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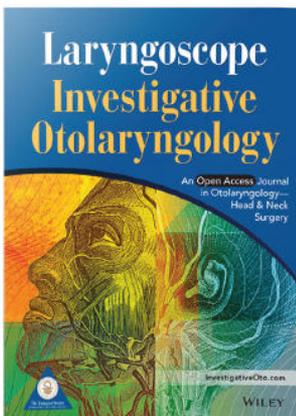


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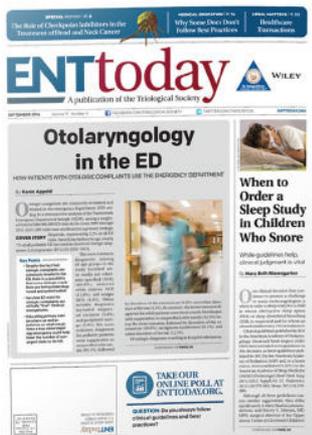
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Antifibrotic Effect of Mitomycin-C on Human Vocal Cord Fibroblasts

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Objective: Acquired laryngotracheal stenosis is a potentially life-threatening situation and a very difficult and challenging problem in laryngology. Therefore, new trends and innovative approaches based on antifibrotic drugs and minimally invasive regimens are being developed to attenuate laryngotracheal fibrosis and scarring. The purpose of this study was to examine the efficacy of mitomycin-C (MMC) to reverse the transforming growth factor (TGF)- β -induced differentiation of MRC-5 fibroblast and human primary vocal cord fibroblasts to reveal the possible applicability of MMC to laryngotracheal fibrotic conditions.

Methods: Human primary fibroblast cells were isolated from vocal cord specimens of patients undergoing total laryngectomy. The established primary vocal cord fibroblast cell cultures as well as the MRC-5 human fibroblast cells were treated with 5 ng/mL TGF- β alone and then with 0.5 μ g/mL MMC for 24 hours. Differentiation of fibroblasts was characterized by α -smooth muscle actin (α -SMA) immunohistochemistry, Western blot analysis, and real-time polymerase chain reaction. Cell motility was assessed by wound-healing assay.

Results: Elevated α -SMA mRNA and protein expression as well as increased cell motility were observed upon TGF- β exposures. However, after MMC treatments the TGF- β -induced fibroblasts exhibited a significant decrease in α -SMA expression and wound-healing activity. Therefore, TGF- β -stimulated fibroblast-myofibroblast transformation was reversed at least in part by MMC treatment. Histopathological examinations of tissue specimens of a laryngotracheal stenosis patient supported these findings.

Conclusion: Antifibrotic effects of MMC were demonstrated on the human MRC-5 cell line and on primary vocal cord fibroblast cultures. These results verify that MMC can be used with success to reverse upper airway stenosis by reverting the myofibroblast phenotype.

Key Words: Laryngotracheal stenosis, antifibrotic effects, mitomycin-C, primary vocal cord fibroblast, α -smooth muscle actin.

Level of Evidence: NA

Laryngoscope, 00:1-8, 2019

INTRODUCTION

In response to injury, inflammation, exposure to toxic substances, or physical trauma, tissues initiate healing processes; however, fibrotic diseases may develop if these reparative mechanisms are misregulated.^{1,2} Pathophysiological progression of fibrotic conditions eventually lead to organ failure; thus, fibrosis can be accounted for millions of deaths worldwide. Generally, fibrotic diseases are characterized by excessive differentiation of fibroblasts to myofibroblasts, resulting in a cell type capable of contraction due to elevated alpha-smooth muscle actin

(α -SMA) expression, as well as of accumulating extracellular matrix components following increased synthesis in the affected tissues. Although fibrosis can occur in various organs, and the underlying etiologies might be substantially different, mechanistically the pleiotropic transforming growth factor β (TGF- β) is the most frequent trigger for fibroblast differentiation in such conditions.

Based on own observations, although the highest incidence of fibrotic disease affects lungs, kidneys, and the liver, the frequency of fibrosis-related disorders in other organs such as the vocal cords and trachea has been progressively and alarmingly increased in recent years. Such an example is iatrogenic laryngotracheal stenosis (LTS), which is a serious end result of uncontrolled tracheal fibrotic processes.³ Previous investigations in our clinic revealed that the pathogenesis of LTS is multifactorial⁴; however, the most common cause is mechanical trauma derived from endotracheal intubation. Depending on the exact anatomical region, a sustained compression of the tracheal tube's cuff on the tracheal wall may cause blood circulation failure, inflammation, scarring in soft tissue, and even cartilage damage. Although minimally invasive treatment modalities have been implemented to manage LTS in most cases,⁵⁻⁷ surgical resection, tracheal

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Editor's Note: This Manuscript was accepted for publication on October 10, 2018.

