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Amino Acid Availability Regulates Type I Procollagen Accumulation in Human Lung Fibroblasts

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Abstract Fibrotic lung diseases are characterized by excessive deposition of type I collagen. Amino acid availability regulates type I collagen mRNA levels in quiescent human lung fibroblasts. In these studies, the effect of amino acid availability on type I collagen protein accumulation in quiescent human lung fibroblasts was examined. Following amino acid deprivation, α1(I) procollagen protein levels were not detected by Western blot analysis in either the intracellular or the extracellular compartments. Fibronectin levels and total protein levels were not affected. Amino acid deprivation resulted in a more pronounced decrease in α1(I) procollagen protein levels than in α1(I) procollagen mRNA levels, suggesting that post-transcriptional events were responsible for the further decrease inα1(I) procollagen protein levels. The addition of transforming growth factor-β to amino acid deprived fibroblasts increased α1(I) procollagen mRNA levels without affecting α1(I) procollagen protein levels, confirming a post-transcriptional site for regulatory control by amino acid deprivation. In the absence of ascorbic acid, α1(I) procollagen protein levels increased in amino acid deprived fibroblasts, but α1(I) procollagen mRNA levels were not affected. The absence of ascorbic acid likely resulted in the accumulation of nonhelical procollagen in the endoplasmic reticulum, indicating that translational mechanisms for α1(I) procollagen were intact. The addition of chloroquine, an inhibitor of lysosomal degradation of proteins, increased α1(I) procollagen protein levels in amino acid deprived fibroblasts. These data suggest that following amino acid deprivation of quiescent fibroblasts, newly synthesized type I collagen was degraded intracellularly, primarily by a process that involved lysosomal proteinases. J. Cell. Biochem. 75:130–137, 1999.

Key words: ascorbic acid; translocation; chloroquine; lysosomes; intracellular degradation

Fibrotic lung diseases are characterized by excessive deposition of type I collagen [Goldstein, 1991]. The rate of accumulation of collagen represents the difference between its rate of synthesis and its rate of degradation [Laurent, 1986]. In pulmonary fibrosis, the rate of collagen synthesis increases [Seyer et al., 1976; Fukuda et al., 1985], however, there have been reports of both increases and decreases in the rate of collagen degradation [Clark et al., 1980; Laurent and McAnulty, 1983].

Secretory proteins, such as collagen, are synthesized on ribosomes attached to the endoplasmic reticulum (ER). The ER is the site of folding of secretory proteins as well as post-translational modification. Newly synthesized proteins that are improperly folded may be retained in the ER and degraded [Lippincott-Schwartz et al., 1988; Lodish, 1988] or they may be translocated through the ER and the Golgi into lysosomes and then degraded [Berg, 1980]. The mechanism of intracellular degradation of type I collagen is incompletely understood.

Individual factors may exert regulatory control at multiple sites in the biosynthetic pathway of type I collagen. For example, transforming growth factor-β (TGF-β) increases the steady-state level of type I collagen mRNA by transcriptional and post-transcriptional mechanisms [Ignotz and Massague, 1986]. TGF-β also increases the synthesis of type I collagen protein and decreases its degradation [McAnulty et al., 1991; Roberts et al., 1992]. Amino acid deprivation decreases type I collagen mRNA levels by decreasing both transcription rate and transcript stability [Krupsky et al., 1997]. In these studies, the effect of amino acid availabil-

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ity on type I collagen protein accumulation in quiescent human lung fibroblasts was examined. Amino acid deprivation decreased α1(I) procollagen protein levels as a result of decreased α1(I) procollagen mRNA levels and increased intracellular protein degradation in lysosomes.

MATERIALS AND METHODS

Cells and Tissue Cultures

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were grown in Dulbecco's modified Eagle medium (DMEM) with 0.37 g sodium bicarbonate per 100 ml, 10% fetal bovine serum (FBS), 100 U penicillin per ml, 10 µg streptomycin per ml, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. After confluence was reached, the FBS content of the medium was reduced to 0.4% for 24 h to render the fibroblasts quiescent. All experiments were then carried out using serum-free DMEM or serum-free amino acid deficient medium. Cell numbers were determined in triplicate with an electronic particle counter (Coulter Counter ZM, Hialeah, FL).

