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# Collagen mRNA Expression in Human Fetal Lung Fibroblasts Treated with Varying Concentrations of Ascorbic Acid, PGE2, and TGF-β1

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**Abstract:**

*Previous experiments have demonstrated that ascorbic acid contributes to the growth of collagen chains. In this study, we have examined the mRNA expression of collagen in Human Fetal Lung (HFL) cells treated with different concentrations of AsA, PGE2, and TGF-β1. The mRNA expression of collagen chains I α 1, I α 2, III α 1, VI α 1, VI α 2, and VI α 3 were all measured using the Taqman PCR method. The results of these studies showed that: 1) AsA modulates collagen gene expression having the greatest effect on Collagen Type III α 1. 2) TGF-β1 increased collagen gene expression, but not identically for all alpha chains for each different collagen type. 3) PGE2 inhibits collagen expression, but not identically for all collagen chains with the greatest effect seen in Collagen Type I α 1. 4) When TGF-β1 and PGE2 were both added to the cell culture mRNA expression was significantly increased showing that TGF-β1 has the dominant role in collagen mRNA expression, although the mechanism for this action is unknown. The reproducibility of the assay should be assessed. It can be concluded that PGE2 may differentially regulate collagen gene expression.*

**Introduction:**

The formation of collagen rich scar tissue is a basic biological response to both internal and external wounds. The degree of scarring is determined by the severity of the wound and the intensity of the cellular response. Understanding the mechanisms behind the formation of excessive scarring is an important part of understanding a variety of diseases. Chronic Obstructive Pulmonary Disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible. It is a progressive disease usually caused by cigarette smoking, but can be caused by other long term irritants such as pollution, dust, or other noxious gases. The airflow limitation is both progressive and associated with an abnormal inflammatory response of the lungs leading to fibrosis. It causes a variety of symptoms including coughing with excessive mucus production, wheezing, shortness of breath, and tightening of the chest. COPD results in less air flowing in and out of the pulmonary system for a variety of reasons. First, the elasticity of the bronchi, bronchioles, and alveolar sacs loose their elasticity. Second, the thin epithelium of the alveoli are often destroyed resulting in inadequate gas exchange. Third, the walls of the bronchi are thickened due to inflammation. Lastly, the airways are clogged due to the overproduction of mucus. The most common course of treatment for COPD is the use of bronchodilators or inhaled glucocorticosteriods. Bronchodilators relieve obstruction by relaxing the muscles controlling the airways; they can either be short or long acting. Glucocorticosteriods reduce airway inflammation. Both treatments are given as inhalants (12).

To understand how to treat COPD you first must understand the general concepts behind the formation of the associated collagen rich scars. Individual collagen chains are synthesized on membrane bound ribosomes and injected into the lumen of the endoplasmic reticulum as larger precursors (pro-alpha chains). Procollagen is formed when selected prolines and lysines are hydroxylated. Each pro-a chain then combines with two others to form a hydrogen bonded, triple strain helical molecule known as procollagen. After secretion of propeptides of fibrillar procollagen molecules are removed by specific proteolytic enzymes outside the cell. The function of propeptides is to guide the intracellular formation of the tristranded collagen molecules. The removal of the procollagen prevents intracellular formation of large collagen fibrils which is harmful to the cell. Fibril formation is driven by the collagen molecules tendency to self-assemble. After fibril formation they are greatly strengthened by the formation of covalent cross-links between lysine residues of the constituent collagen molecules. Understanding the mechanisms behind collagen formation is important because disrupting any of the formative steps will inhibit collagen production.

Collagen is an integral part of the extracellular matrix that surrounds fibroblasts. There are several different collagen chains with a varying number of helical alpha chains that form its structure. While the roles of some collagen chains are widely understood different collagen chains have different roles throughout the body, specifically in would healing. The different alpha chains derive their stability from hydrogen bonding between molecules. Gylcine must be every third amino acid in the chain, and the structure usually follows the pattern Gly – X – Y where X is usually praline and Y is usually 4-Hyp.