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This study received support from the National Research, Development and Innovation Office-NKFIH (GINOP-2.3.2-15-2016-00040).

The authors have no funding, financial relationships, or conflicts of interest to disclose.

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DOI: 10.1002/lary.27657

reconstructions, and end-to-end anastomosis must be applied as a definitive treatment even if these have moderate efficiency due to high risk of granulation tissue formation and restenosis.^{8,9} Considering several factors such as high risk of surgery, postoperative complications, extended hospitalization period after surgery, and substantial costs, new trends and innovative approaches of LTS treatment are mandatory.

For modulation of wound-healing processes and for attenuating scarring, one of the possible solutions described in the scientific literature is the application of antifibrotic drugs on primary lesions. For this purpose, various antifibrotic pharmaceutical agents such as corticosteroids,¹⁰ 5-fluorouracil,¹¹ carnitine, and mitomycin-C (MMC)^{12,13} have already been employed either alone in short-term local surface applications or as at our clinic in combination with preceding laser excision of cicatrice tissue in laryngotracheal stenosis treatment. Although MMC is a traditional chemotherapeutic drug, isolated from *Streptomyces sp.*, it has also been regarded as a compound with high potential to prevent tissue fibrosis. The antifibrotic activity of locally applied MMC is presumably due to its inhibiting effects on fibroblast proliferation¹⁴⁻¹⁶; however, clinical and experimental data considering its application in fibrosis and LTS treatment are still elusive.^{17,18} Although some clinical observations suggest the possible applicability of MMC for laryngotracheal fibrotic conditions, its efficacy on LTS requires more mechanistic and detailed investigations. Thus, using the human MRC-5 fibroblasts cell line and patient-derived primary laryngotracheal fibroblasts, we established TGF- β -induced fibrosis models to investigate the antifibrotic potential of MMC.¹⁹⁻²¹

MATERIALS AND METHODS

Culturing MRC-5 Fibroblasts

The MRC-5 human fibroblast cell line (Sigma-Aldrich, St. Louis, MO) was cultured in Eagle's Minimum Essential Medium (EMEM) complemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.001% streptomycin, and 0.005% penicillin under standard conditions.

Isolation and Culturing of Human Primary Fibroblasts

Human primary fibroblast cells were isolated from vocal cord specimens of human patients undergoing total laryngectomy due to laryngeal cancer. The larynx was removed, and the healthy part of the vocal cord was resected and placed in phosphate-buffered saline immediately. The tissue sample contained epithelium, lamina propria, and muscle tissue. The resected vocal cord was cut into 1 × 1 mm pieces, which were then placed into precoated T25 flasks in culture medium (EMEM supplemented with 10% FBS, 100 U/mL penicillin, 0.01 mg/mL streptomycin, 2 mM L-glutamine) and were kept at 37°C in 5% CO₂ atmosphere and 95% humidity. When fibroblast cultures started to grow, the medium was frequently changed. When 80% of the surface was covered with fibroblasts, the tissue pieces were removed, the cells were allowed to reach confluence, and then cells were trypsinized and passaged to new flasks. Cultured fibroblast was used between 4 to 8 passages.

Immunocytochemistry

To verify that the obtained patient-derived cells were fibroblasts, cells were stained for α -SMA and vimentin. For this, cells were grown on coverslips and fixed by 4% formaldehyde (PFA) solution. Following permeabilization with 0.3% Triton-X-100 (Sigma) solution, the samples were blocked in 5% bovine serum albumin (BSA) (Sigma) and stained with α -SMA- and vimentin-specific primary antibodies (Abcam) overnight. On the following day, fluorophore-conjugated secondary antibodies (Abcam) were applied and cell nuclei were counterstained with DAPI (Sigma). Samples were visualized by an Olympus BX51 fluorescent microscope equipped with Olympus DP70 camera (Tokyo, Japan).

Fibroblast Differentiation and Mitomycin-C Treatment

MRC-5 and primary vocal cord fibroblast cells were treated with 5 ng/mL TGF- β (Abcam) diluted in 2% serum-containing culture medium for 48 hours. After 24 hours, MMC was added to the TGF- β -containing medium in 0.5 μ g/mL concentration (determined by viability measurements), and cells were incubated for an additional 24 hours (Barcelona, Spain).

