Lysosomotropic drugs inhibit maturation of transforming growth factor-β

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Abstract: Transforming growth factor-β (TGFβ) is synthesized as a precursor protein, pro-TGFβ, that must be cleaved by a furin-like proteinase before it becomes biologically active. We hypothesized that alkalinization of the trans-Golgi network (TGN)/endosome system may suppress pro-TGFβ processing and decrease TGFβ secretion. This hypothesis was tested in human A549 alveolar epithelial and T98G glioblastoma cell lines and in C57BL/6 mice. Inhibition of furin-like activity with decanoyl-RVKR chloromethylketone suppressed pro-TGFβ processing, thereby significantly reducing the levels of secreted TGFβ. Brefeldin A, bafilomycin A1, ammonium chloride, and monensin also prevented pro-TGFβ processing. The alkalinizing lysosomotropic drugs chloroquine, hydroxychloroquine, amodiaquine, and azithromycin had a similar effect on the overall production of mature bioactive TGFβ. Reduced levels of secreted TGFβ were also associated with a decrease in Smad2 signaling. Mice treated with chloroquine showed a decrease in bronchoalveolar lavage fluid TGFβ. We conclude that alkalinizing lysosomotropic drugs inhibit pro-TGFβ processing.

Key words: idiopathic pulmonary fibrosis, lysosomotropic drugs, glioblastoma, furin, epithelial cells, convertases.

Résumé : Le facteur de croissance transformant β (TGFβ) est synthétisé en tant que protéine en amont : le pro-TGFβ qui doit être scindé par une protéinase analogue à la furine avant de devenir biologiquement actif. Nous posons donc l'hypothèse selon laquelle l'alcalinisation de l'ensemble réseau trans-golgien/endosome stoppe la production de pro-TGFβ et diminue la sécrétion de TGFβ. Nous avons testé cette hypothèse dans une cellule épithéliale d'une alvéole A549, dans une lignée cellulaire de glioblastome T98G et chez des souris C57BL/6. L'inhibition de l'activité analogue à celle de la furine par la chlorométhylcétone de decanoyl-RVKR arrête la production de pro-TGFβ et cause une diminution significative de la concentration de TGFβ sécrétée. La bréfeldine A, la bafilomycine A1, le chlorure d'ammonium et la monensine arrêtent aussi la production de pro-TGFβ. La chloroquine, l'hydroxychloroquine, l'amodiaquine et l'azithromycine ont aussi un effet similaire sur la production globale de TGFβ bioactif arrivé à terme. La diminution de la sécrétion de TGFβ est aussi associée au fléchissement de la signalisation de Smad-2. Les souris traitées à la chloroquine révèlent une diminution de la concentration de TGFβ dans le liquide de lavage bronchoalvéolaire. En conclusion, les drogues lysosomotropiques au pouvoir alcalinisant arrêtent la production de pro-TGFβ.

Mots-clés : fibrose pulmonaire idiopathique, drogues lysosomotropiques, glioblastome, furine, cellules épithéliales, convertases.

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Introduction

Transforming growth factor-β (TGFβ) plays a central role in the pathophysiology of several diseases, particularly those associated with tissue fibrosis and neoplastic growth (Blobe et al. 2000). Three isoforms of TGFβ are relevant to human biology: TGFβ1, TGFβ2, and TGFβ3 (Sheppard 2006). Increased expression of TGFβ1 occurs in the alveolar epithelial cells of patients with idiopathic pulmonary fibrosis (IPF), whereas TGFβ1 and TGFβ2 are expressed in glioblastoma cells (de Martin et al. 1987; Khalil et al. 1991; Leitlein et al. 2001). Each TGFβ isoform is encoded by a distinct gene encoding a large proprotein that is cleaved by the serine protease furin or a member of the proprotein convertase (PC) family (Dubois et al. 1995).

