INTRODUCTION

Respiratory syncytial virus (RSV), a single-strand negative sense RNA virus, is the most important cause of lower respiratory tract infection in early childhood; especially in preterm neonates with chronic lung diseases like bronchopulmonary dysplasia (BPD), these infections can be clinically severe being associated with high morbidity and mortality. In addition, RSV increases the long-term risk for subsequent wheezing and elevated susceptibility to asthma. Underlying molecular mechanisms are not yet fully known.

Connective tissue growth factor (CTGF), a downstream mediator of transforming growth factor (TGF)-β, is a matricellular signaling modulator and a key molecule in tissue remodeling. As upregulation of CTGF has been detected after mechanical ventilation and hyperoxia in the neonatal lung, in proliferating type II lung epithelial cells, and activated fibroblasts of fibrotic lungs, it has been suggested that CTGF may contribute to BPD. Moreover, a role of RSV in activation of TGF-β gene expression has been described, suggesting a possible involvement of CTGF during RSV infection.

Administration of glucocorticoids to attenuate severe forms of BPD in preterm infants is controversially discussed, and the potential impacts of glucocorticoids as well as RSV infection on remodeling processes of the lung are barely defined. Caffeine, a methylxanthine commonly used to minimize apnea of prematurity, has been associated with the ability to reduce the incidence of BPD.

We recently described increase in CTGF expression by various glucocorticoids in lung epithelial and fibroblast models. In addition, we have shown that TGF-β1-induced upregulation of CTGF can be antagonized by caffeine in A549 lung epithelial cells. As anti-inflammatory agents appear to offer no clinical benefit, we hypothesized that caffeine might counteract detrimental effects of glucocorticoids and RSV infection. Using the human lung epithelial cell line H441 and the fetal lung fibroblast strain IMR-90, the aim of this study was to define if RSV has an impact on the expression of CTGF mRNA in the simultaneous presence of dexamethasone and caffeine.

KEYWORDS

Caffeine, CCN2, dexamethasone, lung remodeling, poly(I:C)
2 | MATERIAL AND METHODS

2.1 | Reagents

Caffeine and dexamethasone were purchased from Sigma-Aldrich (St. Louis, CA). Polyinosinic:polycytidylic acid (poly(I:C)) was from InvivoGen (San Diego, CA). Linear polyethylenimine MW 25 000 (PEI) was purchased from Polysciences Inc. (Warrington, PA).

2.2 | Cells and virus

NCI-H441 (H441) and IMR-90 cells were purchased from ATCC (LGC Standards, Teddington, UK) and cultured as described. Recombinant green fluorescent protein-expressing RSV (rgRSV) was a kind gift of Peter L. Collins (National Institute of Allergy, Immunology, and Infectious Diseases, National Institutes of Health, Bethesda, MD).

2.3 | Infection and transfection

For infection, cells were seeded on six-well plates (Greiner, Frickenhausen, Germany) until 80% confluence was reached and infected with $5 \times 10^5$ pfu of rgRSV. After 2 hour, medium was removed and cells were incubated with substances in growth medium as indicated. Images of cells were captured using a DM IRE 220 microscope (Leica, Solms, Germany). For transfection, $5 \times 10^5$ cells were seeded on six-well plates (Greiner) and 16 hour later transfected with 10 μg poly(I:C) using 20 μg PEI in a total volume of 1 mL Opti-MEM I (Gibco, Thermo Fisher Scientific, Waltham, MA). After 4 hour, medium was removed and cells were treated for 24 hour with substances in growth medium as indicated.

2.4 | RNA extraction, RT-PCR, and quantitative real-time PCR (qPCR)

RNA extraction, RT-PCR, and qPCR were performed as described previously. Levels of mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences for qPCR are listed in Table 1. Mean fold changes in mRNA expression were calculated by the $\Delta\Delta^C_T$ method.

2.5 | Statistical analysis

Results are given as means ± SD. Unless otherwise stated, data were analyzed using one way ANOVA with Bonferroni’s multiple comparison post hoc test. A $p$-value ≤.05 was considered significant. All statistical analyses were performed using Prism® version 6 (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | Impact of rgRSV and dexamethasone on inflammatory markers in H441 and IMR-90 cells

After treatment of lung epithelial cells H441 and fetal lung fibroblasts IMR-90 with rgRSV for 24 hour, virus infection was evident to varying degrees in all lung cell cultures by the expression of GFP (Figure 1A and B). While no increase in IL-6 mRNA expression could be observed in H441 cells (Figure 1C), rgRSV infection increased IL-6 mRNA expression in IMR-90 cells (44.6 ± 2.5-fold, $p < .0001$; Figure 1D). IL-8 mRNA expression was increased in H441 (1.3 ± 0.2-fold, $p = .0243$; Figure 1E) as well as in IMR-90 cells (71.5 ± 18.4-fold, $p < .0001$; Figure 1F). Dexamethasone was able to significantly reduce this rgRSV-mediated increase in IL-6 mRNA in IMR-90 cells ($p < .0001$) and of IL-8 mRNA in H441 and IMR-90 cells ($p < .0001$ for both cell types).