Measurement of Cytotoxicity, Proliferation, and Apoptosis

Impedance-based dynamic monitoring of living adherent cells was carried out with xCELLigence RTCA System (Roche Applied Science, Mannheim, Germany). 5,000 cells/well were loaded onto special 96-well E-plates with built-in gold electrodes and were covered with culture medium. After cells reached steady growth, they were treated with various concentrations (0.1, 0.3, 0.5, 1, 3, 5 μ g/mL) of MMC. To assess the toxicity of MMC (Inibsa) real-time, the impedance of the cells was registered every 10 minutes, and for each time point a cell index was calculated based on the measured values in the presence and in absence of cells. Based on these measurements, a nontoxic concentration for MMC could be determined, which was used throughout further cell treatments.

To detect cell proliferation, MRC-5 cells were seeded to 24-well plates and treated with 5 ng/mL TGF- β and/or MMC diluted in 2% serum containing medium. Cells were collected each day by trypsinization, and the number of cells/well was determined using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA). For cytotoxicity measurements, MRC-5 cells were seeded into 96-well plates in 5,000 cells/well density. On the following day, cells were treated with 5 ng/mL TGF- β diluted in 2% serum containing medium for 48 hours. After 48 hours, MMC was added to the TGF- β -containing medium, and cells were treated for further 48 hours. At the end of the treatment, cell viability was determined using CellTiter Aqueous One cell proliferation assay (Promega, Madison, WI), and absorbance was determined in a Synergy HTX multimode reader. Upon calculating cell viability, values corresponding to nonstimulated cells were considered 100%. For apoptosis detection, MRC-5 cells were seeded into black-wall 96-well plates (Thermo), and the same experimental protocol was used as described above. As a readout, Apo-ONE homogeneous Caspase-3/7 Assay (Promega) was applied using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany).

Western Blot

To analyse α -SMA protein levels, Western blot analysis was performed. MRC-5 and primary vocal cord fibroblast cells were grown in T25 culture flasks. Following treatments with either

TGF- β or MMC or both, cells were scraped, collected by centrifugation, and lysed in Radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Sodium dodecyl sulfate (SDS), 1% Triton-X-100). Protein concentrations were determined using the BCA method, and then 25 μ g total protein from each sample was resolved on 8% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham). The membranes were blocked with 5% nonfat dry milk-TBST solution, and then α -SMA-specific primary antibody (Abcam) was applied overnight in 1:2000 dilution and Tubulin-specific antibody (Sigma, 1:2000) was hybridized to the membranes to ensure equal loading. HRP-conjugated secondary antibodies (Dako) were used, and then membranes were developed using Immobilon Western HRP substrate (Millipore). Chemiluminescent signal was detected in a Li-Cor C-Digit blot scanner (Li-Cor Biotech., Cambridge, U.K.).

Scratch Assay

To measure the migration activity of MRC-5 and primary vocal cord fibroblasts, cells were left to grow in 6-well plates until they reached confluence. Next, cell layers were then treated with 5 ng/mL TGF- β for 24 hours, and then wounds were created using a P200 pipette tip. Cell-free zones were photographed with a phase-contrast microscope, and then 0.5 μ g/mL MMC was added to the cells. After 24-hour treatment, samples were photographed again, and the number of migrating cells were counted using ImageJ1.44 software.

qPCR

For real-time quantitative polymerase chain reaction (RT-qPCR) analysis, 6×10^5 cells were seeded into 6-cm diameter culture dishes. On the following day, cells were treated with TGF- β , and then 24 hours later MMC was added to the culture for an additional 24 hours of treatment. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the guidelines of the manufacturer. Taqman Reverse Transcription Reagent (Applied Biosystems, Foster City, CA) was applied to generate cDNA using 1 μ g of total RNA, and relative levels of TGF- β , COL1A1, PAI-1, and CTGF transcripts were quantified in a Pico Real-Time qPCR System (Thermo Scientific) using gene-specific primers (Table I) and SYBR Green PCR Master Mix (Applied Biosystems). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific messengers was measured to calculate relative amount of each transcript using $\Delta\Delta$ Ct analysis. RT-qPCR measurements were carried out in triplicates.

Immunohistochemistry of Patient Samples

Samples were obtained from a patient undergoing laser excision of the posterior glottis stenosis and receiving topical

MMC treatment 1 week following scar excision. Tissue samples were fixed in 10% formaldehyde and 4- μ m thick paraffin sections were prepared. The specimens were incubated with α -SMA-specific primary antibody (Cellmarque Corp., Rocklin, CA) in 1:300 dilution at pH 9. Throughout the staining, the EnVision FLEX kit on Dako Autostainer Plus (Dako) protocol was used. α -SMA-positive cells were counted by ImageJ1.44 software with a CellCounter plugin on four independent ImageJ/sample.