Furin (EC 3.4.21.75) is a ubiquitous subtilisin-like proprotein convertase that cleaves the TGFβ proprotein to release the N-terminal propeptide known as the latency-associated peptide (LAP) and TGFβ (Dubois et al. 1995, 2001; Thomas 2002). Other convertases of the PC family may also act as pro-TGFβ convertases. Once pro-TGFβ is cleaved, disulfide-linked dimers of LAP and TGFβ associate noncovalently to form the small latency complex, which can be activated by proteases, oxidants, and other mechanisms (Sheppard 2006). Pro-TGFβ, TGFβ, and LAP can each be detected in the extracellular milieu of lung alveolar epithelial cells and glioblastoma cells, where activated TGFβ

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binds to its receptor and initiates a signaling cascade that phosphorylates the Smad proteins leading to gene transcription (Massague et al. 2005).

Furin and the TGFβ proprotein colocalize to the trans-Golgi network (TGN)/endosomal compartment, a mildly acidic compartment, where endoprotease cleavage of furin-like substrates has been shown to occur (Anderson et al. 2002). Since cleavage occurs in the TGN and since furin is active (or activated) in a mildly acidic compartment, we hypothesized that alkalinizing lysosomotropic drugs such as chloroquine and amodiaquine, which are antimalarial agents, hydroxychloroquine, an antirheumatic agent, and azithromycin, an antibiotic, can inhibit TGFβ maturation by preventing PC-dependent proprotein maturation. This hypothesis was verified in relevant cell lines derived from human alveolar epithelium (A549) and glioblastoma tumor (T98G), as well as in C57BL/6 mice.

Materials and methods

Cell lines and reagents

The A549 pulmonary epithelial and the T98G glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, Md.), ATCC Nos.CCL 185 and CRL 1690, respectively. The A549 and T98G cells were grown in 100 millimetre petri dishes or in 6-well culture plates (Linbro Chemical, New Haven, Conn.) in RPMI medium (Gibco, Invitrogen Life Technologies, Burlington, Ont.) supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L glutamine in 5% CO_2 at 37 °C. The A549 and T98G cells were trypsinised and seeded at a density of 1.5×10^6 cells/well in 6-well culture plates and incubated for 24 h before each experiment. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (RVKRcmk) and the control peptide decanoyl-RVRK-chloromethylketone (RVRKcmk) were from Bachem (Torrance, Calif.). Brefeldin A, monensin, ammonium chloride, bafilomycin A1, azithromycin, amodiaquine, chloroquine, and hydroxychloroquine were purchased from Sigma-Aldrich (St. Louis, Mo.).

TGFβ ELISA assay

A549 and T98G cells were incubated in 6-well culture plates in RPMI without FBS for 24 h either alone or with the following: 10 μmol/L RVKRcmk, 10 μmol/L RVRKcmk, 1 μmol/L brefeldin A, 1 μmol/L bafilomycin A1, 10 mmol/L NH_4Cl, 40 mmol/L NH_4Cl, and 1 μmol/L monensin. In addition, both cell lines were incubated in RPMI without FBS for 24 h in the presence of 10, 50, 100, and 200 μmol/L chloroquine, and A549 was also incubated with azithromycin, amodiaquine, and hydroxychloroquine. To determine the kinetics of chloroquine effects on TGFβ1 production, A549 cells were incubated with or without 100 μmol/L chloroquine for 0.25, 0.5, 1, 2, 4, 12, and 24 h. After incubations, the cell supernatants were collected and treated with HCl to convert all mature TGFβ1 (that is, both active and inactive) into a form recognized by the antibody used in the assay. The concentration of HCl-treated human TGFβ1 was measured by an ELISA for human TGFβ1 (R&D Systems, Minneapolis, Minn.). It is important to note that this assay recognizes mature (active and inactive) TGFβ but does not detect pro-TGFβ.