Assessment of Total Protein Levels

Samples for SDS-PAGE were prepared from fibroblast cultures incubated in amino acid deficient or complete medium each containing [^{35}S]methionine at 37°C for 2 h. The cell layer was dissolved in sample buffer at 4°C and SDS-PAGE was performed under reducing conditions using 10% polyacrylamide gels [Laemmli, 1970]. The gels were dried and exposed to X-ray film for autoradiography.

Samples for colorimetric protein assay were prepared from fibroblast cultures incubated in amino acid deficient or complete medium. The cell layer was dissolved in RIPA buffer at 4°C and centrifuged at 14,000*g* for 10 min. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Samples for Western blot analysis were prepared from the cell layer or the medium of fibroblast cultures incubated in amino acid deficient or complete medium. Fibroblast cultures were incubated in the presence of 250 µM ascorbic acid unless otherwise indicated. The cell layer was dissolved in RIPA buffer at 4°C and centrifuged at 14,000*g* for 10 min. The medium was concentrated using Centricon 3 concentrators (Amicon, Beverly, MA). SDS-PAGE was performed under reducing conditions using 7.5% polyacrylamide minigels [Laemmli, 1970]. Minigels were transferred to a nitrocellulose filter, blocked at 4°C overnight with 10% evaporated milk in phosphate-buffered saline with 0.1% tween, and incubated at 21°C for 1 h with a 1:1,000 dilution of a primary antibody: rabbit anti-human α1(I) procollagen (LF-39) [Fisher et al., 1989] (generously provided by Larry W. Fisher, National Institutes of Health, Bethesda, MD) or rabbit anti-human fibronectin (Sigma Chemical Company, St. Louis, MO). A secondary antibody (1:5,000 dilution) conjugated to horseradish peroxidase was added and incubated at 21°C for 1 h. Bands were visualized using Western blot chemiluminescence reagent (NEN Life Sciences Products, Boston, MA).

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by the single-step method employing guanidine thiocyanate-phenol-chloroform extraction [Chomczynski and Sacchi, 1987]. RNA was quantitated by absorbance at 260 nm. Purity was determined by absorbance at 280 nm and 320 nm. RNA (10µg) was electrophoresed on a 1% agarose-6% formaldehyde gel and transferred to a nylon transfer membrane. RNA loading was assessed by ethidium bromide staining of ribosomal bands fractionated on agarose-formaldehyde gels and by cohybridization with a constitutively expressed mRNA that codes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Hybridization was performed using $0.5–1.0 \times 10^6$ CPM/lane labeled probe (SA, $4–10 \times 10^8$ CPM/µg), and the membrane was washed according to methods described by Thomas [1983]. The membrane was exposed to X-ray film for autoradiography at several different times to ensure that the bands could be quantitated by densitometry within the linear range. The α1(I) procollagen probe utilized in these experiments was cloned from rat α1(I) procollagen cDNA that specifically identifies corresponding human α1(I) procollagen mRNA [Genovese et al., 1984].

Statistics

A Student's *t*-test was used for means of unequal sizes. Probability values less than 0.05 were considered significant.

RESULTS

In fibroblasts incubated in amino acid deficient medium for 72 h, α1(I) procollagen mRNA levels decreased by 78% of levels in fibroblasts incubated in complete medium for 72 h (Fig. 1), confirming previous results [Krupsky et al., 1997]. In contrast, α1(I) procollagen protein levels were not detected in either the intracellular or the extracellular compartments following amino acid deprivation (Fig. 2).

Fibronectin levels were not affected by amino acid deprivation (Fig. 3). In addition, amino acid deprivation did not affect total protein levels as assessed by SDS-PAGE and colorimetric protein assay (Fig. 4). Fibroblasts incubated in amino acid deficient or complete medium were short-term labeled with [^{35}S]methionine. Following protein extraction, equal amounts of protein were separated by SDS-PAGE. Autoradiography revealed minimal differences in the band patterns for fibroblasts incubated in amino acid deficient or complete medium.

We previously reported that cystine regulatesα1(I) procollagen mRNA levels [Rishikof et al., 1998a]. In order to determine whether cystine also regulated α1(I) procollagen protein levels, fibroblasts were incubated in complete medium containing varying concentrations of cystine (0 mM to 0.2 mM) (Fig. 5). At cystine concentrations of 0 mM and 0.002 mM, the α1(I) procollagen protein levels were not detected by Western blot analysis. However, at a cystine concentration of 0.02 mM, the α1(I) procollagen protein levels were restored to levels detected in complete medium (cystine 0.2 mM).