Statistical Analysis

Statistical analysis and graphical visualization of the obtained data were carried out in a GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA) using *t* test and two-way analysis of variance (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ and # $P = 0.0006$ indicate statistical significance).

RESULTS

MMC Hampers TGF- β -Triggered MRC-5 Fibroblast Transformation

To test the antifibrotic potential of MMC, we first evaluated its cytotoxicity on MRC-5 human fibroblast cells. These cells represent a generally accepted and widely utilized in vitro model system for studying cellular and molecular mechanisms in the background of fibrotic diseases. MRC-5 cells received increasing concentrations of MMC, and the dose response of the treatments was obtained by real-time cell electronic sensing. Upon MMC exposure, fibroblast cells showed gradually decreasing cell indices suggesting a dose-dependent antiproliferative effect of MMC (Fig. 1A). Differences in the kinetic curves indicated that 5 μ g/mL MMC exhibited serious toxicity throughout the examined time period; however, MMC concentrations between the 0.1 and 1 μ g/mL range proved to be sublethal.

To test that MMC also exhibits similar effects on TGF- β -transformed fibrotic cells, MRC-5 cells were treated with either TGF- β or with MMC (in 0.5 or 5 μ g/mL concentrations) or with TGF- β +MMC combinations, and cell proliferation was monitored for 5 days (Fig. 1B). TGF- β -stimulated fibroblasts showed elevated proliferative activity compared to nonstimulated cells because the TGF- β -exposed cells reached the plateau phase already after 72 hours, whereas control fibroblasts accomplished this cell number only after 120 hours. Similar to what we observed earlier, 0.5 μ g/mL MMC exhibited cytostatic effect, whereas treatments with 5 μ g/mL MMC proved to be cytotoxic on nonstimulated

TABLE I.
Primer Sequences.

Target	Forward Primer	Reverse Primer
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGA-3'
TGF- β	5'-AGCAACAATTCCTGGCGATAC-3'	5'-CTAAGGCCGAAAGCCCTCAAT-3'
COL1A1	5'-CATCTGGTGGTGAGACTTGC-3'	5'-TCCTGGTTTCTCCTTTGG-3'
PAI-1	5'-AGTGGACTTTTCAGAGGTGGAG-3'	5'-GCCGTTGAAGTAGAGGGCATT-3'
CTGF	5'-GGAAATGCTGCGAGGAGTGG-3'	5'-GGCTCTAATCATAGTTGGTCTGG-3'

CTGF = connective tissue growth factor; COL1A1 = collagen alpha-1(1); GAPDH = glyceraldehyde 3-phosphate dehydrogenase; PAI-1 = plasminogen activator inhibitor-1; TGF = transforming growth factor.

