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Effect of Chrysotile Exposure in Human Bronchial Epithelial Cells: Insights on the Pathogenic Mechanisms of Asbestos-Related Diseases

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This paper is dedicated to the memory of Dario Ghigo, who passed away on 7th October 2015.

We all remember our friend as a master in life and science and we hope he will continue to help us from the place where he is now.

Conflict of interest: The authors declare that they have no competing interest.

Abstract

Background: Chrysotile asbestos accounts for more than 90% of the asbestos used worldwide and exposure is associated with asbestosis (asbestos-related fibrosis) and other malignancies, but the molecular mechanisms involved are not fully understood. A common pathogenic mechanism for these malignancies is represented by epithelial-mesenchymal transition (EMT), through which epithelial cells undergo a morphological transformation to take on a mesenchymal phenotype. In the present work we propose that chrysotile asbestos induces EMT through a mechanism involving a Transforming Growth Factor beta (TGF β)-mediated signaling pathway.

Objectives: To investigate the role of chrysotile asbestos in inducing EMT trying to elucidate the molecular mechanisms involved in this event.

Methods: Human bronchial epithelial cells BEAS-2B were incubated with 1 $\mu\text{g}/\text{cm}^2$ chrysotile asbestos for up to 72 h and several markers of EMT were investigated. Experiments with the specific inhibitors for TGF- β , Glycogen Synthase Kinase-3 β (GSK-3 β) and Akt were carried out in order to confirm their involvement in asbestos-induced EMT. Real time PCR, Western blot and gelatin zymographies were performed to detect the mRNA and protein expression changes of these markers.

Results: Chrysotile asbestos activates a TGF- β -mediated signaling pathway, which implicates the contribution of Akt, GSK-3 β , and SNAIL-1. The activation of this pathway in BEAS-2B cells was associated with a decrease in the epithelial markers (E-cadherin and β -catenin), and an increase in the mesenchymal markers (α -smooth muscle actin, vimentin, metalloproteinases and fibronectin).

Conclusions: Our findings suggest that chrysotile asbestos induces EMT, a common event in asbestos-related diseases, at least in part by eliciting the TGF- β -mediated Akt/GSK-3 β /SNAIL-1 pathway.

Introduction

Asbestos is the nonspecific term commonly used to describe any of six types of naturally occurring fibrous silicate minerals that were widely used commercially during the 20th century. Chrysotile asbestos is estimated to account for 90% of asbestos used worldwide (Qi et al. 2013). Since the beginning of the last century, asbestos inhalation has been considered responsible for a number of lung diseases, such as asbestosis (asbestos-induced fibrosis), lung tumors and malignant mesothelioma (MM) (Kamp 2009). Both fibrosis and epithelial tumors are highly dependent on the ability of epithelial cells to transform into mesenchymal cells through a process called epithelial-mesenchymal transition (EMT).

EMT is both a physiological and pathological process: it has been related to early embryonic development and later organogenesis, as well as to wound healing in fibrotic tissues, and tumor development and metastasis in cancer (Kim et al. 2013; Kim and Cho 2014). During EMT, cell-cell adhesion molecules are inactivated and sometimes destroyed, while cell-matrix adhesion increases. Cells undergoing EMT lose epithelial marker proteins, such as the adherent junction proteins E-cadherin and β -catenin and the tight junction protein zonula occludens, and begin to express mesenchymal proteins such as collagen, vimentin, α -smooth muscle actin (α -SMA) and fibronectin (Barrallo-Gimeno and Nieto 2005; Cannito et al. 2010; Moody et al. 2005; Peinado et al. 2004). These events lead to the acquisition of a fibroblast-like and spindle-shaped morphology: cells acquire the capacity to degrade the basement membrane and migrate through the extracellular matrix to populate different territories during either embryonic development or cancer progression, or to adopt a profibrotic myofibroblast nature (Acloque et al. 2009; Cannito et al. 2010; Kalluri and Neilson 2003; Kalluri and Weinberg 2009; Moustakas and Heldin 2012).

However, the spectrum of changes occurring during EMT may vary significantly depending on the epithelial cell type, the surrounding microenvironment and the type of inducer. Extracellular signals able to trigger EMT include growth factors such as transforming growth factor- β (TGF- β), hepatocyte growth factor, platelet-derived growth factor, fibroblast growth factor, and cytokines such as tumor necrosis factor- α (TNF- α) (Cannito et al. 2010; Chen et al. 2014; Farrell et al. 2014; Moustakas and Heldin 2012).

TGF- β is a multifunctional protein capable of regulating cell growth and differentiation as well as stimulating the production of extracellular matrix (Fine and Goldstein 1987). The different roles of TGF- β have been widely explored through the years: TGF- β exerts its biological activity by regulating growth, differentiation, and epithelial transformation in the multistep processes of carcinogenesis, wound healing and embryogenesis (Bhowmick et al. 2001; Perdue and Brody 1994).

Many works in literature have investigated the effects of asbestos: asbestos induces lung fibrosis via increased secretion of TGF- β (Manning et al. 2002), particularly in idiopathic pulmonary fibrosis where TGF- β has been localized in association with bronchiolar epithelial cells and their extracellular matrix (Liu and Brody 2001; Pociask et al. 2004). Casarsa and coworkers stressed the importance of EMT markers in MM prognosis (Casarsa et al. 2011). In another work by Qi et al, they compared the toxicity of two different kinds of asbestos: chrysotile and crocidolite – which is an amphibole asbestos whose exposure is often considered the most oncogenic type of asbestos - (Gibbs and Berry 2008). In their work, Qi et al. suggested that a continuous exposure to crocidolite and chrysotile could cause EMT of human mesothelial cells via High Mobility Group Box 1 and TNF- α signaling. Particularly, they found that repeated exposure to chrysotile

and crocidolite led to similar molecular changes and similar amount of HMGB1 secretion *in vitro* and *in vivo*, with differences in inducing the MM-related biological alterations, according to their biopersistence (Qi et al. 2013). For these reasons, a growing interest about the role of asbestos as an inducer of EMT has recently emerged.

Starting from the strong association of chrysotile exposure with TGF- β activation (Murthy et al. 2015; Pociask et al. 2004), which is in turn associated with EMT induction, in the present work we investigated the role of chrysotile in inducing EMT via TGF- β in a human bronchial epithelial cellular model (BEAS-2B), to improve the knowledge of the molecular bases of asbestos-related lung diseases.

Materials and Methods

Asbestos samples

UICC (Union International Contre le Cancer) chrysotile and UICC crocidolite were sonicated (100 W, 30 s, Labsonic Sonicator; Sartorius Stedim Biotech S.A.) before incubation with cell cultures, to dissociate fiber bundles and allow their better suspension in the culture medium.

Cell cultures

BEAS-2B cells are immortalized human bronchial epithelial cells obtained from American Type Culture Collection (ATCC). They were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Human bronchial epithelial cells (NuLi-1) are a generous gift from Dr. Claudia Voena (Department of Molecular Biotechnology and Health Sciences, University of Torino). NuLi-1 cells were cultured in a serum-free medium (Bronchial Epithelial Cell Growth Medium; Lonza) in Petri dishes pre-coated with 60 μ g/ml solution of

Human Placental Collagen Type IV at least 18 hours in advance, then air-dried and rinsed 2-3 times with PBS.

Human lung adenocarcinoma alveolar epithelial cells (A549) were provided by Istituto Zooprofilattico Sperimentale “Bruno Ubertyni” (Brescia, Italy). They were cultured in Ham’s F12 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin.