3.2 | Effect of rgRSV, dexamethasone, and caffeine on expression of CTGF mRNA in H441 and IMR-90 cells

Infection with rgRSV was able to increase CTGF mRNA expression in H441 (10.5 ± 1.8-fold, $p = .0002$; Figure 2A) but not in IMR-90 cells (Figure 2B). Dexamethasone increased CTGF mRNA expression in H441 (9.5 ± 1.2-fold, $p = .0007$; Figure 2A) and in IMR-90 cells (1.4 ± 0.2-fold, $p = .0006$; Figure 2B). In combination, dexamethasone and rgRSV showed an additive increase in H441 cells (25.7 ± 3.9-fold, $p < .0001$; Figure 2A) but reduced CTGF mRNA levels in IMR-90 cells (0.6 ± 0.1-fold, $p = .0003$; Figure 2B). In H441

| Gene symbol | Sequence accession # | Orientation | Sequence [5’ to 3’] | Amplicon length [bp] |
|-------------|----------------------|-------------|---------------------|---------------------|
| CTGF        | NM_001901.2          | Forward     | ACCCAACTATGATTAGAGCC | 189                 |
|             |                      | Reverse     | TTGCCCTTCTTAATGTTTCTC |                     |
| GAPDH       | NM_002046.5          | Forward     | CCATGGAAGGCGCCTGGG  | 195                 |
|             |                      | Reverse     | CAAAGTTGTCATGGATGACC|                     |
| IL6         | NM_000600.4          | Forward     | AACAAATTCGGTACATCCTC| 167                 |
|             |                      | Reverse     | AAGTCTCCTCATTGAAATCCA|                     |
| CXCL8 (IL8) | NM_000584.3          | Forward     | CAGTGCATAAAGACATACTCC| 198                 |
|             |                      | Reverse     | TTTATGAAATCTCAGCCTC |                     |

CTGF, Connective tissue growth factor.
cells, the presence of caffeine was able to prevent a significant increase in CTGF mRNA by dexamethasone, rgRSV, and their combination (P < .0001 for all comparisons; Figure 2B).

3.3 | Poly(I:C) increases mRNA expression of inflammatory markers and CTGF in H441 cells

24 hour after transfection of H441 cells with poly(I:C), IL-6 (20.7 ± 8.9-fold, P = .0014; Figure 3A), IL-8 (24.3 ± 7.7-fold, P < .0001; Figure 3B), and CTGF mRNA expression was increased (23.2 ± 12.0-fold, P = .0245; Figure 3C). Although CTGF mRNA levels were further elevated by the addition of dexamethasone in poly(I:C)-transfected cells (36.2 ± 17.1-fold), this was not statistically significant (P = .3992) in comparison with cells transfected with poly(I:C) alone.

4 | DISCUSSION

We report an upregulation of CTGF mRNA by RSV infection in H441 lung epithelial cells which was additive to the recently described induction by glucocorticoids.9 Different to work describing a reduction of vanadium pentoxide-induced fibrosis by RSV via reduction of growth factors,12 our data indicate that RSV infection might conceivably be able to induce fibrotic conditions by increasing CTGF mRNA in lung epithelial cells which might be further enhanced by glucocorticoids.3,13

It has been shown that RSV-induced exacerbation of airway hyperresponsiveness in a mouse model was only partially suppressed by steroid treatment.14 According to our observations in H441 cells, the simultaneous presence of glucocorticoids and RSV infection might provoke aggravated remodeling processes in the lower airways via increase in CTGF expression, which has been demonstrated in bleomycin-induced fibrosis.13 Furthermore, in addition to the prevention of glucocorticoid-mediated increase,9 our data suggest that caffeine treatment is able to block RSV—as well as dexamethasone/RSV-caused upregulation of CTGF mRNA expression. Apart from its preventive effects on the development of BPD,8 caffeine might thus also counteract potential pro-fibrotic effects mediated by RSV.