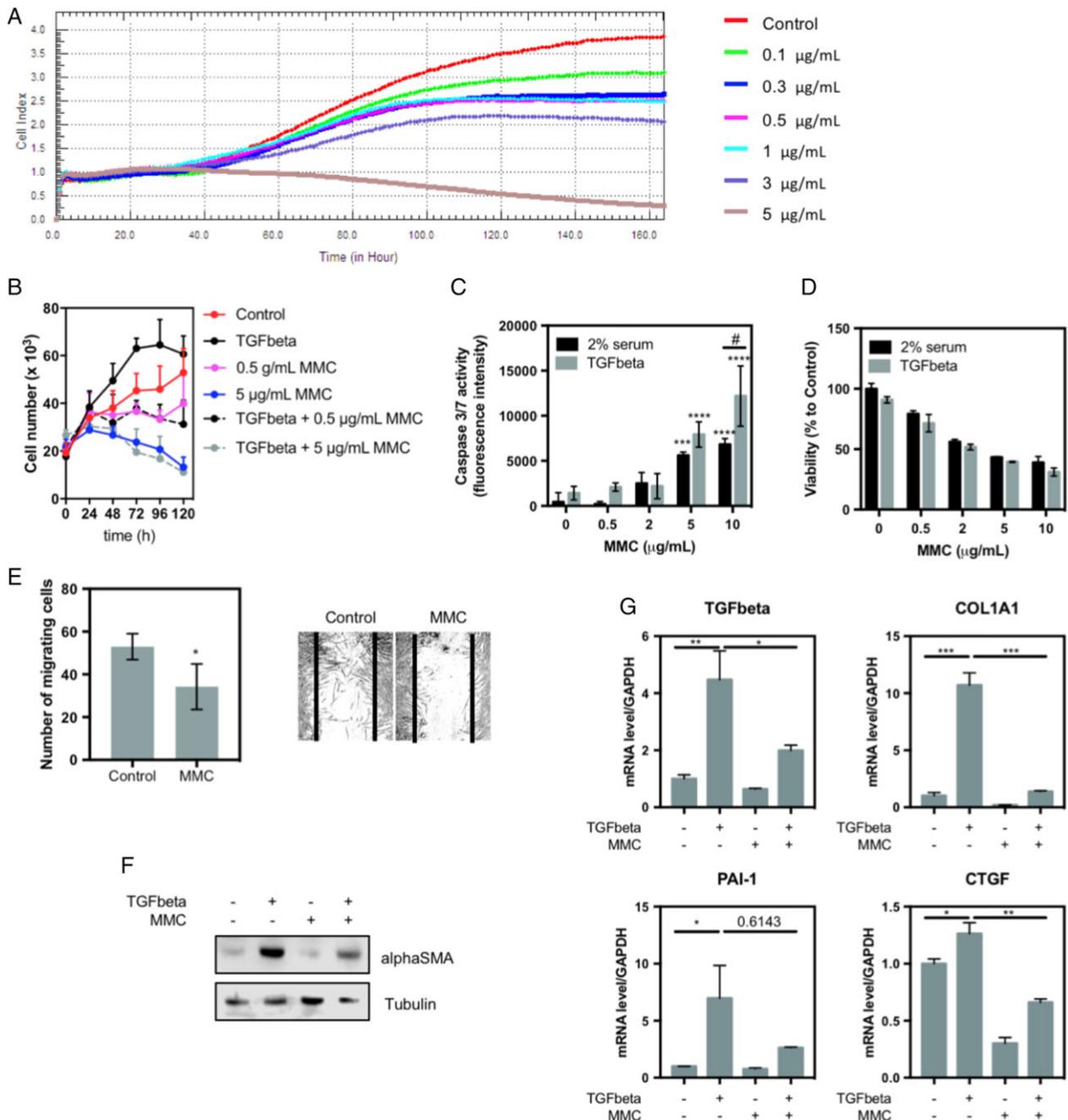


Fig. 1. (A–G) Dose response of MMC treatment (A) and effect of MMC on TGF- β -triggered MRC-5 fibroblast transformation, assessed by proliferation (B), Caspase 3/7 activity (C), viability (D), wound-healing assay (E), Western blot (F), and quantitative polymerase chain reaction (G). MMC = mitomycin-C; SMA = smooth muscle actin; TGF = transforming growth factor.

MRC-5 cells. Most importantly, MMC treatments exerted comparable effects on TGF- β -transformed cells as on nonstimulated cells, proving the antiproliferative activity of MMC on transformed fibroblasts as well. To compare the apoptotic and cytotoxic activity of MMC on fibrotic cells, nonstimulated and TGF- β -transformed cells were treated with MMC in various concentrations for 48 hours, and then Caspase 3/7 activity measurement (Fig. 1C) or cell viability assay (Fig. 1D) was performed. MMC treatments decreased cell viability in a dose-dependent manner both in naïve and TGF- β -transformed fibroblasts. Five and 10 $\mu\text{g/mL}$ MMC

induced significant induction in Caspase 3/7 activity. Moreover, treatments with 10 $\mu\text{g/mL}$ MMC triggered Caspase 3/7 activity with a significantly higher degree in transformed fibroblasts than in the nonstimulated counterparts.

Because elevated cell migration during cicatrix formation is one of the characteristic hallmarks of fibrotic cells, we tested the migration capacity of scratch-activated MRC-5 fibroblasts upon MMC treatment. For this, MRC-5 cells were cultured until they reached confluence, and then wounds were scratched into the cell layers using sterile pipette tips. After scratching, cells were