Western blot analysis

Cell supernatants were concentrated on Amicon 5000 (Millipore, Billerica, Mass.) for 15 min for TGFβ1 Western blot analysis. To obtain cell lysates for phosphorylated Smad2 Western blot analysis, the cells were washed twice with PBS and then lysed in 0.5% (*w*/*v*) sodium deoxycholate (Sigma-Aldrich), 0.1% (*w*/*v*) sodium dodecyl sulfate (SDS) (Sigma-Aldrich), 1% (*v*/*v*) nonionic detergent (Igepal CA-630, Sigma-Aldrich), and protease inhibitor cocktail (1 tablet/50 mL) (Roche, Mannheim, Germany) before being placed on ice for 10 min. The lysates were centrifuged at 13 000*g* for 10 min at 4 °C, and the supernatants collected. The protein concentrations of the cell supernatants and lysates were estimated by using the Bio-Rad protein assay kit. Samples containing 75 μg protein of cell lysates or 5 μg protein of supernatant were resolved on 8% SDS – polyacrylamide gels. The separated proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, Ont.) and analyzed by Western blotting procedures, using 5% (*w*/*v*) bovine serum albumin (BSA) as the blocking agent, and overnight incubation at 4 °C with goat anti-LAP (1:250, R&D Systems) or rabbit anti-Smad2 (pSer465/467) (1:1000, Calbiochem, San Diego, Calif.) polyclonal antibodies. The membranes were then washed and incubated 1 h with anti-rabbit or anti-goat IgG horseradish peroxidase-conjugated (1:3000, Calbiochem) and revealed by chemiluminescence (ECL kit, Amersham, Buckinghamshire, UK).

Chloroquine in mice

C57BL/6 mice (Charles River, St. Constant, Qué.) weighing 25 g were housed in accordance with guidelines from the Canadian Council on Animal Care. The protocol was approved by the institutional animal care committee of the University of Sherbrooke. Animals were treated with daily subcutaneous injections of 50 μL 0.9% saline or 50 μL of 20 mg/mL chloroquine for 7 days. On day 7, bronchoalveolar lavage (BAL) was performed with 3 aliquots (0.9 mL each) of 0.9% saline. BAL fluid was centrifuged, and the supernatant collected to measure TGFβ1 by ELISA and proteins with the Bradford protein assay (Bradford 1976).

Statistics

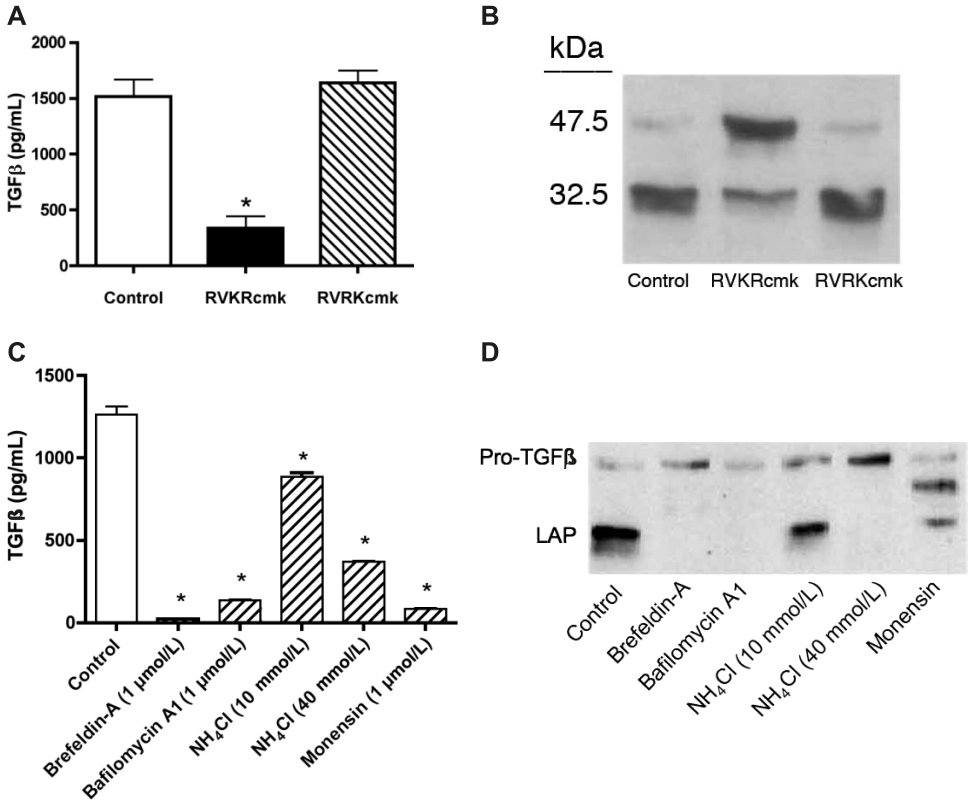
The results are presented as means ± SE. Data with multiple group comparisons were analyzed by using ANOVA and the post hoc Bonferroni test (GraphPad Prism version 4.0, San Diego, Calif.).