All cell cultures were kept in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Experimental conditions

To assess the appropriate concentration and time for the incubation of BEAS-2B cells with chrysotile or crocidolite, dose and time-dependence experiments were performed (data not shown). As a consequence of these preliminary results, we chose to seed 3.5×10^5 or 1.5×10^5 BEAS-2B cells in 100 mm-diameter Petri dishes and incubate them respectively up to 72 h or 7 days in the absence or presence of $1 \mu\text{g}/\text{cm}^2$ chrysotile asbestos or $5 \mu\text{g}/\text{cm}^2$ crocidolite. In the same manner, after preliminary experiments, 1.5×10^5 NuLi-1 cells were seeded in 60 mm-diameter Petri dishes and incubated for 96 h in the absence or presence of $1 \mu\text{g}/\text{cm}^2$ chrysotile. 1.5×10^5 A549 cells were seeded in 100 mm-diameter Petri dishes and then incubated for 96 h with $5 \mu\text{g}/\text{cm}^2$ chrysotile.

The protein content in the cells was detected through the BCA kit (Sigma Chemical Co.). The plasticware for cell cultures was provided by Falcon (Corning Incorporated). For all experiments, ultrapure water (Millipore,) was used.

Cell morphology

At the end of the incubation period, cells were observed at the light microscope, and images were obtained through the Leica Application Suite program (Leica Microsystems).

Measurement of reactive oxygen species (ROS)

BEAS-2B cells were incubated for 30 min, 1, 3 or 6 h in the absence or presence of 1 $\mu\text{g}/\text{cm}^2$ of chrysotile, then cells were loaded for 30 min with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is a cell-permeable probe that is cleaved intracellularly by non-specific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound dichlorofluorescein (DCF) in a 1:1 stoichiometry (Bass et al. 1983). After incubation with DCFH-DA, the cells were washed twice with PBS to remove excess probe, and DCF fluorescence was determined at excitation wavelength of 504 nm and emission wavelength of 529 nm, using a Synergy HT microplate reader (BioTek Instruments). The fluorescence value was normalized by protein concentration and expressed as units of arbitrary fluorescence.

Western blot analysis

Cytosolic and nuclear extracts were obtained using the Active Motif nuclear extraction kit (Active Motif), according to the manufacturer's instructions.

Cytosolic and nuclear extracts were separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore) and probed with the required antibody diluted in PBS-Tween 0.1% with Blocker Non-Fat Dry Milk 5%. After 1 h of incubation the membranes were washed with PBS-Tween 0.1% and then subjected for 1 h to a peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (from sheep; Amersham International), diluted 1:3000 in PBS-Tween 0.1%

with Blocker Non-Fat Dry Milk 5%. The membranes were washed again with PBS-Tween 0.1% and proteins were detected by enhanced chemiluminescence (Perkin Elmer).

Anti-E-cadherin, β -catenin, tubulin, SNAIL-1, TATA-binding protein (TBP) antibodies were all provided by Santa Cruz Biotechnology, Inc.. Tubulin and TBP were used as control loading for the cytosol and the nucleus, respectively. The anti-vimentin antibody was provided by Sigma Chemical Co. The anti- α -SMA antibody was from GeneTex. The anti-Smad2 and p-Smad2 antibodies were from Abcam.

Specific inhibitors

The neutralizing anti-TGF- β antibody was purchased by Abcam and was used at a concentration of 5 μ g/ml; the GSK-3 β inhibitor SB 216763 and the Akt 1/2 Kinase Inhibitor were from Sigma and were both used at a 5 μ M concentration.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was obtained by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), using RiboZol RNA Extraction Reagents (Amresco), following the manufacturer's instructions. Total RNA (0.2 μ g) was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories AG) according to the manufacturer's instructions. qRT-PCR was carried out using IQTM SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. PCR amplification was: 1 cycle of denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C for 30 s, annealing for 30 s, and synthesis at 72°C for 30 s.

The relative expression of each target gene was performed comparing each PCR gene product with the S14 ribosomal subunit product using the Gene Expression Macro (<http://www3.biorad.com/LifeScience/jobs/2004/04-0684/genex.xls>; Bio-Rad).

Quantification of TGF- β secretion by ELISA

After incubation of BEAS-2B cells in the absence or presence of chrysotile, the extracellular medium was collected and centrifuged at 4°C at 13,000 x g for 30 min. To determine the concentration of TGF- β in the supernatant, ELISA was performed according to the kit instructions (Invitrogen Corporation). Absorbance was measured at 450 nm with a Synergy HT microplate reader. The cytokine amount was determined using a standard solution curve and corrected for the content of cell proteins. Results were finally expressed as pg/mg of intracellular proteins.

Gelatin zymography

Since fetal bovine serum contains matrix metalloproteinases (MMPs), the cells were cultured in 1% serum medium only. Afterwards the supernatants were collected, supplemented with Laemmli sample buffer and subjected to SDS-PAGE 10% with 1 mg/ml gelatin under non-denaturing and non-reducing conditions as previously described (Giribaldi et al. 2011).

Statistical analysis

Where appropriate, data in figures are reported as means \pm SEM. The results were analyzed by a one-way Analysis of Variance (ANOVA) and Tukey's test (software: SPSS 11.0 for Windows, SPSS Inc.); $p < 0.05$ was considered significant.

Results

Effect of chrysotile and crocidolite on EMT in BEAS-2B cells

After a 72 h-incubation with 1 $\mu\text{g}/\text{cm}^2$ chrysotile, BEAS-2B cells acquired a spindle-shaped fibroblast-like morphology, similar to the one observed after stimulation with TGF- β , previously documented by Doerner et al. (Doerner and Zuraw 2009), and typical of EMT (Figure 1A). A 72 h-incubation of BEAS-2B cells with chrysotile significantly decreased the protein levels of E-cadherin and β -catenin, compared to untreated cells. In parallel, markers commonly associated with a mesenchymal phenotype, such as α -SMA, vimentin and fibronectin, were found to be significantly increased (Figure 1B). All the markers were analyzed also by qRT-PCR to investigate the changes in the gene expression: these experiments gave results similar to those observed with protein expression (Figure 1C). Since MMPs play a fundamental role in the extracellular matrix remodeling and are markers of EMT (Cannito et al. 2010; Kessenbrock et al. 2010), we investigated their secretion and activity. Cells exposed to chrysotile excreted more MMP-2 and MMP-9 than untreated cells (Figure 1B).

BEAS-2B cells were also incubated for 7 days with 5 $\mu\text{g}/\text{cm}^2$ crocidolite. At the end of the incubation, cells observed by optical microscopy had lost their organization, assuming a fibroblast-like appearance with pointed ends and elongated protrusions (Figure 2A). The expression of E-cadherin and β -catenin was significantly decreased in crocidolite-exposed cells, while the mesenchymal proteins (α -SMA and vimentin) were significantly over-expressed in the same experimental conditions, compared to untreated cells (Figure 2B-C).

Effect of chrysotile on EMT in NuLi and A549 cells

In addition, we performed some experiments in order to confirm the effect of chrysotile in human bronchial epithelial cell line (NuLi-1) and in a human lung adenocarcinoma cell line (A549). After chrysotile incubation, NuLi-1 and A549 cells were observed by optical microscopy, then the expression of epithelial and mesenchymal markers were evaluated by Western blotting. Asbestos-treated cells had lost their organization in compact islets, taking on a tapered and spindle shape with pointed ends and elongated protrusions, assuming a fibroblast-like appearance, whereas control cells showed their typical epithelial morphology. The Western blotting data revealed that the levels of the epithelial markers (E-cadherin and β -catenin) significantly decreased in cells treated with chrysotile, while the mesenchymal proteins (α -SMA and vimentin) were significantly over-expressed in the same experimental conditions, compared to untreated cells (see Supplemental Material, Figures S1-S2).

