ASCORBIC ACID PROMOTES A TGFβ1 – INDUCED MYOFIBROBLAST PHENOTYPE SWITCH

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ABSTRACT

L-ascorbic acid (AA), generally known as vitamin C, is a crucial co-factor for a variety of enzymatic reactions, including prolyl-3-hydroxylase (P3H), prolyl-4-hydroxylase (P4H), and lysyl hydroxylase (LH)-mediated collagen maturation. Here, we investigated whether AA has additional functions in the regulation of the myofibroblast phenotype, besides its function in collagen biosynthesis. We found that AA positively influences TGFβ1-induced expression of $COL1A1$, $ACTA2$, $FN1$, and $COL4A1$. Moreover, we demonstrated that AA is necessary for αSMA stress fiber formation, synthesis and deposition of collagens type I and IV, and the contraction of a 3D collagen lattice. Additionally, AA amplified the expression of several TGFβ1-induced genes, including $DDR1$ and $CCN2$. Finally, we could not show that mechanism of AA action is Smad2 or Smad3 dependent, demanding further investigations.
INTRODUCTION

L-ascorbic acid (AA), generally known as vitamin C, is a water-soluble vitamin with antioxidant properties. Unlike most animal species, humans are unable to synthesize AA due to a mutation in the enzyme L-gulono-1,4-lactone oxidase and therefore depend on uptake from the diet. The world’s first clinical trial by James Lind revealed that AA supplementation through fruits and vegetables is an effective treatment for scurvy, a connective tissue disorder often found in sailors of the 17th and 18th century. The anti-scorbutic action of AA is ascribed to its function as co-factor for three enzyme families involved in the biosynthesis of collagens, namely prolyl-3-hydroxylases (P3H), prolyl-4-hydroxylases (P4H), and lysyl hydroxylases (LH). These α-ketoglutarate-dependent non-heme iron dioxygenases are responsible for the hydroxylation of proline and lysine residues in the assembly of the collagen triple helix. For these enzymes, AA acts as an electron donor in the catalytic cycle by reducing the highly reactive iron species (Fe$^{4+}$ and Fe$^{3+}$) into the catalytically active Fe$^{2+}$. The formation of hydroxyproline (Hyp) is required for the stability of the triple helix; an unstable triple helix is prone to intracellular degradation. Because of its role in collagen biosynthesis, AA has been implicated in the pathophysiology of fibrosis, a chronic pathology characterized by excessive extracellular matrix (ECM) accumulation and cross-linking.

Although it is known for a long time that AA is required as culture medium supplement to enable collagen deposition by human fibroblasts, many studies have overlooked this fact, which may have led to unreliable conclusions. For example, exposure of human fibroblasts to conditioned medium from fetal or adult stem cells results in decreased collagen levels when AA is present in the medium thus showing an anti-fibrotic effect of stem cell conditioned medium, whereas the opposite is seen when AA is absent. That the latter conclusion is incorrect, is illustrated by rodent fibroblasts that react to conditioned medium of stem cells in the same way as AA-exposed human fibroblasts, even in the absence of exogenous AA in the culture medium. In contrast to human fibroblasts, rodent fibroblasts are able to synthesize AA themselves.

The myofibroblast is the key cell in the pathophysiology of fibrosis and it is specialized in the synthesis of ECM components. Chronic organ injury activates effector cells such as fibroblasts and pericytes to adopt a myofibroblast phenotype, under the influence of the pro-fibrotic cytokine transforming growth factor (TGF)β. We asked whether TGFβ1 and AA act in synergy with respect to collagen deposition, and whether AA is involved in the TGFβ1-induced phenotype switch from fibroblasts to myofibroblasts. Our results indicate that AA works in synergy with actions of TGFβ1 both with respect to collagen deposition and in regulating a signature myofibroblast expression profile. We further elucidated that the involved mechanism of the latter is probably independent of canonical Smad signaling.
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METHODS

Cell culture
Human dermal fibroblasts were purchased from ATCC (CCD-1093Sk (ATCC® CRL-2115™), Wesel, Germany), and subcultures were maintained in Eagle’s minimal essential medium (EMEM; Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (complete growth medium) and 10% heat inactivated fetal bovine serum (FBS). For all experiments, cells were seeded at 15,000 cells/cm² in complete growth medium, and left to adhere for 24 hours before serum starvation. In brief, cells were starved in complete growth medium supplemented with 0.5% FBS (bare medium). After 18 hours, cells were stimulated with either 0.17 mmol/L AA (A8960, L-ascorbic acid 2-phosphate sesquimagnesium hydrate; Sigma-Aldrich, Zwijndrecht, the Netherlands), 10 ng/mL TGFβ1 (100-21C; PeproTech Ltd, London, United Kingdom), or both, for up to 6 days and medium was refreshed daily.