Results

Pro-TGFβ processing by furin-like proteinase activity in A549 and T98G cells

TGFβ1 levels in the supernatant from A549 cells were significantly reduced following treatment with RVKRcmk (336.3 ± 108.6 pg/mL) compared with media alone (1,518.0 ± 152.0 pg/mL) or the control peptide RVRKcmk (1639 ± 113.9 pg/mL), as determined by ELISA (Fig. 1A). The cell supernatants were also analyzed by Western blot to determine the apparent molecular ratio of the LAP as an index of TGFβ proprotein processing (shown in Fig. 1B is one representative result of 4 separate experiments). As can be

Fig. 1. Release of TGFβ1 and maturation of cellular pro-TGFβ in A549 cells treated with RVKRcmk, an inhibitor of furin-like proteinase activity (A, B), and agents that disrupt or alkalinize the acidic trans-Golgi network (TGN)/endosomal compartment (C, D). Cells were incubated for 24 h in media alone (control), or with 10 μmol/L RVKRcmk, 10 μmol/L control peptide RVRKcmk, or the indicated concentrations of TGN/endosome modifiers. ELISA determination of TGFβ1 production was performed on the cell supernatant media (A, C), and Western blots were used to identify the migration of the latency-associated peptide (LAP) in its native pro-TGFβ (upper bands) and cleaved LAP (lower bands) forms (B, D). Blots are representative of 4 separate experiments. Asterisk (*) indicates significant difference ($p < 0.001$) from control and RVRKcmk (A) or from control (B), $n = 4$ per condition.



seen, we detected an increase in the intensity of the bands migrating at 47.5 kDa, corresponding to pro-TGFβ and a decrease in the intensity of the bands migrating at 32.5 kDa, corresponding to the LAP. The control peptide RVRKcmk had no effect on pro-TGFβ processing.

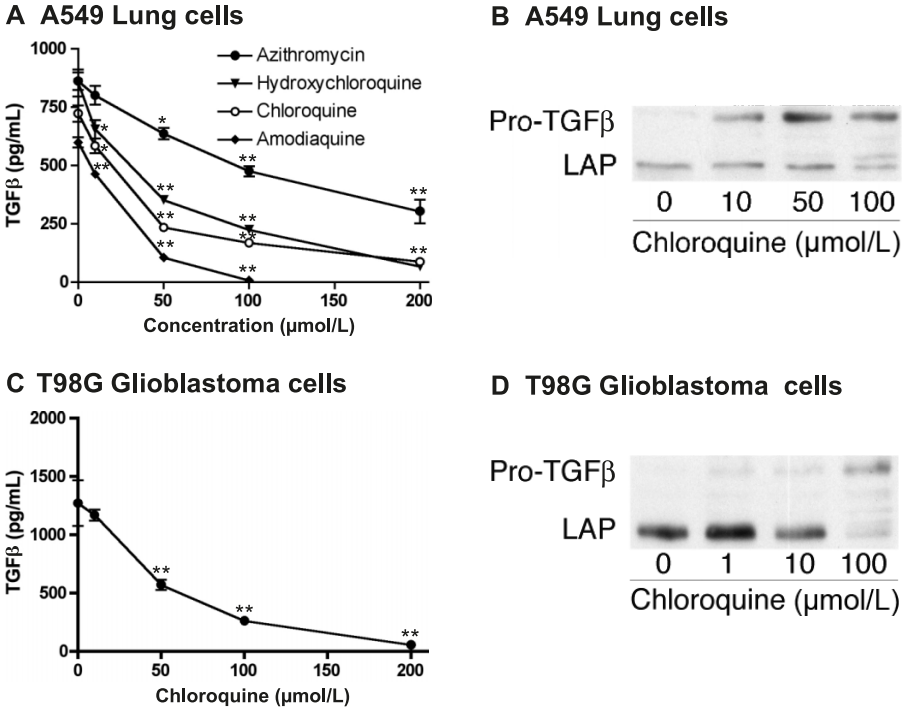
Pro-TGFβ processing depended on the TGN/endosome system and its acidic milieu