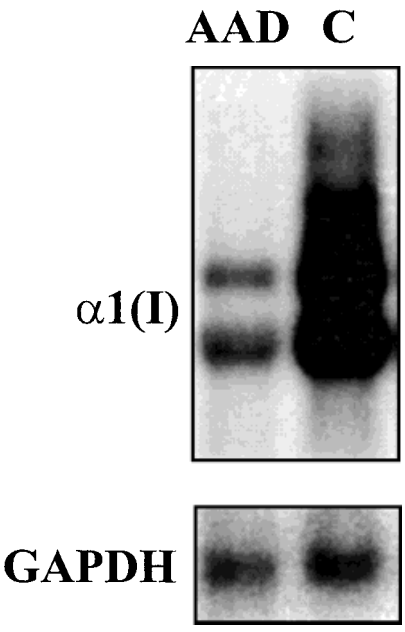

Fig. 1. Effect of amino acid deficiency on α1(I) procollagen mRNA levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium for 24 h. Total RNA was isolated and the expression of α1(I) procollagen mRNA was assessed by Northern blot analysis.

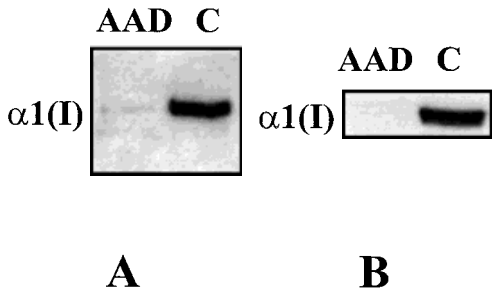

Fig. 2. Effect of amino acid deficiency on intracellular and extracellular α1(I) procollagen protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium for 24 h. Total protein was extracted from **(A)** the cell layer or **(B)** the medium and α1(I) procollagen protein levels were assessed by Western blot analysis.

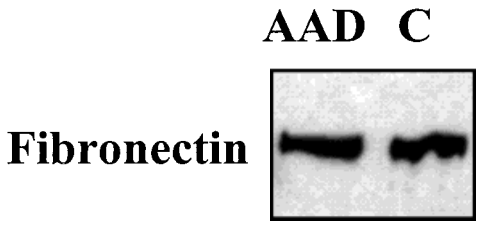

Fig. 3. Effect of amino acid deficiency on fibronectin protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium for 24 h. Total protein was extracted from the cell layer and fibronectin protein levels were assessed by Western blot analysis.

The effect of amino acid deprivation on α1(I) procollagen protein levels was further characterized using TGF-β. In fibroblasts incubated in amino acid deficient medium, TGF-β increased α1(I) procollagen mRNA levels (Fig. 6A), whereas TGF-β did not affect the α1(I) procollagen protein levels (Fig. 6B). The addition of TGF-β to fibroblasts incubated in complete medium increased the α1(I) procollagen protein levels (Fig. 6B).

Ascorbic acid was used to examine the mechanism by which amino acid deprivation de-

