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All Trans-Retinoic Acid Selectively Down-Regulates Matrix Metalloproteinase-9 (MMP-9) and Up-Regulates Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) in Human Bronchoalveolar Lavage Cells

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Abstract

Background: The balance between proteinases and antiproteinases plays an important role in tissue destruction and remodelling. In chronic obstructive pulmonary disease (COPD) and emphysema, an imbalance between matrix metalloproteinases (MMPs) and inhibitors of tissue metalloproteinase (TIMPs) has been reported. Alveolar macrophages are considered to be the main source of MMPs. We therefore have analyzed the effects of free and liposomal all trans-retinoic acid (ATRA) on the expression of MMP-9 and TIMP-1 in bronchoalveolar lavage (BAL) cells from patients with COPD and patients with other lung diseases.

Material and Methods: BAL cells were incubated 1–3 day with either liposomal or free ATRA. Supernatants were tested for MMP-9 and TIMP-1 protein in specific ELISA systems; mRNA analysis was performed by semi-quantitative RT-PCR and by quantitative LightCycler PCR.

Results: We demonstrate that either liposomal or free ATRA selectively down-regulates MMP-9 and up-regulates TIMP-1. At the protein level, MMP-9 is decreased 3-fold and TIMP-1 is increased 3.5-fold compared to the base line with empty liposomes or untreated cells. The ratio of MMP-9 and its inhibitor TIMP-1, which may be crucial to the overall proteolytic potential decreased by factor 8. That this countercurrent effect of ATRA is not due to an altered protein stability but to transcriptional regulation could be demonstrated by RT-PCR. Quantitative LightCycler analysis revealed a 2.5-fold decrease of MMP-9 mRNA and a 4.5 fold increase of TIMP-1 mRNA.

Conclusions: These data suggest that ATRA treatment via its impact on the proteinase/antiproteinase ratio may become a new therapeutic strategy for patients with inflammatory destructive lung diseases.

Abbreviations: ATRA, all trans-retinoic acid; BAL, bronchoalveloar lavage; COPD, chronic obstructive pulmonary disease; MMP, matrix metalloproteinase ; TIMP, tissue inhibitor of metalloproteinase; RT-PCR, reverse transcriptase polymerase chain reaction; PS, phosphabidyl-serine.

Introduction

Matrix metalloproteinases (MMPs) are capable of degrading all components of the extracellular matrix and, therefore, are of crucial importance in processes such as cancer progression, wound healing, and inflammation. MMPs are Zn-dependent enzymes that can be grouped into five subclasses, which include collagenases, gelatinases, matrilysin, transmembrane MMPs, and stromolysins. Their activity is controlled by endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs) that form 1:1 covalent complexes with MMPs (1).

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In inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD), MMPs have been reported to be elevated in induced sputum (2,3). In this disease, inflammation is dominated by alveolar macrophages. In animal models of lung injury, macrophages are an important source of destructive proteinases (4,5). Therefore, we studied MMP and TIMP expression in bronchoalveolar lavage (BAL) cells from patients with different lung diseases including COPD. To do this, we employed all trans-retinoic acid (ATRA) a potent Vitamin A derivate because this compound has been shown to suppress many activities of the monocyte lineage (6–9).

We show herein that liposomal ATRA is effective in decreasing the MMP-9 protease (10) and increasing the TIMP-1 anti-protease (11) in alveolar macrophages. This dual effect of liposomal ATRA may provide a novel therapeutic option for inflammatory and destructive lung diseases.

Material and Methods

Patients and Lavage

BAL was performed on patients with COPD (n = 5), with sarcoidosis (n = 5), with pneumonitis (n = 2), with lung cancer (n = 1), and with Churg-Strauss disease (n = 1). A detailed description of the patients and their MMP-9 and TIMP-1 values at time point zero are given in Table 1. After informed-consent lavage was performed during fiberoptic bronchoscopy by instilling 160 mL of 0.9% saline solution in 20 mL aliquots into the lingula or middle lobe and withdrawing the fluid immediately. Total cell counts were determined, and cytocentrifuge smears were prepared for cytologic analysis. Differential cell counts of 400 cells were made by Giemsa-May-Grünwald staining (Diff-Quick, Dade Behring, Suisse). All patients underwent the lavage procedure for diagnostic purpose. BAL was done during routine diagnostic procedure after informed consent had been obtained.