Among the eleven collagen strains there are five groups of collagen. Fibril-forming (fibrillar) collagen includes types I, II, III, V, and XI. Type I fibrillar collagen is found in a variety of places including the bone, skin, tendons, ligaments, cornea, and internal organs. It accounts for over 90% of the body's collagen. Type III fibrillar collagen is found in the skin, blood vessels, and internal organs. Fibrillar collagen is secreted into the extra cellular space where it assembles into higher order polymers called fibrils. They further aggregate into larger bands called collagen fibers. Fibril-associated collagen includes types IX and XII that decorate the surface of the collagen fibers. Network forming collagen includes types IV and VII. Type IV constitutes a major part of the mature basal lamina as a felt-like sheet, but it has structural

and biosynthetic properties that drastically differ from those of collagen I and III. Unlike collagen I and III, collagen VI is not degraded by metalloproteinases. Even though its functions are not fully understood it is suspected to form an integral part of the anchoring filaments for collagen I fibers and the basement membrane. Additionally, it may have a significant role in cell-binding and have protease inhibitor function (13). Collagen VI also differentiates itself from other collagen types because its large N- and C-propeptides are not removed via proteolytic processing to form mature collagen. Lastly, collagen VI α 3 has a length twice that of the other two alpha chains (3).

Cells mediate collagen fibril formation mechanically and chemically. Fibroblasts mechanically alter the collagen they secrete by pushing it into compact sheets that are then drawn out into cables. Fibroblasts generate long-range order in the extra cellular matrix to create things like tough, dense layers of connective tissue that sheathe and bind together most organs. The diversity of collagen presents a variety of challenges to biomedical research because the role of collagen type and each individual alpha chain must be assessed to understand its mechanism of action.

There are a variety biological factors that regulate collagen production both *in vivo* and *in vitro*. Vitamin C, also known as ascorbic acid (AsA), is obtained from fruits and vegetables. AsA is a water soluble vitamin that plays an integral role in collagen synthesis such as in the bone, cartilage, and gums. It is also an antioxidant, aids in detoxification, and improves iron absorption. A lack of AsA results in scurvy causing degeneration of skin, teeth, and blood vessels. It also results in general weakness, delayed wound healing, and impaired immunity (5). AsA is required for the hydroxylation of proline and lysine in collagen via the enzyme ascorbate. Proline is important because its derivative 4(R)-L-hydroxyproline (4-Hyp) plays an essential role in the folding of collagen and the maintenance of its structure. The addition of 4-Hyp makes the collagen molecule stable and it raises the melting temperature by 28°C in a 10 peptide molecule. Scurvy results when AsA is absent leading to collagen instability and connective tissue problems (5,8).

Prostaglandins are eiconsanoids, 20 carbon long molecules, which are made by most vertebrate cell types and have a wide variety of biological activities. Their main role is in the regulation of pain and inflammatory responses, contraction of smooth muscles, and regulation body temperature (1). PGE2 is a central lipid mediator in inflammation and pain. Evidence suggests that adult fibroblasts treated with PGE2 show collagen I inhibition in vitro. $PGE_2$ transmits its signal through several different G-protein coupled receptors. The four receptors, E1-E4, use a variety of mechanisms: EP1 signals through $G_q$, by inducing increased intracellular $Ca^{2+}$; EP2 and EP4 signal through $G_s$, by activating adenylate cyclase which increases intracellular cAMP; and EP3 primarily signals through $G_i$, has variants that mediate multiple signaling pathways leading to decreased cAMP (9,14). Protein kinase A (PKA), a proven cAMP effector, and its activation alters cellular function by one of two ways. It either directly phosphorylates transcription factors or it indirectly modulates other signaling pathways. PGE2 has been shown to inhibit fibroblast proliferation and collagen expression in patient-derived normal adult lung fibroblasts via E prostanoid-2 receptor (EP2) and cAMP signaling (9). Increased cAMP, due to PGE2, also mediates the activity in embryonic fibroblasts via the EP2 receptor (7). Even 2 fold changes in cAMP concentration were effective at suppressing collagen production in cultured HFL fibroblasts (2).