Poly(I:C), an artificial dsRNA structure, stimulates toll-like receptor-3 signaling and induces an activation of the RNA helicases RIG-I and MDA-5, thereby initiating cytokine production.15 By transfecting H441 cells with poly(I:C), mimicking viral dsRNA replication intermediates, we reveal that the observed increase in CTGF mRNA can be mediated by cytoplasmic dsRNA structures. In contrast to infection with rgRSV, IL-6 and IL-8 mRNA levels were much stronger elevated in the presence of poly(I:C), possibly indicating anti-inflammatory mechanisms mediated by RSV predominantly in H441 cells, as the inflammatory outcome in IMR-90 cells was more pronounced.

However, there are certain limitations of this study to be considered. The fact that increase in CTGF mRNA expression by rgRSV infection in contrast to that by dexamethasone treatment could be observed exclusively in H441 cells indicates a possible restriction to lung epithelial cells, a feature of papillary adenocarcinoma cells, or a consequence of cytopathogenicity. In addition, the time at which
CONCLUSION

We report a transcriptional attribute of increased CTGF mRNA expression in H441 cells following infection with RSV potentially prevalent for and restricted to lung epithelial cells. Caffeine might have the ability to support anti-inflammatory actions of glucocorticoids but antagonize initiation of CTGF expression, thereby pushing the overall outcome from long-term morbidity to healing.

ACKNOWLEDGEMENTS

We thank Peter L. Collins (National Institute of Allergy, Immunology, and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA) for providing rgRSV and Theresa Kreuzahler for excellent technical support.

ORCID

Markus Fehrholz  http://orcid.org/0000-0003-2183-2415

REFERENCES

1. Openshaw PJM, Chiu C, Culley FJ, Johansson C. Protective and harmful immunity to RSV infection. Annu Rev Immunol. 2017;35:501-532.
2. Piedimonte G. RSV infections: state of the art. Cleve Clin J Med. 2015;82:03.
3. Kubota S, Takigawa M. Cellular and molecular actions of CCN2/CTGF and its role under physiological and pathological conditions. Clin Sci. 2015;128:181-196.
4. Alapati D, Rong M, Chen S, et al. Connective tissue growth factor antibody therapy attenuates hyperoxia-induced lung injury in neonatal rats. Am J Respir Cell Mol Biol. 2011;45:1169-1177.
5. Pan LH, Yamauchi K, Uzuki M, et al. Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. Eur Respir J. 2001;17:1220-1227.
6. Mgbemena V, Segovia J, Chang T, Bose S. Kruppel-like factor 6 regulates transforming growth factor-beta gene expression during human respiratory syncytial virus infection. Virol J. 2011;8:409.
7. Rostas SE, McPherson C. Systemic corticosteroids for the prevention of Bronchopulmonary Dysplasia: picking the right drug for the right baby. Neonatal Netw. 2016;35:234-239.
8. Abdel-Hady H, Nasef N, Shabaan AE, Nour I. Caffeine therapy in preterm infants. World J Clin Pediatr. 2015;4:81-93.
9. Fehrholz M, Glaser K, Speer CP, Seidenspinner S, Ottensmeier B, Kunzmann S. Caffeine modulates glucocorticoid-induced expression of CTGF in lung epithelial cells and fibroblasts. Respir Res. 2017;18:017-0535.
10. Fehrholz M, Speer CP, Kunzmann S. Caffeine and rolipram affect smad signalling and TGF-β1 stimulated CTGF and transgelin expression in lung epithelial cells. PLoS One. 2014;9:e97357.
11. Chambers RC, Mercer PF. Mechanisms of alveolar epithelial injury, repair, and fibrosis. Ann Am Thorac Soc 2015;12:S16-S20.
12. Turpin EA, Antao-Menezes A, Cesta MF, et al. Respiratory syncytial virus infection reduces lung inflammation and fibrosis in mice exposed to vanadium pentoxide. Respir Res. 2010;11:20.
13. Yang J, Velikoff M, Canalis E, Horowitz JC, Kim KK. Activated alveolar epithelial cells initiate fibrosis through autocrine and paracrine secretion of connective tissue growth factor. Am J Physiol Lung Cell Mol Physiol. 2014;306:7.
14. Nguyen TH, Maltby S, Simpson JL, et al. TNF-alpha and macrophages are critical for respiratory syncytial virus-induced exacerbations in a mouse model of allergic airways disease. J Immunol. 2016;196:3547-3558.
15. Hafner AM, Corthesy B, Merkle HP. Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. Adv Drug Deliv Rev. 2013;65:1386-1399.

How to cite this article: Kunzmann S, Krempfl C, Seidenspinner S, Glaser K, Speer CP, Fehrholz M. Increase in CTGF mRNA expression by respiratory syncytial virus infection is abrogated by caffeine in lung epithelial cells. Influenza Other Respi Viruses. 2018;12:662-666.
https://doi.org/10.1111/irv.12561