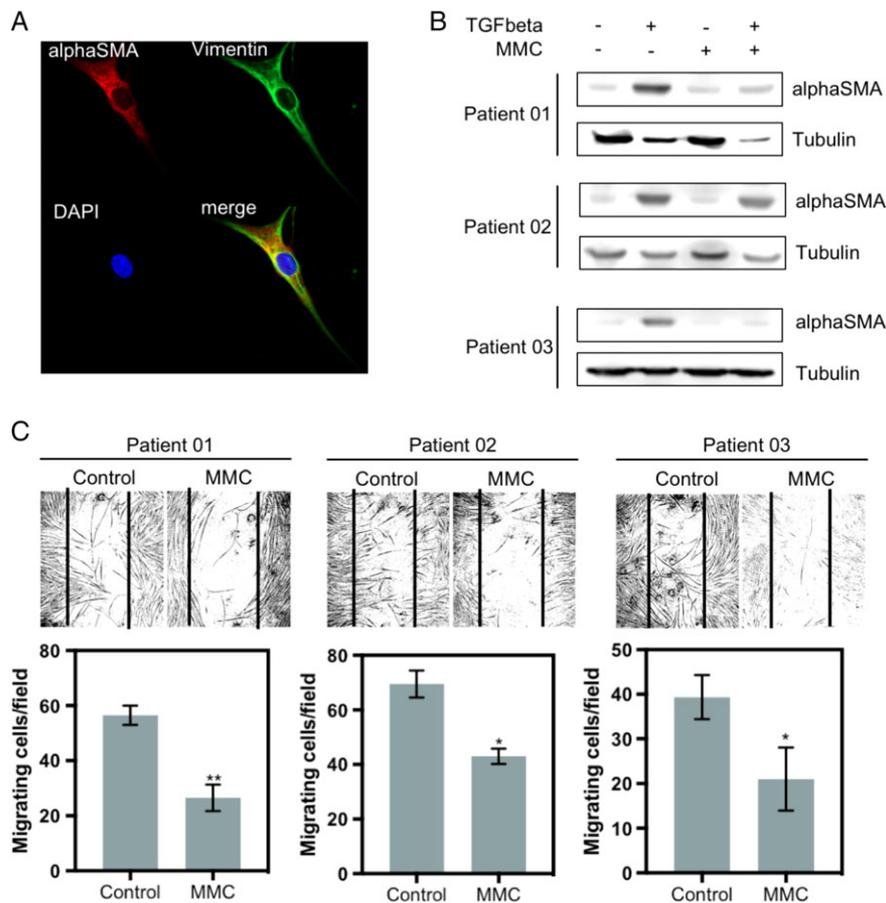


Fig. 2. (A–C) Verification of patient-derived fibroblast cells by immunocytochemistry (A). Effect of MMC on TGF- β -triggered patient-derived primary vocal cord fibroblasts transformation by Western blot (B) and wound-healing assay (C). MMC = mitomycin-C; SMA = smooth muscle actin; TGF = transforming growth factor.

incubated for 24 hours either in the presence or in the absence of 0.5 $\mu\text{g/mL}$ MMC. Cell-free zones were photographed, and then the number of cells that had migrated into the cell-free zones was determined using software-based ImageJ1 analysis. We found that MMC treatment significantly decreased the scratch-triggered migration of MRC-5 fibroblast cells (Fig. 1E).

Because elevated TGF- β secretion is responsible for maintaining fibroblasts in the activated phenotype during the development of fibrotic diseases,²² we tested whether MMC treatment can influence the TGF- β -provoked fibroblast-to-myofibroblast transformation. For this, serum-starved MRC-5 cells were treated with TGF- β in 0.5 ng/mL concentration for 24 hours, and then MMC was added to the culture and the cells were incubated for an additional 24 hours. To detect fibroblast transformation, α -SMA protein expression—a well-recognized marker of fibrotic fibroblasts—was monitored by Western blotting. As expected, TGF- β alone induced substantial fibroblast transformation because we observed highly elevated α -SMA expression in these cells compared to untreated fibroblasts. Although MMC alone did not influence α -SMA expression in nonstimulated fibroblasts, we found that MMC treatment effectively attenuated the fibroblast-transforming effect of TGF- β treatments (Fig. 1F). To verify this

antifibrotic activity of MMC, total RNA was isolated from TGF- β -stimulated and MMC-treated MRC-5 fibroblasts, and mRNA levels of fibrotic marker genes TGF- β , COL1A1, PAI-1, and CTGF were quantified by qPCR measurements. TGF- β alone induced significant upregulation in the expression of every examined fibrosis marker gene, although this effect was significantly abolished when TGF- β -exposed fibroblasts were treated with MMC as well (Fig. 1G). It is also noteworthy that MMC treatment alone resulted in lower expression levels of COL1A1 and CTGF in MMC-exposed cells compared to those of control MRC-5 fibroblasts.