Cells and Culture Conditions

Culture medium consisted of RPMI 1640 (Biochrom #F1415, Berlin) supplemented with L-Glutamine 2mM (Gibco #25030-024), Penicillin 200 U/mL Streptomycin 200 μg/mL (Gibco #15140-114), nonessential amino acids (NEAA) 1-2 $\times$ (Gibco #11140-35), and OPI-supplement (contains oxalacetic acid, sodium pyruvate, and insulin) 10 mL for 1 l (Sigma #O-5003). To avoid any inadvertent LPS contamination, we used culture medium that was filtered through a Gambro Ultrafilter U 2000 (Martinsried, Germany) to remove LPS (12). This medium was found negative for MMP-9 and TIMP-1 protein. BAL cells cultured for 24 h without stimulus show no TNF production, indicating that no LPS is in the cultures. 1×10^6 BAL cells in 1 mL culture medium were seeded in 24 well Costar low-attachment plates (Costar #3473, Bodenheim, Germany), which were pretreated according to the manufacturer's instructions to prevent cell adhesion and differentiation. To these cultures, we added free ATRA, liposomal ATRA, or empty liposomes and cultured the cells for 1–3 days at concentrations of 5×10^{-6} to 5×10^{-8} M. Then, supernatants were harvested, and cells were recovered for mRNA isolation.

Preparation of Liposomes

Di-oleyl-phosphoserine (OOPS) and palmitoyl-oleyl-phosphocholine (POPC) (kindly provided by Dr. L. Tarcsay, Ciba-Geigy, Basel, Switzerland) were dissolved at an OOPS:POPC ratio of 0.43 in chloroform (13). All trans-retinoic acid (#R-2625, Sigma, Deisenhofen, Germany) in 96% ethanol was added at different concentrations. To protect the vitamin A against oxidation, ($\pm$)-α-Tocopherol (#T-3251, Sigma, Deisenhofen, Germany) was added at a final concentration of 200 μg/mL liposomes. Liposomes were generated by evaporating organic solvent followed by adding aequous buffer. This preparation was then reduced to $\leq$0.4 μm in diameter by a three-times passage through polycarbonate filters (Nuclepore 110407, Costar, Bodenheim, Germany) in an extruder device (B002, Lipex Biomembranes, Vancouver, Canada). Resultant unilamelar liposomes were sterile filtered through 0.2 μm and stored up to 4 weeks at 4°C.

RT-PCR

Semiquantitative PCR for mRNA was performed as previuosly described (14). In brief, 2×10^4 cells were lysed in 200 μl RNAclean (AGS, Heidelberg, Germany), and RNA was isolated. The following primers were used:

MMP-2 5′ primer: 5′ TGA CAT CAA GGG CAT TTC AGG AGC 3′
MMP-2 3′ primer: 5′ GTC CGC CAA ATG AAC CGG TCC TTG 3′
Product length: 180 bp (15)

MMP-9 5′ primer: 5′ GTG CTG GGC TGC TGC TTT GCT G 3′
MMP-9 3′ primer: 5′ GTC GCC CTC AAA GGT TTG GAA T 3′
Product length: 303 bp (16)

MMP-12 5′ primer: 5′ATA TGT CGA CAT CAA CAC AT 3′
MMP-12 3′ primer: 5′ ATA AGC AGC TTC AAT GCC AG 3′
Product length: 286 bp (15)

TIMP-1 5′ primer: 5′ GGG GAC ACC AGA AGT CAA CCA GA 3′
TIMP-1 3′ primer: 5′ CTT TTC AGA GCC TTG GAG GAG CT 3′
Product length: 400 bp (16)

α-Enolase 5′ primer: 5′ GTT AGC AAG AAA CTG AAC GTC ACA 3′
α-Enolase 3′ primer: 5′ TGA AGG ACT TGT ACA GGT CAG 3′
Product length: 619 bp (17)

Quantitative PCR

Using the LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, we performed quantitative PCR by using the same primer pairs as noted above. In brief, mRNA was isolated and reverse-transcribed as for conventional RT-PCR. 3 μl of cDNA were used for amplification in the SYBR Green format using the LightCycler–FastStart DNA Master SYBR Green I kit from Roche (Cat. No. 2 239 264, Mannheim, Germany). For quantitative PCR, the LightCycler system offers the advantage of speed and real-time measurement of fluorescent signal during amplification. The SYBR Green dye binds specifically to the minor groove of double stranded DNA. Fluorescence intensity is measured after each amplification cycle. During PCR, a doubling of template molecules occurs in each cycle only during the log-linear phase.