TGF-β1 is especially important in wound healing by stimulating a transformation of fibroblasts to myofibroblasts. It promotes the formation of collagen-rich scar tissue to give additional strength to a healing wound. TGF-β1 is a pleiotrophic cytokine that induces the extra cellular matrix expression and inhibits growth of vascular smooth muscle cells. Evidence suggests adult fibroblasts treated with TGF-β1 have increased collagen gene expression in vitro. TGF-β1 stimulates the production of collagen types I and III in human corpus cavernosum smooth muscle cells (HCCSMC). Exogenous TGF-β1 also resulted in detectable levels of collagen types X and VI. However, the induced collagen growth by TGF-β1 was suppressed by PGE1. These data further show the role of prostaglandins in the regulation of collagen synthesis in the corpus cavernosum. Excess collagen growth in HCCSMC did lead to fibrosis (11). The exact mechanism of increased collagen is not known.

The purpose of this study is to determine the role of AsA, PGE2, and TGF- β1 individually and when combined. In this study, we have examined the mRNA expression of collagen in HFL cells treated with different concentrations of AsA, PGE2, and TGF-β1. Based on previous studies, it was hypothesized that AsA would increase collagen mRNA expression. Furthermore, it was hypothesized that PGE2 would inhibit collagen mRNA expression and TGF-B1 would increase collagen mRNA expression. This study is unique because it deals with how AsA, PGE2, and TGF-β1 regulate three different types of collagen and their various alpha chains. Type VI collagen is not clearly understood so comparing its regulation to other collagen types is a necessary step to determining its role in wound healing. Determining mRNA expression is an important step to understanding collagen synthesis because it is one of the earliest steps in protein production.

**Materials and Methods:**

*Cell Culture:*

The human fetal lung (HFL) fibroblasts were plated in DMEM, 10% fetal bovine serum (FBS), and 1% fungizone. Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were from Invitrogen Life Technologies (Grand Island, NY). The cells were treated with 10 ug/mL of Ascorbic Acid (AsA) and grown for 48 hours in a 37°C incubator. The cells

were then treated with 100 pM TGF-β1, and 1 uM PGE2, and a mixture of TGF-β1 (100 pM) & PGE2 (1 uM). The re-treated cells were then incubated for another 24 hours. All of the cells used were between passage 8 and 15.

*Extraction & RNA Harvest:*
The cells were then extracted using Trizol from Invitrogen Life Technologies (Cat. No. 15596-026). The Trizol extraction method maintains the structure of RNA while disrupting cells and dissolving its cellular components. Chloroform was added, followed by centrifugation which separated the solution into an aqueous upper phase and an organic lower phase. RNA was exclusively in the aqueous phase and was recovered by precipitation with isopropyl alcohol. In order to check the concentration of RNA the isolated RNA had an $A_{260}/A_{280}$ ratio of >1.8 in TE (10). Once the concentration of RNA was known the sample was then treated with DNAse. The concentration and purity of RNA determined the amount that needed to be added to the 10x buffer, DNAse, and RNAse free water. This treatment assured that the only nucleic acids present in the sample were RNA.