Effect of chrysotile on TGF- β secretion and ROS production in BEAS-2B cells

Since the previously shown markers (E-cadherin, β -catenin, α -SMA, vimentin, fibronectin and MMPs) are strongly associated with TGF- β -induced EMT (Cannito et al. 2010; Kessenbrock 2010; Kondo 2004), we investigated the possible involvement of TGF- β in the effects observed in our cellular model. TGF- β levels were assessed in the supernatants of BEAS-2B cells after 30 min, 1, 3, and 6 h of incubation with 1 $\mu\text{g}/\text{cm}^2$ chrysotile. TGF- β levels significantly increased after 1 h of incubation, then decreased to a level that was not significantly different from controls at 6 h (Figure 3A). When incubation was extended to 72 h, TGF- β levels were increased in both treated and untreated cells, but were significantly higher in treated cells (244.75 ± 11.75 pg/mg

proteins) compared with the control cells (164.77 ± 12.62 pg/mg proteins) ($p < 0.05$ for means \pm SEM from three independent experiments, see Figure 3A).

Chrysotile exposure has already been associated with the increased activation of TGF- β (Liu and Brody 2001; Manning et al. 2002), and ROS generated by the iron contained in asbestos can mediate the biological activity of TGF- β (Pociask et al. 2004). As shown in Supplemental Material (See Supplemental Material, Figure S3), we detected a significant production of ROS after 30 minutes of incubation of BEAS-2B cells with chrysotile: ROS remain high until 1 h of incubation and then slowly decrease to reach the control level after 6 h. These results are consistent with a mechanism whereby ROS cause the early activation of TGF- β .

A neutralizing anti-TGF- β antibody was used to confirm whether TGF- β was a mediator of the changes E-cadherin, β -catenin, α -SMA and vimentin protein levels. E-cadherin and β -catenin levels were significantly lower and α -SMA and vimentin significantly higher in the cells treated with chrysotile compared to untreated cells. The co-incubation of BEAS-2B cells with both chrysotile and the TGF- β blocking antibody partially restored the protein expression levels (Figure 3B-C). No change in the proteins expression was observed in BEAS-2B cells incubated with neutralizing anti-TGF- β antibody alone (see Supplemental Material, Figure S4).

Effect of chrysotile exposure on the Smad2-dependent and independent pathways

TGF- β is responsible for the activation of a canonical pathway mediated by the intracellular effectors Smad proteins (Xie et al. 2014). In order to investigate whether any change in the levels of Smad2 and phospho-Smad2 (p-Smad2) proteins occurred in our cellular model, we performed time-dependence experiments in which BEAS-2B cells were incubated with $1 \mu\text{g}/\text{cm}^2$ chrysotile

up to 6 h, and evaluated the expression of Smad2 and p-Smad2 in the cytosolic and nuclear fractions at each time point by Western blotting. As shown in Supplemental Material, Figure S5 (panel A), after 30 min of incubation with chrysotile the level of p-Smad2 decreased in the cytoplasm and contemporarily increased in the nuclei. The increase is evident and significant after 30 min and it is significant up to 6 h (Supplemental Material, Figure S5, panel B).

However, to improve our knowledge about the mechanisms leading to the down-regulation of E-cadherin, which is typical of EMT (Lamouille et al. 2014), we analyzed the involvement of the transcription factor SNAIL-1. SNAIL-1 subcellular localization and degradation are highly dependent on glycogen synthase kinase-3 β (GSK-3 β) (Zhou et al. 2004). Normally, GSK-3 β induces phosphorylation of nuclear SNAIL-1 mediating its nuclear export and subsequent cytosolic degradation; nevertheless phosphorylation and functional inactivation of GSK-3 β is crucial to stabilize SNAIL-1 into the nucleus, where it down-regulates the E-cadherin gene (Zhou et al. 2004).

In our experimental model, to assess the role of both GSK-3 β and SNAIL-1, we performed time-dependence experiments. A 6 h exposure to chrysotile resulted in GSK-3 β phosphorylation and SNAIL-1 accumulation in the nucleus (Figure 4A). To confirm the role of GSK-3 β in allowing the nuclear stabilization of SNAIL-1, we performed time-dependence experiments where the co-incubation of BEAS-2B cells with chrysotile and SB216763, a specific inhibitor of GSK-3 β (Coghlan et al. 2000), prevented the increase of SNAIL-1 in the nucleus (Figure 4B). Moreover, after a 72 h-incubation of BEAS-2B cells with both chrysotile and SB216763, the immunoblot analysis showed that, while chrysotile increased SNAIL-1 levels in the nucleus and decreased E-cadherin levels in the cytosol, the co-incubation with the GSK-3 β inhibitor partially reversed both

the nuclear increase of SNAIL-1 and the cytosolic decrease of E-cadherin induced by chrysotile, thus resulting in a statistically significant difference compared to both chrysotile and control levels (Figure 4C).

GSK-3 β is a ubiquitously expressed serine-threonine kinase involved in many different signaling pathways (Pap and Cooper 1998). Among these, we hypothesized that GSK-3 β phosphorylation could result from the activation of the Smad-independent pathway of TGF- β signaling that would involve Akt as an upstream kinase for GSK-3 β . The ratio between phosphorylated and non-phosphorylated Akt (p-Akt/Akt) was increased after 30 min-3 h of incubation of BEAS-2B cells with chrysotile, but was not significantly different from control levels at 6 h (Figure 5A). The involvement of Akt in this pathway was confirmed by incubating the BEAS-2B cells with chrysotile together with the specific Akt 1/2 Kinase Inhibitor. Results showed that the Akt inhibitor kept unaltered both phosphorylation levels of GSK-3 β and nuclear stabilization of SNAIL-1, compared to the control levels (Figure 5B). Furthermore, while chrysotile increased SNAIL-1 in the nucleus and decreased E-cadherin in the cytosol after a 72 h-incubation, the co-incubation with the specific Akt Inhibitor partially restored the control levels of both proteins (Figure 5C).

Discussion

The carcinogenicity of asbestos is well documented (IARC 2012) and consequently many countries all over the world have banned all forms of the asbestos commercially employed. However, at least 125 million workers worldwide are still exposed to asbestos fibers, among them 1.2 million are in the European Union (IARC 2012). Furthermore, its use continues in developing countries and exposure to asbestos in non-occupational settings and in the general

environment remains a serious health concern (Birnbaum et al. 2010). Moreover, it is also problematic that countries with growing economies, such as China, India, and Russia, are the largest consumers of asbestos (LaDou 2004). Furthermore, asbestos is widespread in the environment, where fibers occur in natural deposit and as contaminant of other minerals. Fibers can be released from the weathering of asbestos-bearing rocks, as well as through anthropogenic activities (IARC 2012).

Since all forms of asbestos (chrysotile, crocidolite, amosite, tremolite, actinolite, and anthophyllite) have been associated with an increased risk of lung cancer and mesothelioma (IARC 2012), it is estimated that asbestos-related pathologies will increase in the next years, both because of the long latency period between asbestos exposure and the onset of any related pathologies and because of the absence of restrictions in the use of asbestos in many countries of the World (Robinson 2012).