Although the LightCycler displays signals from every cycle, the instrument uses fluorescent signals only generated during this informative log-linear phase to calculate the relative amount of template DNA.

Assays for MMP-9 and TIMP-1

For determination of MMP-9 and TIMP-1 protein, 1×10^6 BAL cells were incubated for 1–3 days in the presence or absence of liposomal or free all trans-retinoic acid. Cell-free supernatants were then tested for protein concentration with commercial ELISA kit systems for MMP-9 (BIOTRAK, code RPN 2614, Amersham Pharmacia, Freiburg, Germany) and TIMP-1 (BIOTRAK, code RPN 2611, Amersham Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. The MMP-9 ELISA assay detects free MMP-9, proMMP-9, and the MMP-9/TIMP-1 complex. For measuring specific MMP-9 enzyme activity in supernatants of BAL cells or in BAL cell lysates, we used the "MMP-9 activity assay" system by Amersham Pharmacia, Freiburg, Germany (code RPN 2630) according to the manufacturer's instructions.

Statistics

For statistical analysis of the data, we used the Student's *t*-test.

Results

Effect of ATRA on MMP-9 and TIMP-1 Protein Levels

Bronchoalveolar lavage cells were incubated 3 days at 5 μM with free ATRA, with ATRA incorporated into liposomes, with empty liposomes or without any additional reagent. When supernatants were tested for MMP-9 protein by ELISA, both free and liposomal ATRA substantially reduced protein levels. In average of 14 lavage samples MMP-9 protein was reduced 3 $\pm$ 1.3–fold by free ATRA ($p < 0.001$). Addition of empty liposomes did not significantly reduce the MMP-9 level, but ATRA-containing liposomes again reduced the protein 3.1 $\pm$ 1.6–fold ($p < 0.001$). For both free and liposomal ATRA, the average reduction was 66% (Figure 1A). When looking at enzyme activity using the MMP-9 activity assay from Amersham Pharmacia (code RPN 2630), we found that free ATRA reduced MMP-9 from 100% in untreated cells (range: 6–160 ng/mL) to 36.6 $\pm$ 18.3% (range: 2.9–36.7 ng/mL) (n = 8; $p < 0.01$). Determination of MMP-9 in cell lysates revealed only low levels on day 3, such that we found 63 $\pm$ 70% of the activity detected in supernatants (n = 4). Treatment with ATRA did not increase intracellular MMP-9 activity; rather, it decreased intracellular MMP-9 by 36.5 $\pm$ 17.3%, as well. This argues against the possibility that ATRA reduces extracellular MMP-9 by inducing enzyme accumulation within the cells.

When BAL supernatants of the MMP-9 antagonist TIMP-1 were analyzed by ELISA (Figure 1B), then the level in untreated cultures was increased 3.6 $\pm$ 1.9–fold by free ATRA ($p < 0.001$). Again, empty liposomes did not substantially affect TIMP-1 protein levels in supernatants, but liposomal ATRA increased TIMP-1 to 3.4 $\pm$ 1.5–fold ($p < 0.001$). The increase of TIMP-1 protein in the supernatant was 330% and