MMC Exhibits Antifibrotic Effects in Patient-Derived Primary Vocal Cord Fibroblasts

Because we found that MMC displays a remarkable antifibrotic activity in MRC-5 fibroblast cells, we decided to test its efficiency on patient-derived primary vocal cord fibroblast cells as well. Three patients attending our clinic underwent total laryngectomy due to laryngeal cancer. Healthy parts of vocal cords were used to initiate primary human fibroblast cultures. Primary cells showed fibroblast characteristics such as spindle-like morphology; moreover, they stained positive for the fibroblast markers

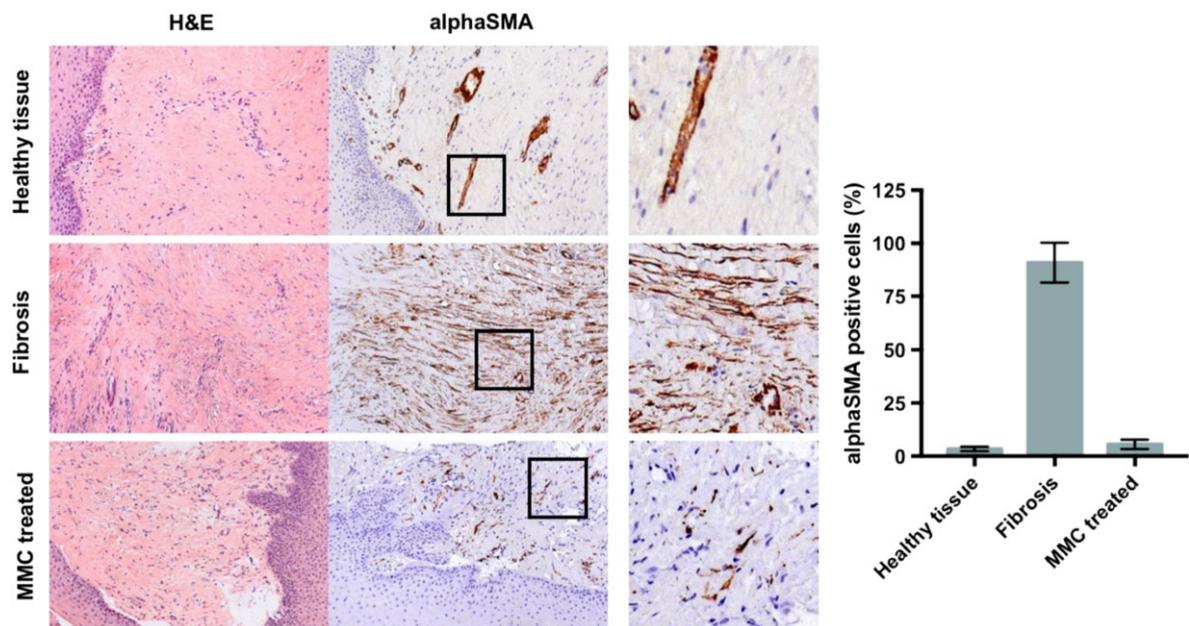


Fig. 3. Topical MMC treatment reduces laryngeal fibrosis in vivo verified by α -SMA immunohistochemistry. H&E = hematoxylin and eosin; MMC = mitomycin-C; SMA = smooth muscle actin.

α -SMA and vimentin and thus were proven to be primary fibroblast cultures (Fig. 2A).

To test the efficacy of MMC on the obtained primary fibroblasts, first, wound healing assays were performed. Primary fibroblasts were left to grow, and wounds were scratched when cells reached confluence. Similar to MRC-5 fibroblasts, patient-derived cells were also treated with MMC in 0.5 μ g/mL concentration for 24 hours, and then the number of cells that migrated to cell-free zones were counted. We found that MMC treatment significantly inhibited scratch-induced migration of all three of the examined patient-derived primary fibroblasts (Fig. 2B).

To examine whether MMC can influence the fibrosis-mediating potential of TGF- β in primary fibroblasts, likewise to the MRC-5 fibroblast cell line, serum-starved primary cells were treated with 5 ng/mL TGF- β . Twenty-four hours later, MMC was added to the medium in 0.5 μ g/mL concentration, and cells were incubated for further 24 hours. We detected the expression of the fibrotic fibroblast and myofibroblast marker α -SMA by Western blotting, which indicated that TGF- β triggered considerable fibroblast activation in all three of the treated primary fibroblast lines. Western blot data show that MMC treatment in primary fibroblasts hindered the fibrosis-inducing effect of TGF- β treatment in all three of the examined cases. Moreover, in cells obtained from patient 03, MMC treatments were able to completely restore the basal phenotype of TGF- β -stimulated fibroblasts because α -SMA level of the TGF- β +MMC-treated cells was comparable to that of the nontreated control cells (Fig. 2C).