The present results represent the first steps in understanding some of the molecular mechanisms involved in EMT, a common event in all asbestos-related pathologies (Batra and Antony 2001; Guarino et al. 2009; Qi et al. 2013; Tamminen et al. 2012). Increasing the knowledge about the molecular mechanisms underlying such a complex scenario can be of great interest in order to discover crucial alterations in the cellular microenvironment that can be eventually used as biomarkers of risk for asbestos-associated pathologies.

Asbestos exposure has been associated with the development of fibrosis, lung tumors and MM (Hodgson and Darnton 2000). In all these pathological conditions EMT plays a fundamental role (Kalluri and Weinberg 2009). In many cellular models, TGF- β is known to be one of the main

EMT inducers and it has been already reported to mediate asbestos-induced fibrosis (Pociask et al. 2004; Sullivan et al. 2008). However, despite this important evidence, we are not aware of a clear mechanism connecting chrysotile and EMT induction in the current literature.

In the present study, we provide information about one of the possible mechanisms through which chrysotile triggers EMT in a lung epithelial cellular model. ROS play an important role in asbestos-mediated regulation of different signal transduction pathways (Kamp 2009; Mossman and Churg 1998; Shukla et al. 2003), and they can work as intracellular effectors, in part responsible for the molecular response to oxidative stress. Despite this, few studies have focused on the ability of asbestos to induce EMT through ROS-mediated mechanisms. In 2012, Tamminen et al. carried out a study on A549 cells using crocidolite (Tamminen et al. 2012) and suggested that crocidolite-mediated ROS production is able to induce EMT through a mechanism involving the MAPK/ERK signaling pathway, but not dependent on the activation of TGF- β signaling. In another work, Sullivan and coworkers (2008) proposed a model where asbestos elicits TNF- α expression. According to their findings, TNF- α controls fibrogenesis by regulating TGF- β expression and asbestos-induced ROS trigger lung fibrosis by activating latent TGF- β .

Moreover, Kim et al. analyzed the ROS-induced EMT in human malignant mesothelioma (HMM) cells and, based on their findings, they suggested that oxidative stress induced by H₂O₂ may play a critical role in HMM carcinogenesis, which would involve TGF- β , hypoxia inducible factor-1 α and some genes related to the capacity of cells to preserve their undifferentiated phenotype (stemness genes) (Kim et al. 2013; Potten and Loeffler 1990). Qi and coworkers observed that chrysotile has limited transforming potential *in vitro* compared with crocidolite, and stated that the morphological and molecular alterations induced by both crocidolite and

chrysotile are suggestive of EMT on human mesothelial cells. They also hypothesized that chrysotile induces transient effects because of its different biopersistence compared to that of crocidolite (Qi et al. 2013). Starting from these data, we asked whether chrysotile could be able to induce EMT and which mechanism could be involved in such a transformation.

In epithelial cells, E-cadherin is known to mediate cell-cell tight junctions that are stabilized by β -catenin (Stappert and Kemler 1994); the loss or downregulation of E-cadherin during EMT results in destabilization of the cadherin/catenin complex and disassembly of adherens junctions (Pectasides et al. 2014). In our cellular model, the expression levels of E-cadherin and β -catenin significantly decreased after chrysotile exposure, confirming the loss of epithelial characteristics of BEAS-2B. At the same time, mesenchymal proteins such as α -SMA and vimentin were found to be increased, suggesting the onset of cytoskeleton-related rearrangements typical of EMT (Lamouille et al. 2014). Furthermore, the increased deposition of fibronectin and the extrusion of both MMP-2 and MMP-9 in cells exposed to chrysotile suggest important changes in the surrounding microenvironment that make the extracellular matrix more suitable to be degraded and invaded (Peinado et al. 2003). We confirmed similar results in other cell lines (NuLi-1 and A549 cells), and such a cellular transformation was also observed when we exposed BEAS-2B cells to crocidolite, one of the most carcinogenic asbestos fiber (Takeuchi and Morimoto 1994). Indeed, cells lost their organization assuming a fibroblast-like appearance, confirming the loss of epithelial morphology and the gain of a mesenchymal phenotype.

As previously reported, chrysotile is known to induce TGF- β activation in a cell-free model (Pociask et al. 2004). In our cellular model, the ELISA assay on the cell supernatants confirmed a significant increase of TGF- β levels after 1 h of incubation with chrysotile, then diminished but

was still significantly increased after 3 h, and returned near to baseline levels at 6 h. Since BEAS-2B cells surviving chrysotile exposure for 72 h developed a morphology suggestive of EMT (spindle-shaped morphology and alterations in the epithelial and mesenchymal marker proteins) (Cannito et al. 2010), we measured the TGF- β levels also after 72 h incubation with chrysotile, i.e. after the time corresponding to the one we used to detect EMT markers. The TGF- β levels were significantly higher in treated cells compared to control cells.

Chrysotile exposure and ROS generated by the iron contained in asbestos have already been associated with the increased activation of TGF- β (Liu and Brody 2001; Manning et al. 2002; Pociask et al. 2004). ROS can activate the latent form of TGF- β (Pociask et al. 2004), so the early ROS production we revealed in BEAS-2B cells with chrysotile may be responsible for the first spike of TGF- β observed. TGF- β can also be activated by many different proteases, including MMPs (Yu and Stamenkovic 2000), whose secretion and activity were increased after chrysotile exposure. Therefore, it is reasonable to think that, since the bioavailability of active TGF- β ligand is greatly dependent on its activation, the alterations caused by chrysotile in the microenvironment of our cellular model (increased production of ROS and secretion of MMPs) could be responsible for the late activation of TGF- β we detected. Thus, we hypothesize that the initial increase in ROS following chrysotile exposure activates latent TGF- β , which in turn activates the Akt/GSK-3 β /SNAIL-1 signaling pathway, while the increase in TGF- β secretion 72 h after initial exposure may result from a continuous autocrine cell stimulation, and may reinforce signaling mechanisms involved in EMT promotion, as summarized in Figure 6. TGF- β involvement in our cellular model was confirmed by using an anti-TGF- β neutralizing antibody. The blocking antibody partially restored the expression levels of epithelial and mesenchymal

markers induced by chrysotile alone, thus suggesting an important role for TGF- β in the reported EMT-related morphological alterations.

Moreover, it is well known that TGF- β is responsible for the activation of a canonical pathway mediated by the intracellular effectors Smad proteins (Xie et al. 2014). According to the literature, once TGF- β binds its receptor TGF- β RI, the recruitment of phosphorylated Smad2 and Smad3 proteins occurs; then the phosphorylated Smad2/3 binds Smad4 to form a Smad heterocomplex to mediate signal transduction. TGF- β /Smad signaling pathway can induce or enhance EMT, invasion, and metastasis (Xie et al. 2014). Our findings support the involvement of the TGF- β -mediated Smad-dependent canonical pathway. However, to increase our knowledge about the molecular mechanisms involved in these TGF- β - and chrysotile-mediated modifications, we investigated the non-canonical Akt/GSK-3 β /SNAIL-1 pathway. For this reason, we referred to previous works in literature reporting TGF- β to be responsible for the up-regulation of the transcription factor SNAIL-1, that is in turn implicated in E-cadherin gene downregulation (Peinado et al. 2003; Zhou et al. 2004). SNAIL-1 persistence into the nucleus is required for the downregulation of E-cadherin gene (Zhou et al. 2004). We observed a time-dependent stabilization of SNAIL-1 into the nucleus of BEAS-2B cells incubated with chrysotile. This event may be result of the phosphorylation of GSK-3 β that is a crucial, and often central, component of many cellular functions, contributing to the regulation of apoptosis, cell cycle, cell polarity and migration, gene expression, and many other functions, including the response to inflammatory stimuli (Jope et al. 2007).