*Taqman PCR:*
Samples were then treated with reverse transcriptase and run on a Mini-cycler using a protocol for high reverse transcription. Samples were then stored at 4°C until being plated in 96-well plates. Collagen primers and probes were then added to their respective column for all six alpha chains tested. Taqman PCR was then performed to obtain cycle threshold. Each cDNA sample was run in duplicate for every PCR. The values were then calculated using the following formulas. Amount of target, normalized to endogenous reference and relative to a calibrator is given by: $2^{-\Delta\Delta CT}$
Derivation of Formula, Exponential Amplification of PCR Xn = Xo x (1+Ex)n
Xn = number of target molecules at cycle n
Xo = initial number of target molecules
EX = efficiency of target amplification
n = number of cycles
Cycle Threshold indicates fractional cycle number at which amount of target reaches a fixed threshold:
XT=Xox(1+EX)CT1x=KX
XT = threshold number of target molecules
CT,X = threshold cycle for target amplification
KX = constant

**Results:**
When HFL fibroblasts were treated with AsA, an increase in collagen production was found for all alpha chains tested. The most significant increase was found in collagen III α 1 (29.20), followed by II α 2 (10.17), VI α 3 (8.49), II α 1 (8.43), VI α 2 (5.20), then VI α 1 (5.26) (Fig. 1). Even the small increase in VI α 1 shows double the amount produced in non treated HFL fibroblasts.

When HFL fibroblasts were treated with TGF- β1, an increase in collagen production was found in all but one of the collagen strains tested. The largest increase in expression was found in collagen III α 1 (20.32), then I α 1 (18.53), VI α 1 (3.77), I α 2 (3.32), and VI α 2 (2.67) (Fig. 2). However, collagen VI α 3 actually showed a reduction in mRNA expression (0.87). These values are set with 1.00 representing HFL fibroblasts treated only with AsA. For example, in collagen III α 1 the addition of TGF-β1 resulted in an 18.53 fold increase in addition to the 8.43 increase caused by AsA.

PGE2 inhibition was found in four of the six alpha chains tested. The inhibitory effects were the largest in collagen VI α 3 (0.20), followed by III α 1 (0.27), VI α 2 (0.36), and I α 2 (0.47). An increase in mRNA expression was found in collagen VI α 1 (10.44) followed by I α 1 (2.21) (Fig. 3). Collagen VI α 1 shows a significant increase mRNA expression compared to the inhibitory effects shown by VI α 3. The mRNA increase or decrease was compared to cells treated with AsA as the control value of 1.00.

When both PGE2 and TGF- β1were both given to HFL fibroblasts an increase in collagen production was found.
The most significant percentage increase in collagen mRNA expression was found in type VI collagen. The VI α 1 (508.51%), followed by VI α 2 (428.90), VI α 3 (372. show a large increase compared to the other collagen types tested. Increase in mRNA expression was also found for collagen I α 2 (295.82%), followed by III α 1 (52.16%), then I α 1 (41.06%) (Fig. 4).

**Effect of AsA on mRNA expression in HFL Fibroblasts**:

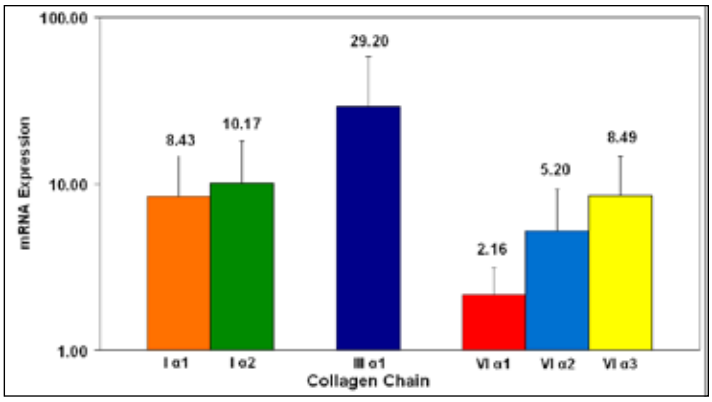
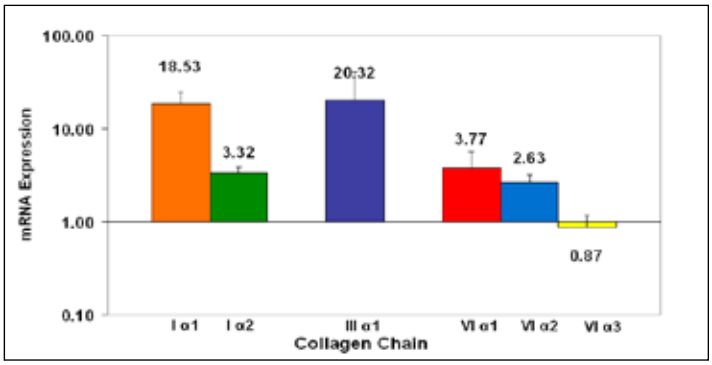