To verify whether the acidic milieu in the TGN/endosome is important for pro-TGFβ maturation, we measured secreted TGFβ1 after incubating cells with several agents known to alkalinize TGN/endosomal pH (Fig. 1C). Extremely low levels of mature TGFβ1 were detected in the supernatants of A549 cells incubated with the TGN-disrupting brefeldin A at 1 μmol/L (21.8 ± 1.5 pg/mL) compared with control (1264.0 ± 48.5 pg/mL). In addition, the other agents also significantly decreased levels of TGFβ1 in the cell media. Inhibition of the vesicular ATPase proton pump with bafilomycin A1, addition of the lysosomotropic weak base NH_4Cl, and dissipation of pH gradients with the ionophore monensin each resulted in a marked decrease of TGFβ1 secretion (Fig. 1C) (bafilomycin A1, 137.5 ± 5.1 pg/mL; 10 mmol/L NH_4Cl, 887.8 ± 21.1 pg/mL; 40 mmol/L NH_4Cl, 373.3 pg/mL; monensin, 86.8 ± 3.3 pg/mL). The mechanism by which these conditions suppressed mature TGFβ secretion was associated with inhibition of the proprotein processing, as shown by a marked reduction in the intensity of the cleaved peptide identified as LAP when compared with control (Fig. 1D) (one representative result of 4 separate experiments).

Drugs that are lysosomotropic weak bases suppressed TGFβ1 maturation

Chloroquine, hydroxychloroquine, amodiaquine, and azithromycin are weak bases that bear a tropism for the acidic TGN/endosomal compartment. Each of these drugs showed a concentration-dependent suppression of TGFβ1 processing by either alveolar epithelial cells (Fig. 2A) or glioblastoma cells (Fig. 2C). For A549 cells, the values at 50 μmol/L were (in pg/mL): amodiaquine 105.6 ± 3.3, control (media alone) 598.3± 21.4; chloroquine 234.2 ± 5.9, control 721.9 ± 34.7; hydroxychloroquine 351.4 ± 19.1, control 853.0 ± 58.5; azithromycin 635.5 ± 24.5, control 861.3 ± 37.2. For T98G cells, the value at 50 μmol/L chloroquine was 571.4 ± 44.0 pg/mL versus control 1273.3 ± 196.7 pg/mL. As can be seen, the increasing concentrations of chloroquine were associated with an increase in the pro-TGFβ band on Western blots (corresponding to the band migrating at 47.5 kDa) from each cell line, indicating that proprotein processing was decreased (Figs. 2B and 2D, one representative result of 4 separate experiments).

Fig. 2. Alkalinizing lysosomotropic drugs affect TGFβ1 proteolytic processing of pro-TGFβ protein in A549 cells (A, B) and T98G glioblastoma cells (C, D) at 24 h. The TGFβ1 concentrations in the cell media were measured by ELISA (A, C). Pro-TGFβ processing was assessed by Western blot using an antibody that recognizes the latency-associated peptide (B, D). Significant at *, $p < 0.05$ and **, $p < 0.01$ vs. control (media alone), $n = 4$. Blots are representative of at least 4 separate experiments.