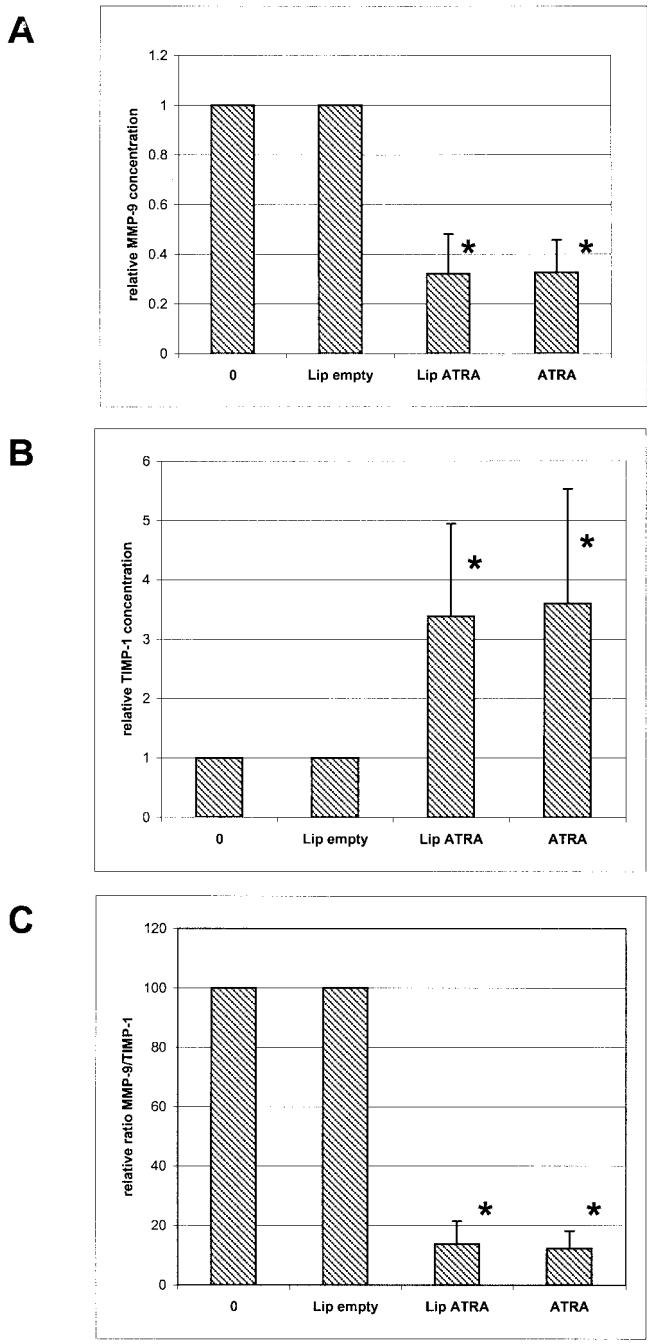


Fig. 1. Effect of ATRA on MMP-9 and TIMP-1 protein levels. BAL cells were cultured for 3 days without and with ATRA at 5×10^{-6} M and MMP-9 and TIMP-1 were determined in supernatants by ELISA (n = 14). (A) MMP-9: 100% values were 20.7 $\pm$ 14.7 ng/mL for cells treated with empty liposomes and 18.3 $\pm$ 14.5 ng/mL for untreated cells. (B) TIMP-1: 100% values were 451 $\pm$ 386 ng/mL for cells treated with empty liposomes and 462 $\pm$ 469 ng/mL for untreated cells. (C) Relative changes in ratio MMP-9/TIMP-1. * = $p < 0.05$

360% for free and liposomal ATRA, respectively. Hence, both free and liposomal ATRA decreased the MMP-9 metalloproteinase and at the same time increased TIMP-1. Because the balance of these two proteins is critical to matrix destruction, the ratio of MMP-9 and TIMP-1 may adequately reflect the proteolytic potential. As shown in Figure 1C the MMP-9/TIMP-1 ratio is reduced by approximately 85% by both preparations of ATRA ($p < 0.001$).