We detected an increased phosphorylation of GSK-3 β accompanied by the simultaneous increase of SNAIL-1 in the nucleus. The involvement of GSK-3 β in the regulation of SNAIL-1 was

confirmed by using SB216763, a specific inhibitor of GSK-3 β : in the presence of this compound, SNAIL-1 increase in the nucleus was partially prevented, and the E-cadherin was increased, but not “completely restored”.

The GSK-3 β phosphorylation is reported to be involved in many different intracellular pathways, including Wnt (Wu et al. 2012), lipid kinase phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt (Liang and Slingerland 2003), and ERK1/2 MAPK pathways (Kim et al. 2003). We investigated whether Akt could be involved as an upstream kinase for GSK-3 β : after chrysotile exposure the p-Akt/Akt ratio increased in a time-dependent manner reaching a peak after 1 h of incubation and then started to decrease after 3 h. To confirm the role of Akt in GSK-3 β phosphorylation and the subsequent steps of the pathway, we co-incubated BEAS-2B cells with chrysotile and a specific Akt inhibitor. After this co-incubation, levels of GSK-3 β phosphorylation and SNAIL-1 accumulation in the nucleus were comparable to controls. Furthermore, after a long-term (72 h) exposure, the Akt inhibitor partially blocked both SNAIL-1 accumulation in the nucleus and the E-cadherin decrease observed after exposure to chrysotile only.

To our knowledge, we are the first to report that chrysotile induces EMT in BEAS-2B cells through a molecular mechanism that seems to involve TGF- β and its intracellular effectors Akt/GSK-3 β /SNAIL-1. However, the non-total recovery of the investigated epithelial and mesenchymal markers and of the intracellular mediators can be explained considering TGF- β as just one of the possible mediators of the asbestos-induced EMT event in these cells.

Conclusions

Asbestos is a complex stimulus that elicits a variety of cellular responses through multiple molecular pathways. Thus, different multiple mechanisms may be activated as a consequence of asbestos exposure. Additional mechanisms may also be identified, but our findings suggest that chrysotile is able to trigger EMT in BEAS-2B cells, at least in part, through a molecular mechanism involving TGF- β and its intracellular effectors Akt/GSK-3 β /SNAIL-1. This work suggests just one of the possible molecular mechanisms supporting the morphological transformations typical of EMT involved in asbestos-related diseases, so it is conceivable that further mechanisms will be unveiled in the future.

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Figure Legends

Figure 1: Chrysotile asbestos effect on cell morphology and EMT marker protein expression levels in BEAS-2B cells. BEAS-2B cells were cultured for 72 h without (Ctrl) or with 1 $\mu\text{g}/\text{cm}^2$ chrysotile (Chry). (A) Representative microscope images are shown (10x; scale bar = 40 μm). (B) Expression of epithelial (E-cadherin, β -catenin) and mesenchymal (α -SMA, vimentin, fibronectin) markers checked by Western blotting and evaluation of MMP-2 and MMP-9 activity checked by zymography. Tubulin was used as loading control. The image is representative of three independent experiments giving similar results. Densitometry data are presented as the percent decrease or increase in the protein expression levels vs respective control. Significance vs respective control: * $p < 0.005$; ** $p < 0.001$; *** $p < 0.0001$. (C) Relative gene expression of the proteins E-cadherin, β -catenin, α -SMA, vimentin, fibronectin (FN) checked by qRT-PCR. Data are expressed in units of relative mRNA expression compared to control cells ($n = 3$). Significance vs respective control: * $p < 0.02$; ** $p < 0.001$; *** $p < 0.0001$.

Figure 2: Effect of crocidolite exposure on cell morphology and alterations in proteins involved in EMT in BEAS-2B cells. BEAS-2B cells were cultured for 7 days without (Ctrl) or with 5 $\mu\text{g}/\text{cm}^2$ crocidolite (CROC). (A) Representative microscope images are shown (10x; scale bar = 40 μm). (B-C) Expression of epithelial (E-cadherin, β -catenin) and mesenchymal (α -SMA, vimentin) markers checked by Western blotting. Tubulin was used as loading control. The image is representative of three independent experiments giving similar results. Densitometry data are presented as the percent decrease or increase in the protein expression levels vs respective control. Significance vs respective control: * $p < 0.0001$.

Figure 3: TGF- β secretion and neutralizing TGF- β antibody effect in BEAS-2B cells exposed to chrysotile. (A) BEAS-2B cells were incubated in the absence (black squares) or presence (white squares) of 1 $\mu\text{g}/\text{cm}^2$ chrysotile for 30 min, 1, 3, 6 and 72 h. At the end of the incubation, supernatants were collected and TGF- β levels were detected through an ELISA kit. Data are shown as mean \pm SEM (n = 3). TGF- β levels are reported as pg/mg of intracellular proteins. Significance vs respective control: *p<0.05; **p<0.02. (B-C) BEAS-2B cells were incubated without (Ctrl) or with 1 $\mu\text{g}/\text{cm}^2$ chrysotile (Chry) or with chrysotile and 5 ng/ml of neutralizing anti-TGF- β antibody for 72 h (Chry + Ab). Expression of epithelial (E-cadherin, β -catenin) and mesenchymal (α -SMA, vimentin) markers was checked by Western blotting. Tubulin was used as loading control. The image is representative of three independent experiments. Densitometry data are presented as the percent decrease or increase vs control cells. Significance vs respective control: *p<0.005; **p<0.001; ***p<0.0001. Significance vs chrysotile: $^{\circ}$ p<0.005; $^{\circ\circ}$ p<0.001; $^{\circ\circ\circ}$ p<0.0001. No statistical difference has been detected for cells co-incubated with chrysotile and TGF- β antibody compared to control cells.

Figure 4: Evaluation of the role of GSK-3 β /SNAIL-1 pathway in E-cadherin gene modulation in BEAS-2B cells. The images are representative of three independent experiments giving similar results. (A) BEAS-2B cells were incubated in the absence (Ctrl, 6 h) or presence of 1 $\mu\text{g}/\text{cm}^2$ chrysotile for 30 min, 1, 3 and 6 h (top panel). The expression of phosphorylated GSK-3 β (p-GSK-3 β) and the accumulation of SNAIL-1 in the nuclei of BEAS-2B cells were examined by Western blotting. P-GSK-3 β to GSK-3 β ratio values are provided (bottom panel, left). Densitometry data concerning SNAIL-1 accumulation in the nuclei are presented as the percent decrease or increase in the protein expression levels vs

control (Ctrl, 6h) (bottom panel, right). Significance *vs* control (Ctrl, 6h): * $p < 0.0001$. **(B)** BEAS-2B cells were treated without (Ctrl, 6 h) or with chrysotile (Chry, $1 \mu\text{g}/\text{cm}^2$) together with the specific GSK-3 β inhibitor SB216763 ($5 \mu\text{M}$) for 30 min, 1, 3 and 6 h. Tubulin and TBP were used as control loading for the cytosol and the nucleus, respectively. **(C)** BEAS-2B cells were treated for 72 h without (Ctrl) or with chrysotile ($1 \mu\text{g}/\text{cm}^2$) both in the absence (Chry) or presence (Chry + SB) of SB216763 ($5 \mu\text{M}$) (left panel). Experiments were performed in triplicate and densitometry data are presented as the percent decrease or increase in the protein expression levels *vs* respective control (right panel). Significance *vs* respective control: * $p < 0.005$; ** $p < 0.0001$. Significance *vs* chrysotile: $^{\circ}p < 0.001$.