Kinetics of chloroquine effect on TGFβ1 production

Incubation of A549 cells with 100 μmol/L chloroquine decreased the concentration of TGFβ1 detectable in the A549 supernatants as early as 60 min and this effect was sustained at 24 h (Fig. 3). The values (in pg/mL) at 60 min were chloroquine 14.8 ± 1.3 compared with control 28.9 ± 0.7; at 24 h, chloroquine 145.2 ± 7.8 compared with control 1121.8 ± 82.1.

Inhibition of pro-TGFβ processing suppressed Smad2 phosphorylation

Active TGFβ in the extracellular space binds to its receptor and initiates phosphorylation of Smad proteins, as observed in the A549 lysates illustrated in the Western blot specific for phosphorylated Smad2 protein (Fig. 3C). Inhibition of pro-TGFβ processing with RVKRcmk markedly suppressed Smad2 phosphorylation. Similarly, 100 μmol/L chloroquine induced a decrease in Smad2 phosphorylation in A549 cells.

Chloroquine decreased TGFβ in BAL fluid of mice

Mice treated with daily subcutaneous injections of 1 mg chloroquine showed a significant decrease in BAL fluid TGFβ levels (Fig. 4). The values shown are chloroquine 315.0 ± 22.5 pg/mg protein and control 430.3 ± 45.3 pg/mg protein. We observed no adverse effects of the chloroquine administration.

Discussion

Proteases of the subtilisin-like proprotein convertase family, such as furin, are involved in TGFβ maturation and may therefore be considered as important targets in many diseases. The furin-like proteinase inhibitor RVKRcmk has been reported to suppress TGFβ1 and TGFβ2 release into the extracellular milieu of glioblastoma cells (Leitlein et al. 2001). Our observations in alveolar epithelial cells indicated that RVKRcmk alone is sufficient to markedly suppress production of bioactive TGFβ (by abolishing processing) and consequently Smad2 phosphorylation. The effect was specific to the RVKR sequence, a recognition site for furin-mediated cleavage, since RVRKcmk had no effect. Thus, because furin-like proteinase activity is located mostly in the TGN/endosomal system, we focused our investigations on interventions affecting this cellular compartment.

Brefeldin A, which disrupts the Golgi apparatus and the TGN/endosome compartment, abolished pro-TGFβ processing and consequently lowered the levels of bioactive TGFβ1 in the media of treated cells. We then hypothesized that agents affecting the mildly acidic TGN/endosomal compartment may also decrease TGFβ1 maturation by preventing cleavage of the TGFβ proprotein. This was confirmed by using 3 different approaches to alkalinize the TGN/endosomal pH: (*i*) a vesicular ATPase proton pump inhibitor, bafilomycin A1, (*ii*) a lysosomotropic weak base, ammonium chloride, and (*iii*) an ionophore that dissipates vesicular pH, monensin. Our results indicated that alkalinization of the mildly acidic TGN/endosomal compartment can be an effective approach to control TGFβ processing in mammalian cells.

Recently, Perkett et al. have reported that bronchial epithelial cells expressing the ΔF508 mutant of cystic fibrosis transmembrane conductance regulator (CFTR) produce increased amounts of TGFβ and that this abnormal production

Fig. 3. Time course of chloroquine-mediated inhibition of TGFβ1 accumulation in the extracellular milieu and effect of chloroquine on Smad2 phosphorylation. A549 cells were incubated with media alone (control) or 100 μmol/L chloroquine for (A) 0–120 min and (B) 6–24 h. (C) Western blots of cells incubated 24 h in the presence of 10 μmol/L RVKRcmk or 0–100 μmol/L chloroquine, washed, and lysed. Specific antibodies against phosphorylated Smad2 protein were used to determine the state of Smad2 phosphorylation. Significant at *, $p < 0.05$ and **, $p < 0.01$ vs. control, $n = 4$ per condition.