RNA extraction and quantitative Real-Time PCR
For gene expression analysis, total RNA was isolated at day 2 and day 6 using the Tissue Total RNA mini kit (Favorgen Biotech Corp., Taiwan). RNA quantity and purity were determined with UV spectrophotometry (NanoDrop Technologies, Wilmington, USA). RNA was reverse transcribed using the RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Landsmeer, the Netherlands), according to manufacturer’s instructions. Real-time PCR was performed with SYBR green PCR master mix (Roche, Basel, Switzerland) using a VIIA7 thermal cycling system (Applied Biosystems, Waltham, MA, USA). The thermal cycling conditions were 2 minutes at 95°C, followed by 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C, for a total of 40 cycles. Primers were designed and tested to have a calculated 95–105% reaction efficiency. mRNA expression levels of genes from the collagen biosynthesis pathway and other ECM components were analyzed with a custom made microfluidic card-based low density array (Applied Biosystems) and a VIIA7 thermal cycling system, as described previously23.

Immunofluorescence
For immunofluorescence of Smad2, cells were washed twice with PBS and fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10 minutes. For immunofluorescence of smooth muscle α-actin (αSMA), collagen type I, procollagen type I, and collagen type IV, cells were washed twice in PBS and fixed with ice-cold methanol/acetone (1:1) for 10 minutes at -20°C. Methanol/acetone fixed cells were first dried and later rehydrated with PBS before use. For all immunofluorescent stainings except collagen type I and IV, fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and incubated with 2.2% bovine serum albumin (BSA) for 30 minutes. Next, cells were incubated for 1h with primary antibodies: mouse monoclonal to αSMA (Clone 1A4, 0.28 µg/mL; Dako, Glostrup, Denmark), mouse monoclonal to collagen type I (ab90395, 1 µg/mL; Abcam, Cambridge, United Kingdom), goat polyclonal to pro-collagen type I (sc-8782, 2 µg/mL; Santa Cruz Biotechnology, Dallas, TX, USA), or mouse monoclonal
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Figure 1. Ascorbic acid synergizes with TGFβ1 to govern collagen production. (A) Relative mRNA expression of COL1A1, and FN1. Cells were exposed to bare medium or TGFβ1 for 2 or 6 days with or without addition of AA. (B) Representative immunofluorescent confocal photomicrographs of procollagen type 1 and collagen type 1α1 staining on day 2 and 6. Original magnification 630×; scale bar = 100 µm (C) Immunoblot on complete cell lysates for pro-collagen type 1 and tropo-collagen type 1α1 after 2 and 6 days. Data are represented as mean ± SD. Two-way ANOVA with Bonferroni post-test. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. AA, ascorbic acid; kD, kilo Dalton; TGFβ1, transforming growth factor β1.

Immunoblotting
Cells were lyzed with RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and sonicated. The DC protein assay (Bio-Rad, Hercules, CA, USA) was used to quantify protein concentrations and equal amounts of protein (20 µg/ lane) were subjected to SDS gel electrophoresis on stain free TGX mini-PROTEAN pre-cast gels. Before protein transfer, hydrogels were put under UV to activate stain free trihalo components in the gel, and gel images were taken for total protein quantification and normalization as described before24. After activation, protein transfer to a nitrocellulose membrane was performed using the semidyry Transblot Turbo system (Bio-Rad). Membranes were blocked in 5% skimmed milk in Tris-buffered saline + 0.1% Tween 20 and incubated overnight with primary antibodies: mouse monoclonal to αSMA (Clone 1A4, 0.28 µg/mL; Dako) and goat polyclonal to collagen type I (sc-8783, 2 µg/mL; Santa Cruz Biotechnology). Next day,
after three washes with TBST, membranes were incubated with goat-anti-mouse HRP (P0447, 1 µg/mL; Dako) or rabbit-anti-goat HRP (P0049, 0.5 µg/mL; Dako) for 1 hour at RT. Protein bands were visualized with chemiluminescence (ECL, Thermo Fisher Scientific) and a ChemiDoc imaging system (Bio-Rad). Image analysis was performed with ImageJ version 5.125.