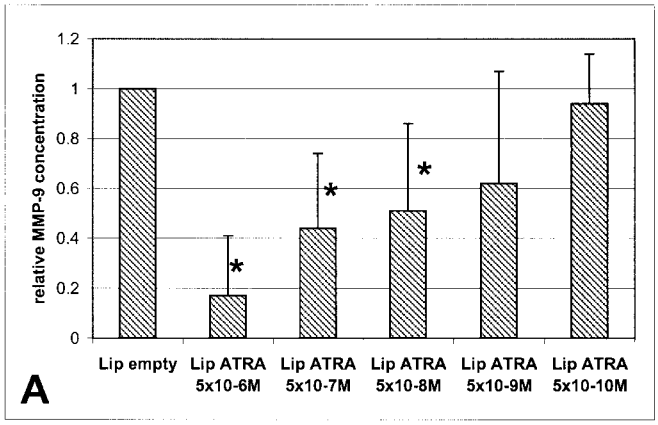
This effect of ATRA on the MMP-9–TIMP-1 system was a consistent finding when comparing BAL cells from patients with fibrosis, sarcoidosis, and COPD (data not shown). When performing dose-response analysis for liposomal ATRA, the standard dose of 5×10^{-6} M was most effective, but the 100-fold lower dose of 5×10^{-8} M still reduced the MMP-9 levels by factor 2 (Figure 2A). Although not significant, liposomal ATRA still had an effect at a dose of 5×10^{-9} M and activity was lost at 5×10^{-10} M. Also induction of TIMP-1 was more than 3-fold with liposomal ATRA at 5×10^{-6} M, 5×10^{-7} M, and 5×10^{-8} M (Figure 2B). There still was activity of ATRA at 5×10^{-9} M; however, little activity on TIMP-1 protein remained at 5×10^{-10} M.

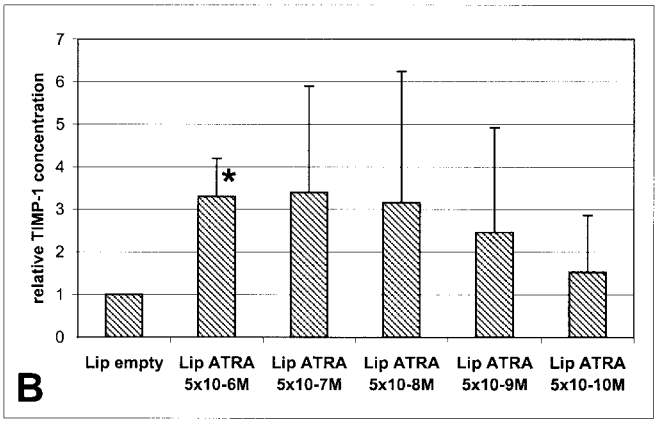
The potency of liposomal ATRA is evident when looking at the MMP-9:TIMP-1 ratio (Figure 2C). The low dose of 5×10^{-8} M still reduced the value by 71% compared to 93.5% for the highest dose of 5×10^{-6} M (Figure 2C). Even at 5×10^{-9} M of liposomal ATRA, the ratio was still reduced by more than 50%.

Effect of ATRA on MMP-9 and TIMP-1 mRNA Levels

To analyze whether ATRA affects the proteolytic potential of BAL cells by regulating transcript levels, we studied mRNA for metalloproteinases and the TIMP-1 inhibitor by RT-PCR. A representative example in Figure 3 demonstrates a near-complete disappearance of MMP-9 mRNA after incubation of BAL cells with free and liposomal ATRA. At the same time, no substantial change occurred in levels of MMP-2 and MMP-12 transcripts similar to the house keeping gene alpha enolase (Figure 3). By contrast, there was a slight increase in the intensity of the TIMP-1 band after culture in the presence of ATRA. These findings were consistently observed with BAL cells from seven patients.

For a more precise study of mRNA levels, we then turned to LightCycler analysis for real-time monitoring of cDNA synthesis. As shown in Figure 4A, linear amplification of MMP-9 cDNA in ATRA-treated cells occured at a higher cycle number of approximately 24 cycles compared to the control cultured cells, which reached the same amount of amplified cDNA after ap-