Figure 1. The role of AsA in HFL was tested alone to ensure that it induced collagen formation. AsA increased mRNA collagen expression for all experimental chains tested. The effect was the greatest on collagen III α 1.

**Effect of TGF-β1 on HFL Fibroblasts:**

Figure 2. (top of next page) TGF-β1 resulted in increased production of all collagen chains except collagen VI α 3.




**Effect of PGE2 on HFL fibroblasts:**

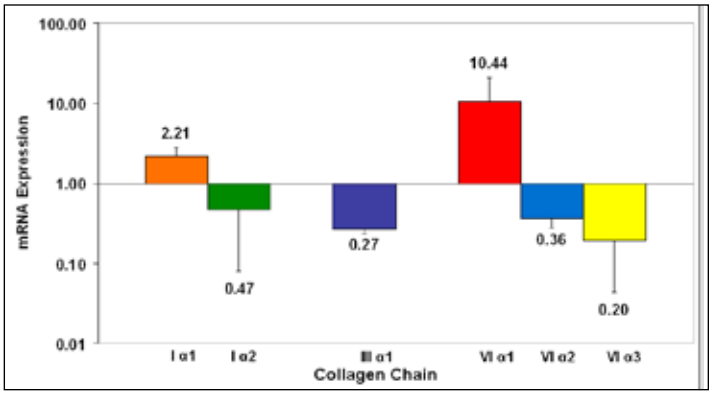



Figure 3. PGE2 inhibited collagen mRNA production in at least one alpha chain of each collagen molecule. The largest effect was on collagen VI α 3. However, collagen I α 1 and collagen VI α 1 were both increased with treatment.

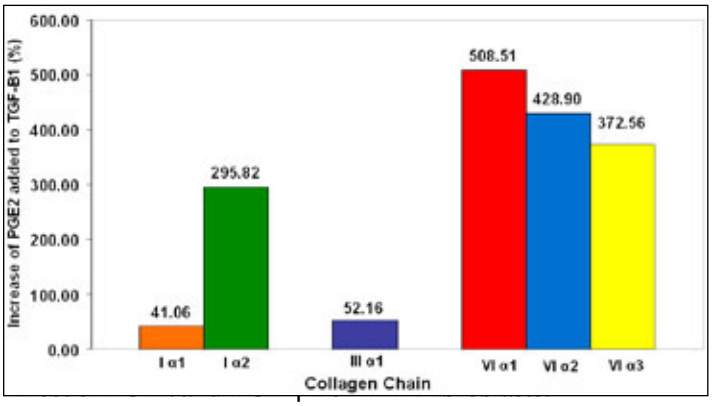



Figure 4. Simultaneous dosage of HFL fibroblasts with both PGE2 and TGF-β1 led to a dramatic increase in collagen mRNA production. Collagen VI showed the most significant expression.