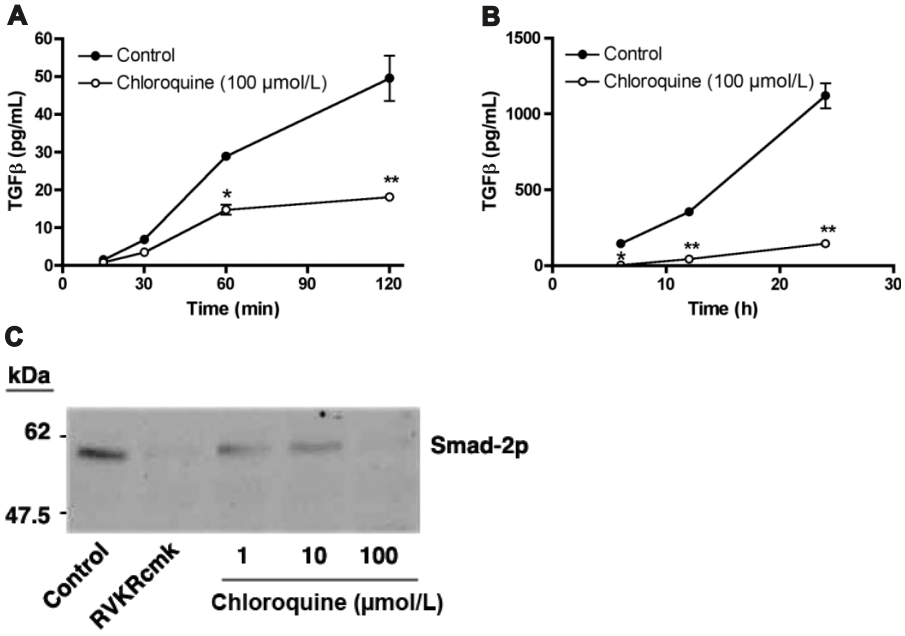
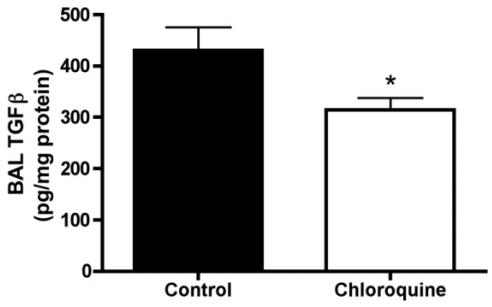


Fig. 4. TGFβ in bronchoalveolar lavage fluid (BAL) from the lungs of mice treated with daily subcutaneous injections of 0.9% saline (control) or 1 mg chloroquine for 7 days. Levels of TGFβ in BAL were determined by ELISA. Asterisk (*) indicates significant difference ($p < 0.05$) from control, $n = 11$ per group.



can be blocked by chloroquine (Perkett et al. 2006). The current study provides evidence that chloroquine decreases pro-TGFβ post-translational processing. Several drugs are lysosomotropic weak bases. These include chloroquine, amodiaquine, hydroxychloroquine, and azithromycin, all of which have a long history of use in humans and a known safety profile. Each of these drugs showed a concentration-dependent inhibition of pro-TGFβ protein processing and TGFβ1 secretion in A549 and T98G cells. Our results indicated that the effects of chloroquine are not specific to cells expressing ΔF508 CFTR, and we speculate that this class of drugs may have the potential for broader application in other conditions associated with furin-mediated proprotein cleavage.

The suppressive effect of chloroquine on pro-TGFβ processing was observed within the first 2 h after treatment. The effect of the lysosomotropic drugs was not through a generalized decrease in protein secretion because increased pro-TGFβ protein was detected by Western blot in the cell supernatants. Although our work did not allow us to identify the mechanism by which lysosomotropic weak bases prevent pro-TGFβ processing and TGFβ1 secretion, one possibility is that an increase of the TGN/endosomal pH may affect the capacity of furin to complete its autocatalytic activation. Furin is synthesized as an inactive proprotein, and the first step in its activation consists of an autocatalytic prodomain excision from the mature protein in the ER, a prerequisite for the efficient furin egress from this compartment. After the initial cleavage, the prodomain remains tightly associated with the convertase, preventing protein activity. When this stable furin–prodomain complex reaches the TGN, the increase in H^+ and (or) Ca^{2+} concentration triggers a secondary cleavage, resulting in the dissociation of the prosegment and, consequently, the activation of the enzyme (Anderson et al. 1997; Bassi et al. 2005; Leduc et al. 1992). These steps in furin activation occur within 2 h (Anderson et al. 1997). Almost complete inhibition of furin cleavage has been reported with chloroquine in the Madin–Darby bovine kidney (MDBK) cell line (Vey et al. 1994). Although other mechanisms may be involved, the rapid inhibition of TGFβ1 secretion we observed is consistent with a pH-dependent interference of furin activation. The dependence of protein maturation on endosomal pH is not specific to pro-TGFβ, as other TGN proteins such as TGN38 and foreign viral proteins undergo pH-dependent processing with furin-like proteases (Chapman and Munro 1994; Li et al. 2008; Yu et al. 2008).