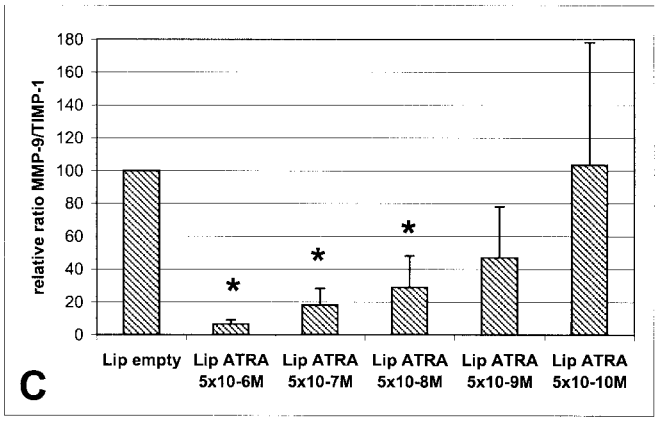


Fig. 2. Dose–response analysis for the effect of liposomal ATRA on MMP-9 and TIMP-1 protein levels. BAL cells were cultured for 3 days without and with liposmal ATRA at 5×10^{-6} M to 5×10^{-10} M and MMP-9 and TIMP-1 were determined in supernatants by ELISA (n = 5). (A) MMP-9: 100% value was 28 ± 35.7 ng/mL in cells treated with empty liposomes. (B) TIMP-1: 100% value was 754 ± 661 ng/mL in cells treated with empty liposomes. (C) relative changes in ratio MMP-9/TIMP-1. * = $p < 0.05$

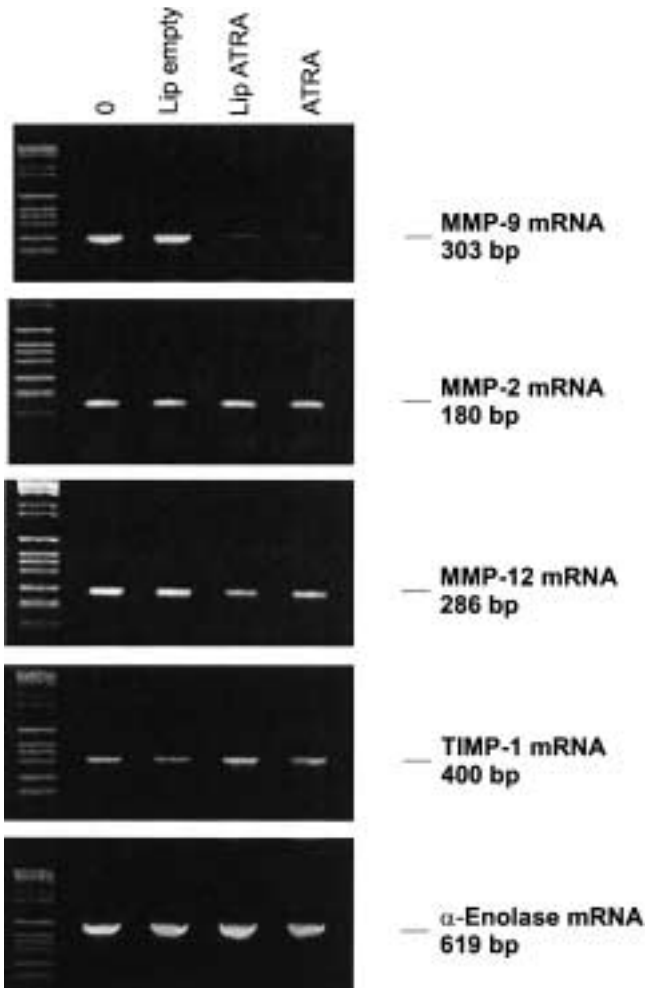


Fig. 3. Effect of ATRA on MMPs and TIMP-1 mRNA. BAL cells from patient I.S. were cultured for 3 days without and with ATRA at 5×10^{-6} M and mRNA levels for MMP-2, MMP-9, MMP-12, TIMP-1, and α-Enolase were determined by RT-PCR followed by gel electrophoresis. Shown is 1 example out of 11.

proximately 22 cycles. This reflects a decrease in MMP-9 mRNA abundance by factor 2.2 for liposomal ATRA and by factor 3.8 for free ATRA in this representative experiment. In average of three BAL samples, a 2.6-fold and 2.5-fold reduction of MMP-9 mRNA was seen for free and liposomal ATRA, respectively. LightCycler analysis for TIMP-1 revealed a linear amplification for the ATRA-treated cells already at approximately 20 cycles as compared to approximately 22 cycles for control-treated cells (Figure 4B). This reflects an increase of TIMP-1 mRNA by factor 5.1 for liposomal and by factor 5.5 for free ATRA. In an average of three BAL samples, this increase was 4.5-fold for liposomal and 4-fold for free ATRA ($p < 0.06$). When comparing mRNA and protein level in a given patient then the effect of ATRA on both MMP-9 and TIMP-1 was of the same magnitude (data not shown).