Collagen lattice contraction assay
After three days of stimulation, dermal fibroblasts were seeded in collagen lattices with a final concentration of 2.4 mg/mL rat tail collagen type I (354249; BD, San Jose, CA, USA), 1x PBS, 20 mM HEPES, 5.8 mM NaOH, 50% EMEM complete growth medium, and 5% FBS. Cells were seeded at a concentration of 2×10⁵/mL gel. Cells were allowed to pre-stress the collagen lattice 3 days prior to detachment, while continuing stimulation with either or both TGF-β1 and AA. At time point t = 0 min, gels were released from the well rim and allowed to contract. Well plates were scanned at multiple time points on a flatbed scanner. Collagen lattice contraction was calculated using ImageJ version 5.1.

Transient transfection and luciferase assay
For the measurement of Smad2/3 transcriptional activity, cells were transfected with 2 µg plasmid DNA containing four copies of a Smad-binding element (SBE4-luc, Addgene #16495)²⁶ using Lipofectamine LTX and PLUS reagent (ThermoFisher Scientific) in bare EMEM. After 24 hours, cells were starved for 4 hours in EMEM with 0.5% FBS (bare medium) and subsequently stimulated with either bare medium, TGF-β1, AA, or both for 18 hours. Cells were lysed and luciferase activity was detected.

Figure 2. Ascorbic acid amplifies TGFβ1-induced αSMA expression. (A) Relative mRNA expression of ACTA2. Cells were exposed to bare medium or TGFβ1 for 2 or 6 days with or without addition of AA. (B) Representative immunofluorescent confocal photomicrographs of αSMA staining on day 2 and 6. Nuclei are visualized with DAPI. Original magnification 630×; scale bar = 100 µm. (C) Immunoblot on complete cell lysates for αSMA after 2 and 6 days. Data are represented as mean ± SD. Two-way ANOVA with Bonferroni post-test. ** p<0.01; **** p<0.0001. AA, ascorbic acid; kD, kilo Dalton; αSMA, α smooth muscle actin; TGFβ1, transforming growth factor β1.
using a luciferase assay system (E1500; Promega, Leiden, the Netherlands) according to the manufacturer’s instructions. The average fold-change was calculated from three independent experiments and normalized against total protein concentration.

Statistics
All data were tested with one-way ANOVA combined with Bonferroni post hoc testing using GraphPad Prism version 7.01 for Windows (Graphpad, La Jolla, CA, USA).

RESULTS
Ascorbic acid and TGFβ1 work in synergy with respect to (pro)collagen type I deposition
To determine whether AA affects mRNA expression levels of COL1A1, we cultured human dermal fibroblasts in the presence or absence of AA and/or TGFβ1 for 2 and 6 days. No changes were seen in COL1A1 mRNA levels at day 2 when either AA or TGFβ1 are added, whereas at day 6 an increase was seen for AA (but not for TGFβ1 alone). In contrast, higher mRNA levels of COL1A1 were seen at day 2 and especially at day 6 when AA and TGF-β1 were added together (Figure 1A). Another extracellular matrix molecule, fibronectin (FN1), was—in contrast to (pro)collagen type I—not affected by AA (Figure 1A). We next investigated the presence of procollagen by means of immunofluorescence with an antibody that recognizes to the α1(I) N-propeptide. Intracellular procollagen was present under all conditions, whereas extracellular procollagen was only seen at day 2 and 6 when AA or AA+TGFβ1 was present (Figure 1B). Staining with an antibody that recognizes native (triple helical) but not denatured (pro)collagen showed the absence of triple helical (pro)collagen in the absence of AA, whereas both intra- and extracellular triple helical (pro)collagen was seen at day 6 when AA was present and at day 2 and 6 when AA + TGFβ1 was present (Figure 1B).
Figure 4. Ascorbic acid synergizes with TGFβ1 to mediate expression of ECM components. (A) Heat map of a microfluidic card-based low density array-based mRNA expression. Cells were exposed to bare medium or TGFβ1 for 2 or 6 days with or without addition of AA. Heat map shows fold induction over bare treatment (-AA -TGFβ1). (B) Relative mRNA expression of COL4A1, CCN2, and DDR1. (C) Representative immunofluorescent confocal photomicrographs of collagen type IV staining. Original magnification 630×; scale bar = 50 µm. Data are represented as mean ± SD. Two-way ANOVA with Bonferroni post-test. * p<0.05; *** p<0.001; **** p<0.0001. AA, ascorbic acid; TGFβ1, transforming growth factor β1.