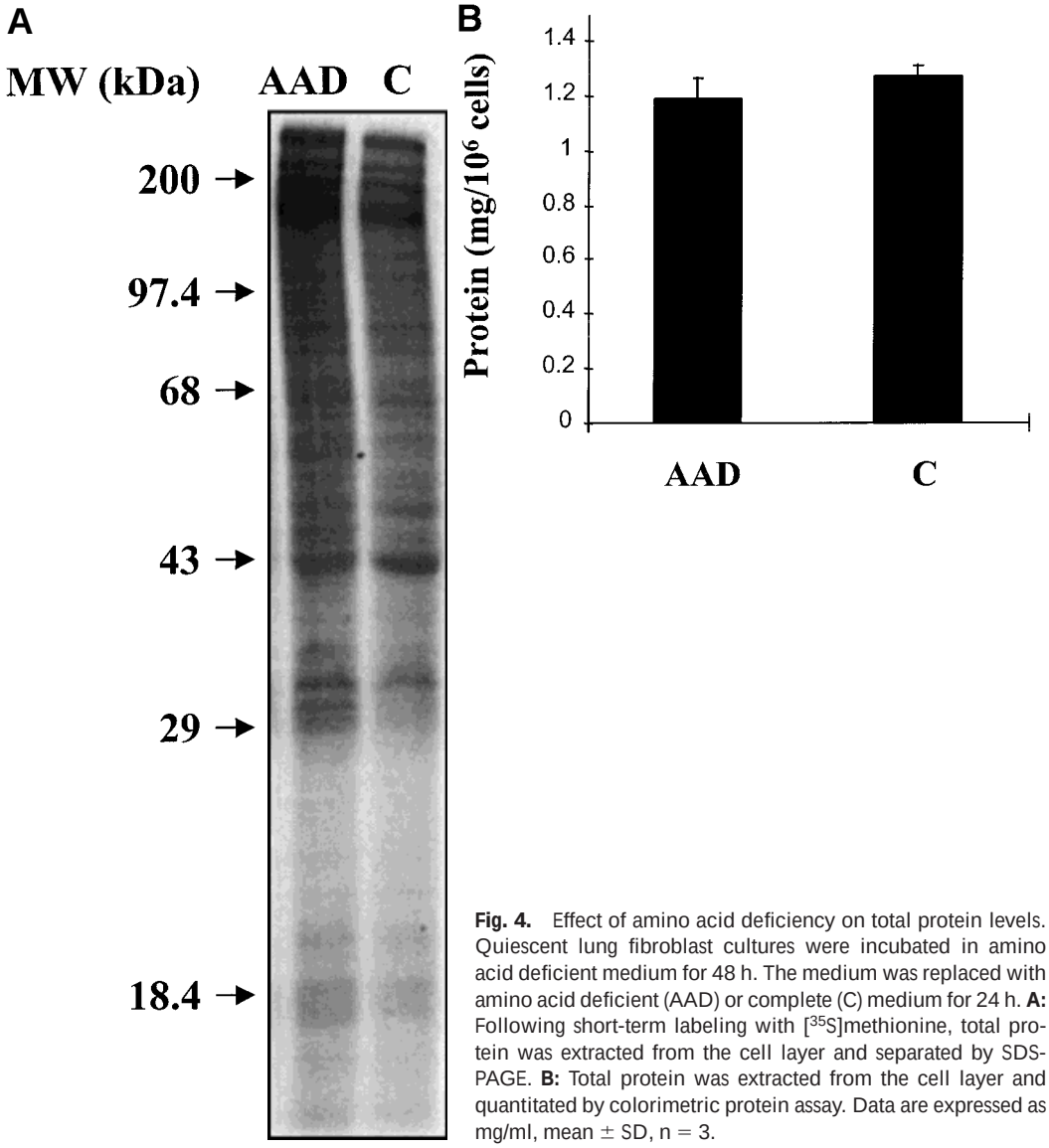

Fig. 4. Effect of amino acid deficiency on total protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium for 24 h. **A:** Following short-term labeling with [^{35}S]methionine, total protein was extracted from the cell layer and separated by SDS-PAGE. **B:** Total protein was extracted from the cell layer and quantitated by colorimetric protein assay. Data are expressed as mg/ml, mean ± SD, n = 3.

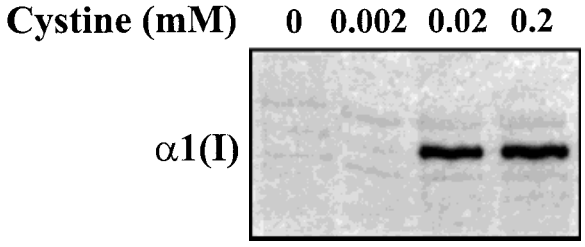

Fig. 5. Effect of varying concentrations of cystine on α1(I) procollagen protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with complete medium containing cystine at the indicated concentrations for 24 h. Total protein was extracted from the cell layer and α1(I) procollagen protein levels were assessed by Western blot analysis.

creased α1(I) procollagen protein levels. The addition of ascorbic acid to fibroblasts incubated in either amino acid deficient or complete medium did not affect α1(I) procollagen mRNA levels (Fig. 7A). In contrast, ascorbic acid decreased α1(I) procollagen protein levels in fibroblasts incubated in either amino acid deficient or complete medium (Fig. 7B).