Amplification for the alpha-enolase house keeping gene led to identical amplification curves for all 4 types of AM cultures (Figure 4C). These data demonstrate that both preparations of ATRA regulate MMP-9 and TIMP-1 proteins at the transcript level.

Discussion

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease of the airways that occurs in about 10% of cigarette smokers and is one of the leading causes of death in developed countries (18). The remodelling and fibrosis of the small airways, which strongly reduces the forced expiratory volume, characterizes COPD. Also the inflammatory process in sarcoidosis may lead to irreversible fibrosis. This remodelling process is governed by proteases, such as the matrix metalloproteinases (MMPs), and their inhibitors. In fact, MMPs and TIMPs are increased in COPD in induced sputum (2), in sarcoidosis, and in patient serum (Frankenberger et al., unpublished). The MMPs and the TIMP inhibitor are produced primarily by airway epithelial cells, neutrophils, and macrophages (19).

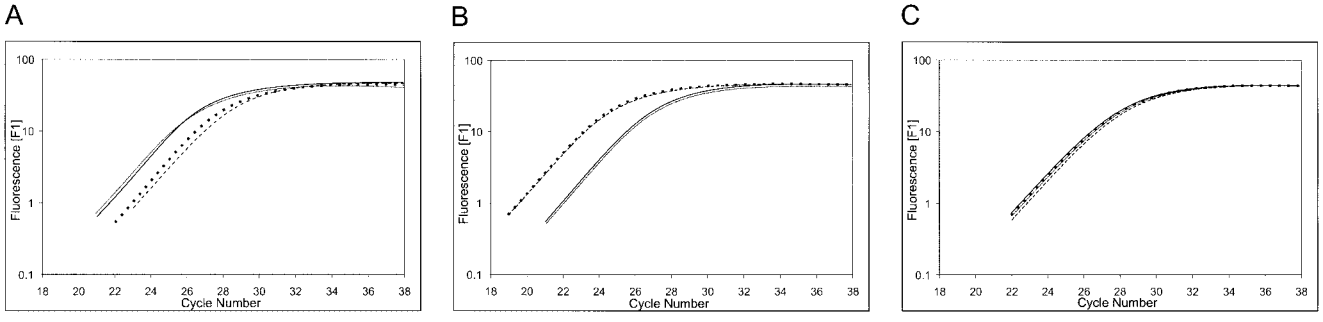


Fig. 4. LightCycler analysis of the effect of liposomal ATRA on MMP-9 and TIMP-1 mRNA. BAL cells from patient K.-H. K. were cultured for 3 days without and with ATRA at 5×10^{-6} M and mRNA levels for MMP-9 (A), TIMP-1 (B) and α-Enolase (C) were determined RT-PCR using cDNA amplification by LightCycler technology using SYBR-green fluorescence. Shown is one example out of three. (..... untreated; _____ Lip empty; **....** Lip ATRA; ------- ATRA)

In the present report, we have studied expression of MMPs and TIMP-1 in bronchoalveolar lavage cells obtained from patients with inflammatory lung diseases. Because lymphocytes express little MMP activity and because macrophages form the majority of cells in our samples, we assume that alveolar macrophages are the main source of MMPs in our studies. Because we did not have the opportunity to study healthy donors, we cannot draw any conclusions regarding a possibly disturbed proteinase–antiproteinase balance in the patient samples. There was, however, a broad range in the MMP-9 protein levels among the patients. Patients with COPD and the other patients did not show any obvious differences for both MMP and TIMP after *in vitro* culture (*data not shown*).

We have studied the effect of Vitamin A in the form of the all trans-retinoic-acid (ATRA) derivative. Vitamin A is an essential vitamin involved in various processes in the body, including embryonic development, vision, cell differentiation, and gene expression (20). Vitamin A acts via the RAR and RXR nuclear receptors, which after ligand binding can bind to specific motifs in the promoters of various genes leading to either transactivation or repression (21). Vitamin A also has pronounced effects on the activity of leukocytes, including anti-inflammatory effects such as the down-regulation of adhesion molecules (6) and cytokines (7,8).