1B). The immunofluorescence data regarding the amount of (pro)collagen confirm that AA and TGFβ1 work in synergy. The immunofluorescence data were verified with immunoblotting using an antibody recognizing both the native and denatured triple helical part of the procollagen α1(I) chain (tropo-collagen). It indeed shows the presence of procollagen under all culture conditions, and furthermore a prominent presence of collagen at day 6 when AA or AA + TGFβ1 were present (Figure 1C).

Ascorbic acid facilitates the TGFβ1-induced differentiation of fibroblasts into myofibroblasts
We wondered, since AA and TGFβ1 work in synergy with respect to mRNA levels of COL1A1 and protein levels of procollagen and collagen, whether AA is also involved in TGFβ1-induced myofibroblast formation. A marker of myofibroblasts is the presence
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of αSMA stress fibers, which are absent in quiescent fibroblasts. We first measured mRNA (ACTA2) and protein levels of αSMA, and found that AA or TGFβ1 alone did not significantly increase ACTA2 mRNA levels, but that the combination AA + TGFβ1 does result in a major increase (Figure 2A). Staining for αSMA showed a modest formation of stress fibers at day 6 under the influence of TGFβ1, whereas an abundance of stress fibers was observed with the combination AA + TGFβ1 (Figure 2B). Incubation with AA alone did not result in the formation of stress fibers. Immunoblots showed elevated levels of αSMA at day 2 and 6 in the presence of TGFβ1 or AA + TGFβ1, but not with AA alone (Figure 2C). These data show that AA facilitates the TGFβ1-induced myofibroblast formation. Since αSMA stress fibers contribute to myofibroblast contractility, we assessed the impact of AA combined with TGFβ1 on myofibroblast contractility with a collagen lattice contraction assay. Combined stimulation with AA and TGFβ1 indeed resulted in increased contraction in a 3D collagen lattice (Figure 3A, B).

AA enhances the expression of COL4A1, CCN2 and DDR1

Since AA seems to be involved in the TGFβ1-induced myofibroblast phenotype, we investigated whether AA is involved in the expression of other genes known to be upregulated in myofibroblasts. We analyzed the expression of genes coding for various ECM components together with proteins and enzymes involved in collagen synthesis and degradation. Microfluidic card-based low density array analysis revealed that compared to bare medium or AA alone, TGFβ1 increases the expression of multiple genes, including COL4A1, P4HA2, P4HA3, and COL5A1 (Figure 4A). Moreover, combined stimulation with TGFβ1 and AA lead to further upregulation of COL4A1 (Figure 4A, B). The pro-fibrotic gene CCN2 showed a similar upregulation when AA and TGFβ1 are combined (Figure 4B). These data suggest that AA increases the expression of some but not all TGFβ1-responsive genes. Immunofluorescence analysis confirmed the increase of collagen type IV after TGFβ1 stimulation and that AA enhances the synthesis of collagen type IV compared to TGFβ1 alone (Figure 4C). An example of a gene that is non-responsive towards TGFβ1 alone but that is expressed on combined exposure to TGFβ1 and AA is the collagen receptor DDR1 (Figure 4A, B).

Ascorbic acid mediated myofibroblast phenotype switch is Smad2/3 independent

Smad2, together with Smad3, are the major transcriptional effectors of the canonical TGFβ1 signaling cascade, and have been shown to govern the expression of several collagens and αSMA. To investigate the relationship between AA and Smad2/3 transcriptional activity, we transiently transfected fibroblasts with a SBE4-luc promoter construct containing four copies of a Smad-binding element in front of a luciferase reporter gene. TGFβ1 alone increased the transcriptional activity of Smad2/3 compared to bare medium and AA alone (Figure 5A). However, combined stimulation of TGFβ1 and AA did not further enhance luciferase activity significantly. Moreover, immunofluorescence analysis revealed that AA addition does not enhance the nuclear accumulation of Smad2 compared to TGFβ1 alone (Figure 5B). Thus, whether the effects of AA on the expression of signature myofibroblast genes are dependent of Smad2 and Smad3, remains inconclusive.
DISCUSSION

We investigated the effect of AA in the presence or absence of TGFβ1 on collagen deposition at day 2 and 6 into more detail by means of quantitative RT-PCR and with antibodies directed toward the N-propeptide of procollagen type I, the native (triple helical) structure of the collagenous part of procollagen type I (thus recognizing only native collagen and procollagen), and an antibody recognizing procollagen and collagen type I in both its native and denatured state. This resulted in some unexpected findings. First, AA alone does not change mRNA levels of COL1A1 on day 2, but does increase COL1A1 expression on day 6, although not significantly. Similarly, AA resulted in a major increase of COL1A1 mRNA levels at day 6 in combination with TGFβ1, whereas this was not seen at day 2. Second, procollagen was observed in all experimental conditions (bare medium; TGFβ1; AA; AA + TGFβ1) intracellularly, whereas extracellular procollagen was observed only in the presence of AA. Third, native procollagen (or native collagen) was barely observable under control or TGFβ1 conditions, but was clearly seen in the presence of AA. The presence of extracellular procollagen or extracellular native collagen/procollagen was most obvious when AA was combined with TGFβ1.