Chloroquine, and other lysosomotropic agents, are concentrated in tissues. In animals, concentrations of chloro-

quine 10–700 times the plasma concentration may be detected in several tissues, including the lung and brain (Adelusi and Salako 1982). In the current study, TGFβ levels in mice at the alveolar epithelial surface, as determined by BAL, were decreased after 1 week of subcutaneous administration of chloroquine at a dose that appeared to be well tolerated. The decrease was more modest in vivo than in vitro, most likely due to the lower tissue concentrations of the drug. Further studies are needed to determine whether similar results can be achieved in humans.

Inhibition of furin markedly decreases astrocytoma proliferation and invasiveness in vitro and thus has been identified as a promising therapeutic target for glioblastoma multiforme, a severe form of malignant astrocytic tumor (Mercapide et al. 2002; Wick et al. 2006). One indication that clinically meaningful inhibition of furin may be achievable by chloroquine in vivo comes from a clinical trial of chloroquine as an adjuvant therapy in patients with glioblastoma (Sotelo et al. 2006). The median survival of patients treated for glioblastoma with surgery, conventional chemotherapy, and radiation therapy was 11 months, whereas it increased to 24 months in patients who were given a daily dose of 150 mg chloroquine. The authors state that the results may be attributable to the antimutagenic properties of chloroquine (Briceno et al. 2003; Sotelo et al. 2006). We suggest that this remarkable improvement in survival may also be related, at least in part, to decreased pro-TGFβ processing.

TGFβ has been clearly shown to be sufficient to induce all of the histologic features of the IPF lung (Xu et al. 2003). Current therapies for adults with IPF are largely ineffective, possibly because none are known to affect TGFβ synthesis or cell signaling (Gauldie 2002). In a recent clinical trial, however, patients with IPF showed modest improvement in some lung function parameters and had fewer respiratory exacerbations while taking the drug pirfenidone for 9 months (Azuma et al. 2005). Pirfenidone has recently been shown to inhibit furin in a malignant glioma cell line (Burghardt et al. 2007). The inhibition of pro-TGFβ maturation we report in the current study was observed with chloroquine and amodiaquine concentrations 100-fold lower than those of pirfenidone.

The decrease in TGFβ processing we report is most likely due to a class effect of the tested drugs, and in this context, other alkalinizing lysosomotropic drugs may also be of interest. In addition, alkalinizing lysosomotropic compounds may have the potential to treat patients with several diseases, including influenza (Ooi et al. 2006), in which furin-related processing may be important.

In summary, we have observed that the lysosomotropic drugs chloroquine, hydroxychloroquine, amodiaquine, and azithromycin suppress production of bioactive TGFβ in lung alveolar epithelial cells and glioblastoma cells. Systemic chloroquine administration also decreases levels of TGFβ in BAL fluid of mice. The mechanism would appear to be related to inhibition of pro-TGFβ protein maturation and likely occurs through suppression of furin-like proteinase activity in the TGN/endosomal compartment. There is a pressing need for effective therapies in several fatal diseases characterized by an excess of TGFβ activity, such as IPF and glioblastoma multiforme. Because of their availability, low-cost, and well-defined safety profile, alkalinizing lysosomotropic drugs warrant consideration for clinical studies of TGFβ-related diseases.

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