Figure 5: Evaluation of Akt involvement in GSK-3 β regulation in BEAS-2B cells. The images are representative of three independent experiments giving similar results. **(A)** BEAS-2B cells were incubated in the absence (Ctrl, 6 h) or presence of chrysotile ($1 \mu\text{g}/\text{cm}^2$) from 30 min up to 6 hours (left panel). Differences in the p-Akt (phosphorylated Akt) to Akt ratio are presented in the right panel. Significance *vs* control: * $p < 0.0001$. **(B)** BEAS-2B cells were treated without (Ctrl) or with chrysotile (Chry, $1 \mu\text{g}/\text{cm}^2$) together with the Akt inhibitor ($5 \mu\text{M}$) from 30 min up to 6 h. p-GSK-3 β = phosphorylated GSK-3 β . Tubulin and TBP were used as control loading. Differences in the p-GSK-3 β to GSK-3 β ratio and densitometry data as percent increase or decrease in SNAIL-1 expression levels are presented in the right panel. **(C)** BEAS-2B cells were treated for 72 h without (Ctrl) or with chrysotile alone (Chry, $1 \mu\text{g}/\text{cm}^2$) or together with $5 \mu\text{M}$ Akt inhibitor (left panel). Tubulin and TBP were used as control loading. Densitometry data are presented as the percent decrease or increase in the protein expression levels *vs* respective control (right panel). Concerning SNAIL-1 densitometry data, no statistical difference has been detected for cells co-incubated with

chrysotile and Akt Inhibitor compared to control cells. Significance vs respective control: * $p < 0.0001$. Significance vs chrysotile: ° $p < 0.0001$.

Figure 6: Selected mechanisms potentially involved in TGF- β mediated EMT. (A) Normally, GSK-3 β -mediated phosphorylation of nuclear SNAIL-1 allows its nuclear export and subsequent cytosolic degradation (Zhou et al. 2004). **(B)** In our study on BEAS-2B cells, chrysotile exposure induced early production of ROS (1) which we hypothesize will subsequently activate the latent form of TGF- β (2). Upon release, TGF- β is stabilized and directly presented to its receptors, which then associate and activate a variety of signaling pathways. Both Smad-mediated (3) and non-Smad-mediated (6) pathways are involved (Peinado et al. 2003; Xie et al. 2014). Increased levels of TGF- β result in the activation of Smad-mediated pathway, suppression of epithelial genes (e.g., E-cadherin) as well as induction of EMT mesenchymal markers (4), through activation/induction of and co-association with a variety of transcription factors (including SNAIL-1) (5). We hypothesize that the non-Smad-mediated pathway leads to Akt activation (6) and GSK-3 β inactivation (7), induction of SNAIL-1 nuclear stabilization and genes target promotion (8), thus contributing to EMT. In addition, we hypothesize that MMPs secreted by cells (9) may promote the late activation of TGF- β (10), resulting in a potentially continuous autocrine cell stimulation that may further reinforce signaling for EMT promotion (11).

Figure 1.

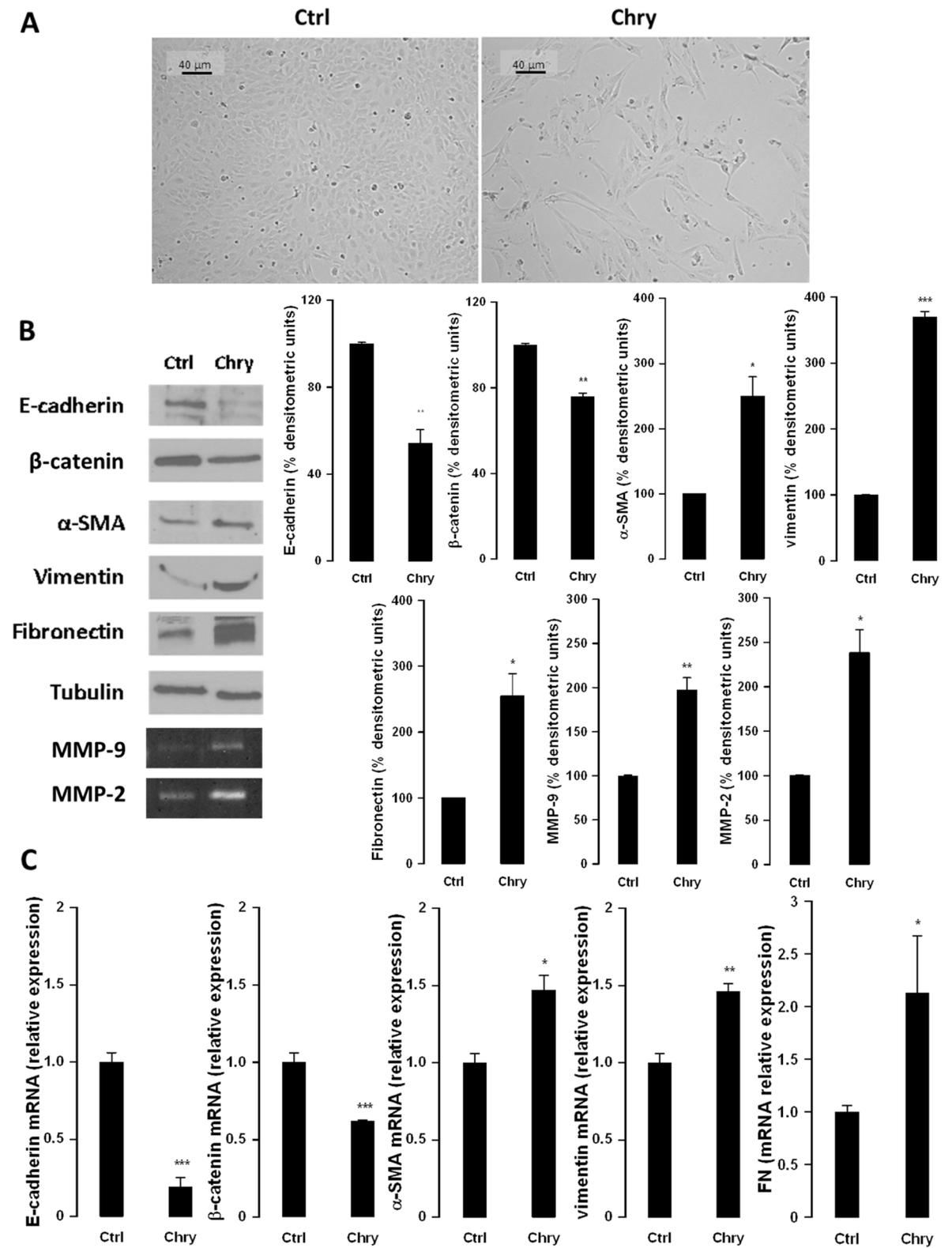


Figure 2.

