruction. This hypothesis is supported by our finding that the sustained expression of Bcl-2 in transgenic mice, where the metallothionein promoter is activated with zinc water, prolongs the presence of GCM compared with wild-type mice.

Increased expression of Bcl-2 after inflammatory responses may be crucial to sustain a transient increase of mucous cell numbers, for the epithelium to have sufficient mucus to protect injury by further insults. However, dysregulated expression of Bcl-2 may sustain GCM to cause disease. Studies have demonstrated that, in patients with CF and in horses with reactive airway obstruction, mucous secretions accumulate in airways and cause obstruction (35, 36). Bcl-2 is also expressed in approximately 50% of mucous cells in horses with reactive airway obstruction (Y.T., personal observation). Thus, our studies showing Bcl-2 expression in mice, rats, horses, and humans suggest that resolution of GCM by this mechanism is conserved among species and support the hypothesis that targeting Bcl-2 for downregulation may be useful to reduce mucous cell numbers and alleviate mucous secretions.

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