In our experiments, we treated BAL cells with Vitamin A and found a strong reduction in protein amounts and MMP-9 enzyme activity. ATRA was equally effective in cells with low and with high MMP-9 levels (*data not shown*). There are reports of an opposite effect of Vitamin A: MMP-1 was found reduced in rabbit fibroblasts (22) and MMP-9 was increased in a neuroblastoma cell line (23). This latter effect may be rather indirect in that it is a consequence of induction of a differentiation program that includes up-regulation of MMP-9. The majority of studies did, however, find a reduction of collagenases by Vitamin A in fibroblasts and synovial cells (24–26).

On the other hand, we found a pronounced induction of the TIMP-1 protein by Vitamin A. This contrasts findings by Overall (27), who reported on a decreased expression of TIMP-1 by rat bone cells but is consistent with the report by Wright and colleagues, who demonstrated an increased production of TIMP-1 protein by synovial fibroblasts (28). Also, Braunhut et al. found an increase in TIMP-1 in Vitamin A–treated endothelial cells (29). It is unclear why such disparate effects of Vitamin A are found for both MMPs and TIMP-1. Species differences or differences in tissue types may account for these findings. Because tissue macrophages are the cells of interest in the context of destructive diseases such as COPD, targeting Vitamin A to these cells may be helpful. For this purpose, we used liposomes containing phosphatidyl-serine (PS) side chains attached to the palmitoyl-oleyl-acyl-chains (30) that bind to PS-receptors. These receptors are specifically expressed on monocytes and macrophages (31) to allow for phagocytosis of apoptotic cells. Previous studies have used this liposomal formulation for macrophage activation (32,33) and differentiation induction in myelomonocytic leukemia (13). In the present study, liposomal ATRA at 5×10^{-6} M was as effective as free ATRA in inducing TIMP-1 and reducing MMP-9. In more detailed dose-response analysis liposomal ATRA still had a strong effect when added at 100-fold lower concentrations to the alveolar macrophages indicating that this compound is extremely potent with respect to regulation of proteinase expression.

Our studies reported herein focussed on BAL cells. Considering the contradicting reports on the effects of RA in different tissues, we also looked at the effects of liposomal ATRA on peripheral blood mononuclear cells. In experiments not shown, we could demonstrate a similar down-regulation of MMP-9 and an up-regulation of TIMP-1 in these blood cells from healthy donors.

Because RA exerts much of its activity by regulating gene expression, we have asked whether ATRA does act on transcript levels for MMPs and TIMP-1. Using a semiquantitative RT-PCR approach, we found, in fact, a down-regulation of MMP-9 and a moderate up-regulation of TIMP-1 mRNA. When using the more precise LightCycler analysis for quantitative RT-PCR, we found an average 2.6-fold decrease of MMP-9 transcripts and a clearcut 4.3-fold increase of TIMP-1 mRNA. Since this increase was not obvious in the standard semiquantitative approach, this latter finding stresses the need for a quantitative approach when judging transcript levels in PCR.

The molecular mechanism of RA in MMP-1 expression involved both RAR and RXR (20). Little is known about the promotor elements involved in MMP-9 and TIMP-1 regulation by retinoids, but we assume that a direct action of retinoic acid receptors is operative here as well.

Retinoic acid derivatives are already in clinical use for different diseases, such as acute leukemia, for which ATRA is used as a differentiating agent (34), or for skin disease, where isotretinoin is applied for acne therapy (35). We suggest that liposomal ATRA may be a new option for treatment of COPD and other destructive lung diseases and this is based on the current finding of its pronounced effect on the metalloproteinase–antiproteinase balance. This treatment may prevent the ongoing destruction of the tissue architecture of small peripheral airways that occurs in COPD. In addition, COPD is associated with emphysema and, in animal model Vitamin A reduced elastase-induced emphysema (36). Recently, a role for MMPs in this process was suggested based on a knockout model in which emphysema and fibrosis were concomitant with an up-regulation of macrophage MMPs including MMP-9 (37). The concurrent decrease of MMP-9 and increase of TIMP-1

may be a much more promising approach as compared to treatment with only proteinase inhibitors (19). Furthermore, the anti-inflammatory actions of retinoic acid (7,8,38) may contribute to an effective treatment in COPD and other inflammatory and destructive lung diseases.

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