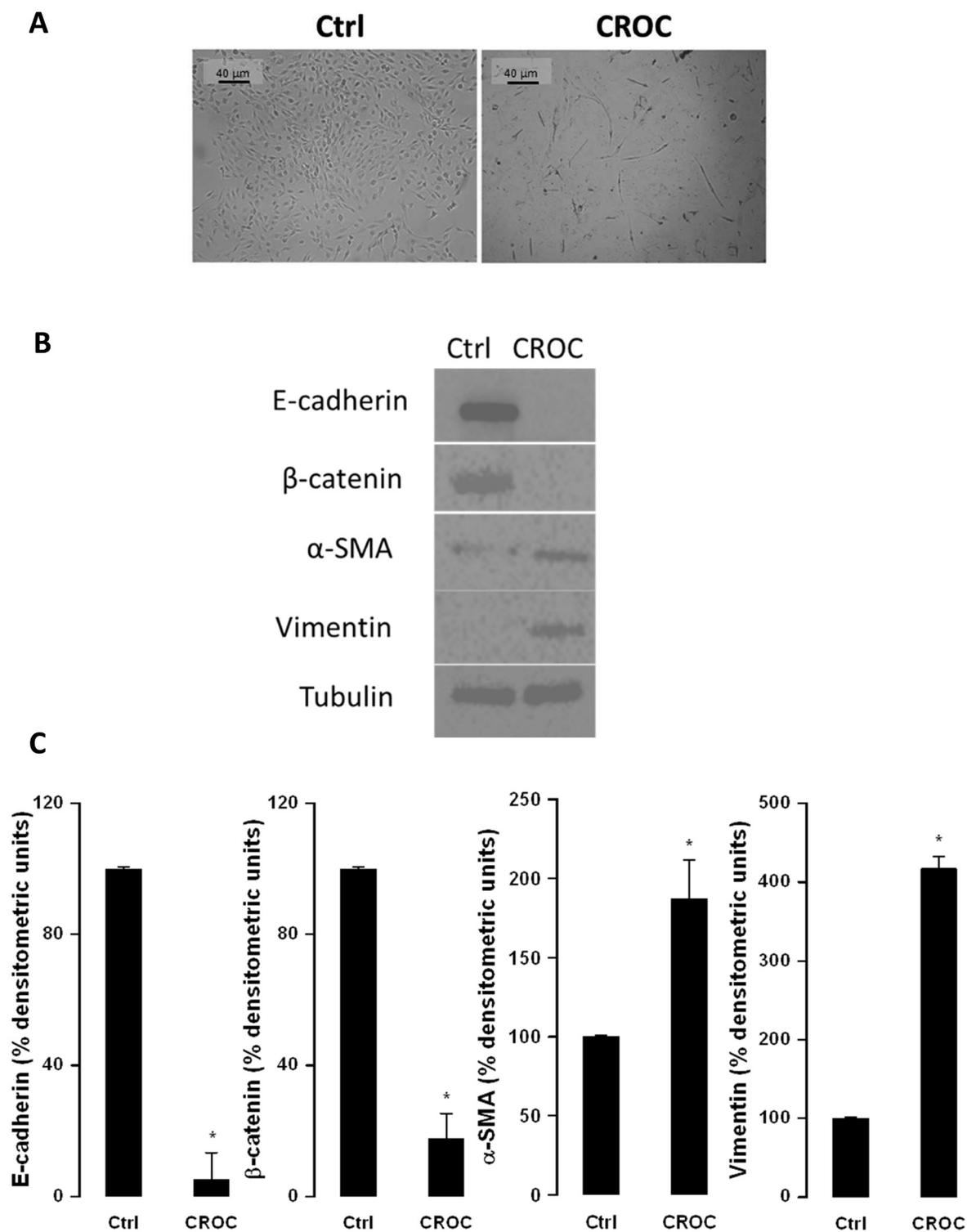


Figure 3.

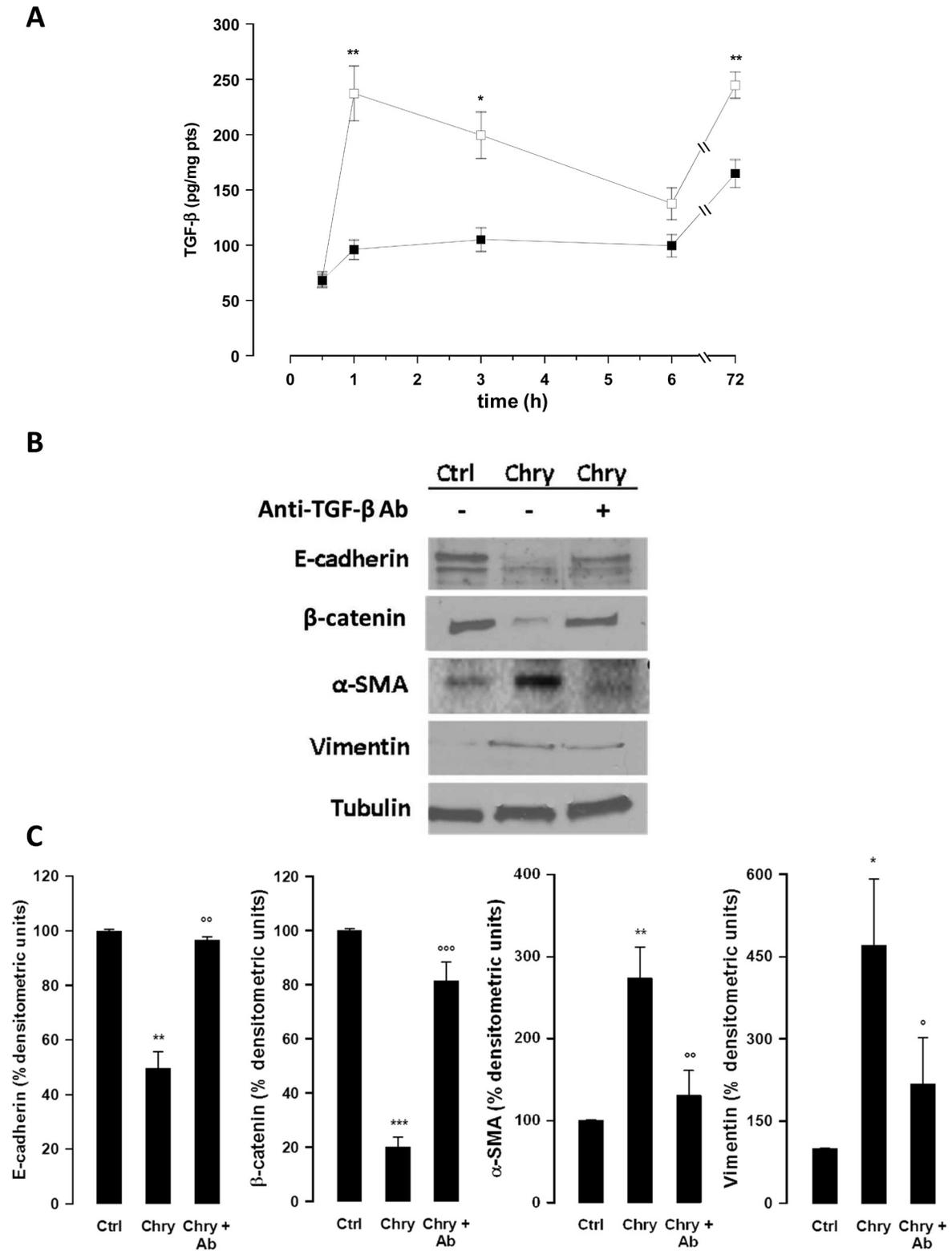


Figure 4.