**Discussion:**

The results of this study indicate that AsA induces collagen transcription in HFL fibroblasts. It was hypothesized that AsA would increase collagen mRNA expression, and AsA had the greatest effect on Collagen Type III α 1 (Fig. 1). These results suggest that AsA plays an integral role in the formation of the collagenous extra cellular matrix as procollagen is transformed to collagen. The important factor to determine is the proper dosage of AsA for cell culture. Previous cell culture techniques provided data that 10 ug/mL of AsA was the optimal concentration for experimental purposes (12). However, more analysis is needed to understand the definitive role of AsA in the formation of the extracellular matrix. In an earlier study the recommended AsA concentration was between 25-50 ug/mL and used a concentration of 20% FBS (4). Tests with other collagen strains are necessary to determine if AsA induces collagen mRNA production in all fibrillar collagen chains. In addition, fibril-associated, network forming, and transmembrane collagens should be tested to see if similar results are obtained. The viability of AsA may have affected results of the experiments as it has a half life of only a few hours. It may have been more effective to add fresh AsA to the medium each twelve hours to ensure that AsA activity was maintained throughout the experiment.

In a study by Chan, et al, AsA did stimulate collagen I and II synthesis reaching a maximum at day two (6). AsA maintained a high specific rate of production until day 10 and afterwards collagen production declined. In addition, the study found after only a 10 hour treatment of AsA deficient cells induced collagen synthesis. AsA increased collagen mRNA expression in collagen I α 1, I α 2, and III α 1. However, the increase in collagen mRNA (2-3 fold) did not correspond to the increased collagen protein synthesis (6-7 fold). These data show that there are transcriptional modifications as well as post-translational modifications that account for the increase of collagen expression in 2-year old human child fibroblasts (6). These data show that there may be additional factors to AsA induced collagen expression that are beyond the scope of the current study. In order to definitively conclude AsA's role in inducing collagen expression in all of the alpha chains tested additional information on the amount of protein produced needs to be assessed. Currently, it can be observed that AsA increased collagen expression differentially in the six alpha chains tested.

From the present study it can be concluded that TGF-β1 increased collagen gene expression, but not identically for all alpha chains for each different collagen type (Fig. 2). mRNA expression in collagen I and III chains was induced. These data show a similar effect to the previous experiments with HCCSMC which show increased collagen I and III production (11). More experiments need to be performed before conclusive observations can be made on the role of TGF-β1 in HFL fibroblasts. Not only does the mRNA need to be measured, but the protein levels should also be tested using Western blot analysis. The differential result from collagen VI is not definitive. Collagen VI is not included in the four main categories of collagen and its role is not as intricately understood. Its inclusion in the experiment was to obtain experimental data to be used in further experiments to further understand its role in the formation of the extra cellular membrane.

At present there is evidence suggesting that PGE2 inhibits collagen expression, but not identically for all collagen chains. The greatest effect was seen in collagen VI α 3 (Fig. 3). The differential regulation of collagen I alpha chains does not provide enough analysis to determine if PGE2 does or does not inhibit expression of collagen in HFL fibroblasts. The mRNA expression of collagen I α 2 is inhibited but collagen I α 1 mRNA is induced. Physiologically, the inhibition of collagen I α 2 mRNA may be reason enough to believe that PGE2 does inhibit collagen production. If one of the integral alpha chains of collagen I is missing then it would be logical that it could not form its triple helix structure effectively. However, further analysis using Western blot techniques is necessary to draw any concrete conclusions as to the amount of collagen I expressed in HFL fibroblasts treated with PGE2. A similar argument can be made for collagen VI α 1, but still without a definitive conclusion. It can be concluded that PGE2 may differentially regulate collagen gene expression.

When TGF-β1 and PGE2 were both added to the cell culture mRNA expression was increased showing that when the two factors are added together they produce a more pronounced effect. It cannot be determined if TGF-β1 has a dominant role in collagen mRNA expression because the mechanism for this action with PGE2 is unknown. Prostaglandins have a wide variety of roles in modulating the body and especially different receptors. Even though PGE1 was shown to inhibit the effects of TGF-β1 in HCCSMC, PGE2 uses different EP receptors for its mechanism of action (11,14). Furthermore, it cannot be assumed that TGF-β1 has the same effect on two different cell types. In order to understand the induced collagen mRNA in HFL fibroblasts expression protein analysis should be performed to see if post translational modifications inhibit such a pronounced induction of collagen production.

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