From the protein data one can conclude that, although procollagen is present in all conditions, the procollagen is only present in its triple helical form when AA is present. We did not observe extracellular procollagen in the absence of AA, indicating that the non-native procollagen is not excreted by the cell and/or is immediately degraded in the extracellular space. The immunoblot revealed, that at day 6 the non-native procollagen was not processed into collagen (i.e. the N-propeptides and C-propeptides were not cleaved off), whereas tropo-collagen was seen in the presence of AA. Indeed, cleavage of the N-propeptides occurs only when the procollagen is in its native state. The native state is facilitated by the presence of Hyp. Absence of AA results in a severe underhydroxylation of proline residues, since AA is a co-factor for prolyl hydroxylase. It should be stressed that most antibodies towards collagen type I react with both native and denatured collagen proteins. Studies carried out with human fibroblasts in the absence of AA will detect collagen with such antibodies, but by far the majority of this “collagen” actually represents non-native procollagen. Not knowing this will clearly lead to unreliable conclusions, and this can unfortunately be readily observed in the existing literature.

It is well-known that TGFβ1 promotes the differentiation of fibroblasts into myofibroblasts. Much to our surprise, we observed that this process is facilitated by AA, as shown by the dramatically increased ACTA2 mRNA levels when TGFβ1 was combined with AA. mRNA levels of ACTA2 were not increased in the presence of AA alone, so there is a clear synergy between AA and TGFβ1. This was also obvious at the protein level: staining for αSMA stress fibers revealed much more myofibroblasts at day 6 compared to AA or TGFβ1 alone, which indeed resulted in an increased contraction of a 3D collagen lattice. Thus, AA works in synergy with TGFβ1, facilitating
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Figure 5. Ascorbic acid does not affect Smad2/3 signaling. (A) Smad binding element (SBE) promoter activity luciferase assay. (B) Representative confocal immunofluorescent photomicrographs of Smad2 staining. Original magnification 630x; scale bar = 50 µm. Data are represented as mean ± SD. Two-way ANOVA with Bonferroni post-test. ** p<0.01. AA, ascorbic acid; AU, arbitrary units; TGFβ1, transforming growth factor β1.

The pro-fibrotic properties of TGFβ1. However, not all TGFβ1-responsive genes were additionally upregulated by AA, indicating that the action of AA may not be regulated via Smad2/3, being the canonical TGFβ1 pathway. Indeed, we observed that AA did not enhance the nuclear translocation of Smad2, and no significant increase in activity was observed with a luciferase reporter containing four copies of a Smad-binding element.

The heat map shows that the addition of AA alone results in a change in the expression pattern of only a few genes, but that AA in combination with TGFβ1 is involved in the general enhancement of the myofibroblast expression profile in dermal fibroblasts. However, how exactly AA amplifies the TGFβ1-induced myofibroblast phenotype remains elusive. We speculate that this is likely due to the function of AA in epigenetics. Several studies highlighted that AA is involved in the process of active demethylation of cytosine (5mC), mediated by the ten-eleven translocation (Tet) methylcytosine dioxygenases enzymes Tet1, Tet2 and Tet3. Conventionally, 5mC is regarded as mark for the transcriptionally repressed chromatin, and DNA methylation of lineage-specific loci govern cellular differentiation programs. Similar to the prolyl hydroxylases P3H and P4H, AA acts as electron donor for Tets and reduces Fe3+ to Fe2+. Moreover, it is thought that AA also acts as cofactor for the Jumonji C-domain containing histone demethylases (jmjC). Methylation of histones is described as another tier of chromatin remodeling, which is associated with either activation of repression of transcription. The importance of AA in determining the epigenetic landscape has also emerged in the reprogramming of induced pluripotent stem cells, which are unable to be fully reprogrammed in the absence of AA.

In conclusion, we have not only shown why AA is crucial in the process of collagen production, but also that AA facilitates the TGFβ1-induced adoption of a myofibroblast phenotype. Thus, AA is involved in fibrotic processes at multiple levels. Finally, we speculate that AA induces epigenetic changes, thereby regulating expression of multiple myofibroblast-related genes.
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