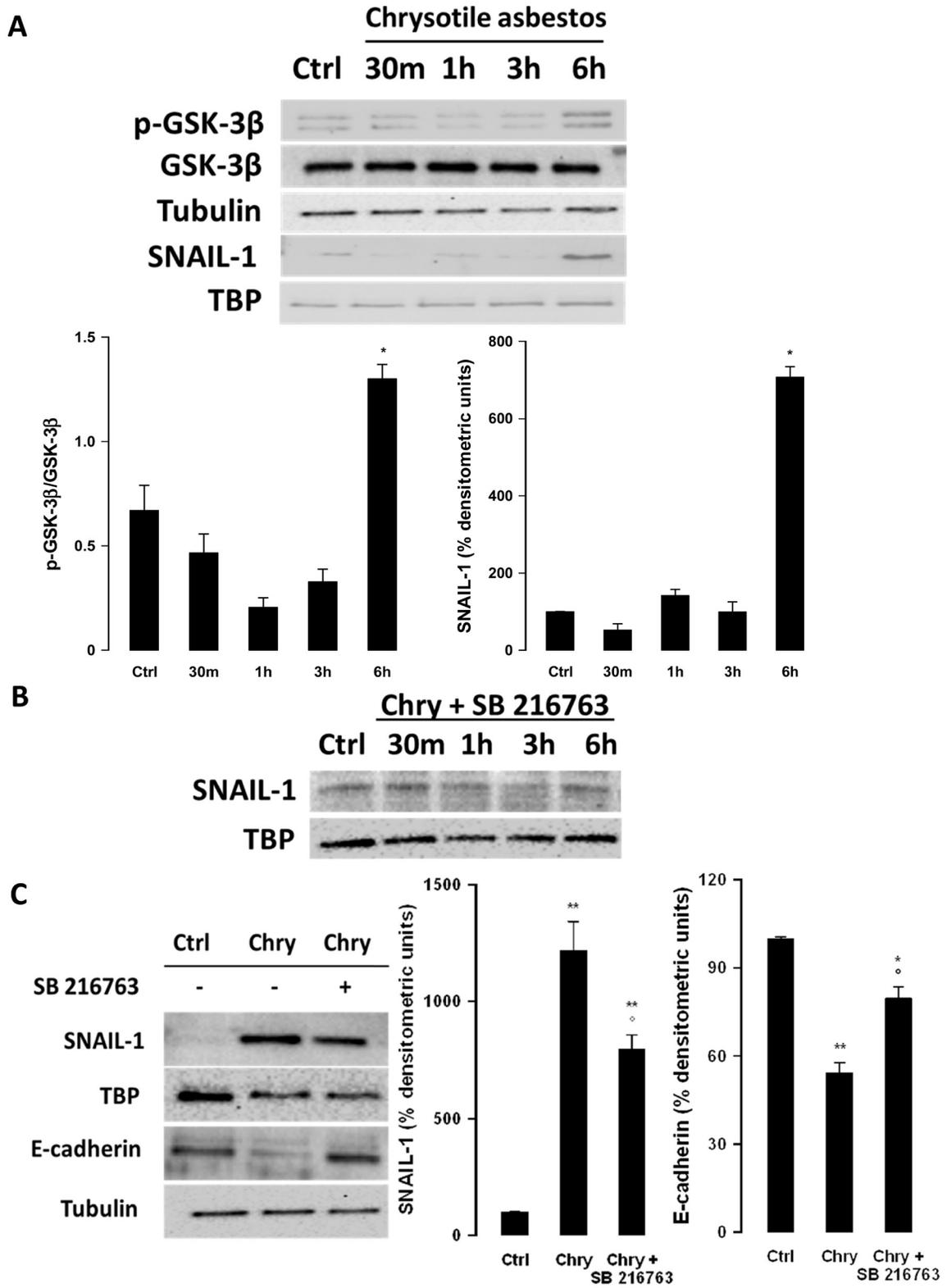


Figure 5.

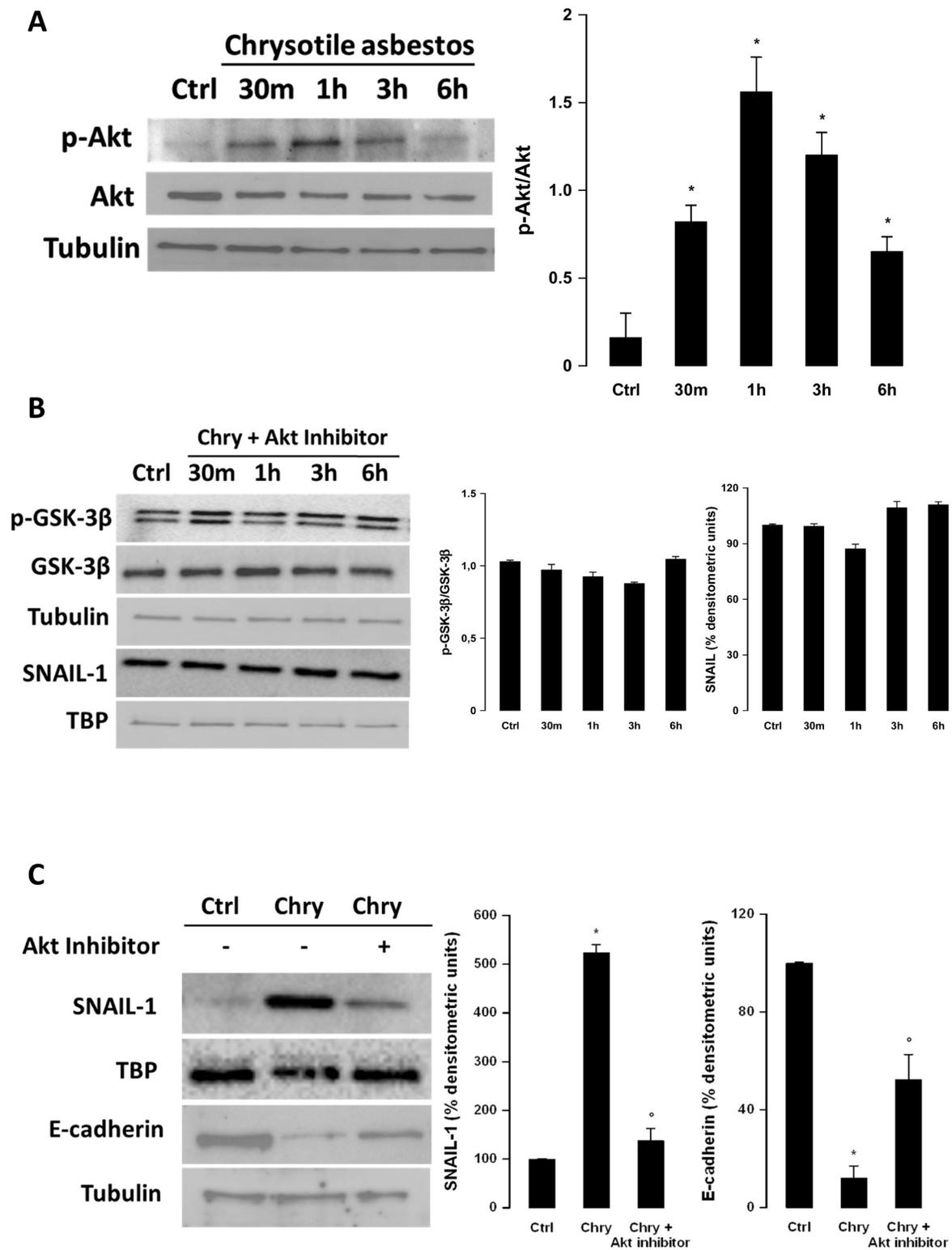


Figure 6.