Topical MMC Treatment Reduces Laryngeal Fibrosis In Vivo

Histopathological verification of highly effective MMC treatment on a patient diagnosed with posterior

commissure stenosis is shown in Figure 3 as a representative case. Prior to MMC treatment, tissue samples were obtained from the fibrotic area and also from the healthy adjacent tissue. Then, the patient received topical MMC treatment two times: after 1 week and 2 weeks after laser resection of posterior commissure stenosis. Two weeks after the second topical MMC treatment, specimens were resected from MMC-treated tissue and subjected to post-treatment histopathology. As expected, the healthy tissue region stained negative to the fibrosis marker α -SMA, whereas a strong positive staining was detected in the fibrotic area. Strikingly, after MMC treatment markedly lower expression of the fibrosis marker α -SMA was observed following immunohistochemical staining and subsequent pathological examination, and the staining intensity was comparable to that of the healthy tissue sample (Fig. 3.). Quantification of α -SMA-positive cells in tissue sections verified the high expression of α -SMA in fibrotic tissues, which was suppressed by MMC treatment.

DISCUSSION

Acquired laryngotracheal stenosis is a potentially life-threatening condition, induced mainly by traumatic and prolonged endotracheal intubation.²³ In most cases, LTS complications significantly influence the patient's life qualities because symptoms may vary from mild dyspnea and stridor to serious respiratory failure.²⁴ In the last decades, due to a significant increase in intensive care interventions worldwide, the frequency of iatrogenic LTS events has been substantially elevated and thus a demand for novel treatment modalities.²⁵

In our study, we proved that MMC is effective in attenuating TGF- β -induced fibroblast-to-myofibroblast transformation not only on the well-established human

MRC-5 fibroblast model system but also on patient-derived primary vocal cord fibroblasts. To verify this antifibrotic activity and the inhibitory action of MMC on fibroblast differentiation, the proliferation, wound-healing activity, apoptotic potential, α -SMA expression, and the mRNA levels of fibrotic marker genes (TGF- β , COL1A1, PAI-1, and CTGF) were quantified in TGF- β -stimulated and MMC-treated fibroblasts, respectively. Importantly, we demonstrated the beneficial effect of topical MMC treatment using histological staining results of a patient with posterior commissure stenosis, for which we observed a markedly lower expression level of the fibrosis marker α -SMA after MMC treatment and thereby confirmed the in vivo competence of MMC.

The antifibrotic potential of MMC has been tested in various human and animal in vivo, as well as in numerous in vitro model systems. MMC was shown to reduce pericardial fibrosis after cardiac surgery in rabbits²⁶ and to decrease the postoperative fibrosis after glaucoma surgery.²⁷ Its antifibrotic effect was confirmed in human Tenon's capsule fibroblast cultures in vitro,²⁸ in a rat Peyronie's disease model,²⁹ and in an experimentally induced urethral stricture model in rats.³⁰ Furthermore, MMC could prevent bronchial stenosis in a young cystic fibrosis patient following bilateral lung transplantation.³¹

We believe that the antifibrotic effect of MMC also can be exploited by LTS patients. According to our hypothesis, the maximal in vivo antifibrotic activity can be achieved if the treatment with MMC occurs in the granulation phase of the wound-healing process, which is when fibroblast proliferation is activated.^{32–37} In fact, rudimentary tissue generation relies mainly on fibroblasts that migrate into the wound area and ultimately transform into myofibroblasts, which is induced by the action of TGF- β .^{38–41} The presence of myofibroblasts in the regenerating tissue is a positive marker of progressive fibrosis, although their number usually decreases as the wound is closed. Experimental and clinical observations imply that topical MMC application should be started not earlier than 1 week after laser excision of stenotic tissue to inhibit myofibroblast overproduction and thereby prevent restenosis. Moreover, we expect that MMC treatment can reduce fibrosis with the highest efficiency by patients with LTS in a long-term application regime, in contrast to the moderate fibrosis inhibiting effect of early, short-term MMC application observed by Li et al.⁴² Considering the high-risk surgical procedures, the extended hospitalization periods, and various postoperative complications of tracheal stenosis, prevention of restenosis and minimalization of surgical intervention with the topical application of MMC might be successful treatment modality for laryngotracheal stenosis.

CONCLUSION

We demonstrated that MMC has the potential to reverse the TGF β -induced myofibroblast differentiation because we observed significant decrease in α -SMA expression and wound-healing activity in MMC-treated cells. Antifibrotic effects of MMC also were verified on human primary vocal cord fibroblast cell cultures. Our

results support that MMC can be applied to suppress upper airway stenosis by overturning the myofibroblast phenotype.

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