The effect of amino acid deprivation on α1(I) procollagen protein levels was examined further using chloroquine, an inhibitor of lysosomal degradation of peptides [Wibo and Poole, 1974; Amenta et al., 1978]. The addition of chloroquine to fibroblasts incubated in amino acid deficient medium in the presence of ascorbic acid resulted in dose dependent increases in

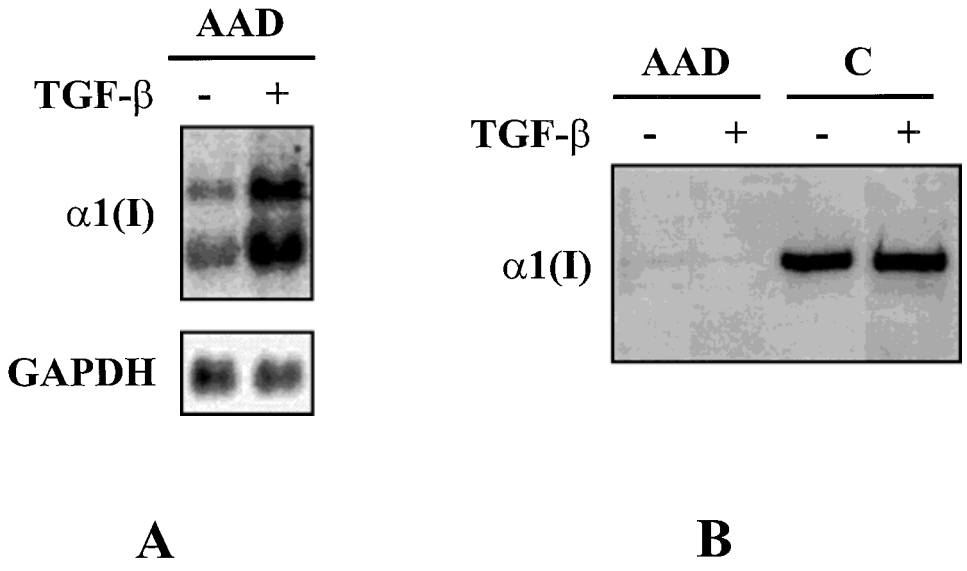

Fig. 6. Effect of TGF-β on α1(I) procollagen mRNA and protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium each in the presence or absence of TGF-β (1 ng/ml) for 24 h. **A:** Total RNA was isolated and the expression of α1(I) procollagen mRNA was assessed by Northern blot analysis. **B:** Total protein was extracted from the cell layer and α1(I) procollagen protein levels were assessed by Western blot analysis.

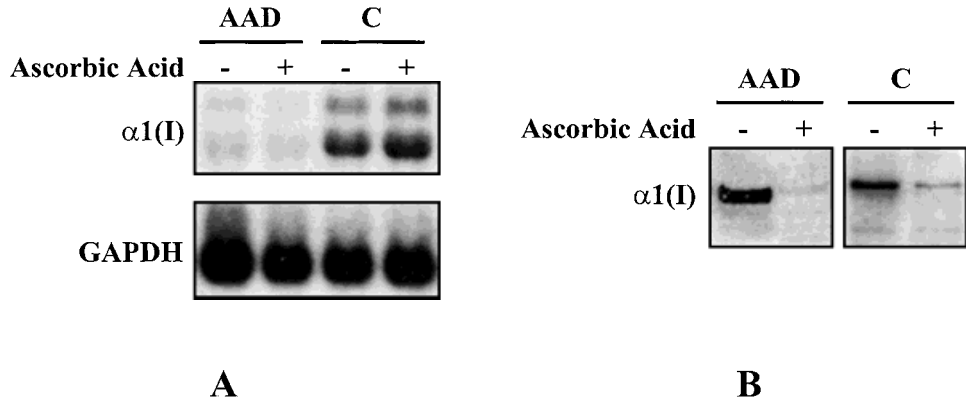

Fig. 7. Effect of ascorbic acid on α1(I) procollagen mRNA and protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient (AAD) or complete (C) medium each in the presence or absence of ascorbic acid (250 μM) for 24 h. **A:** Total RNA was isolated and the expression of α1(I) procollagen mRNA was assessed by Northern blot analysis. **B:** Total protein was extracted from the cell layer and α1(I) procollagen protein levels were assessed by Western blot analysis.

α1(I) procollagen protein levels (Fig. 8). The addition of chloroquine to fibroblasts incubated in amino acid deficient medium in the absence of ascorbic acid did not affect α1(I) procollagen protein levels (Fig. 8).

DISCUSSION

The effect of amino acid deprivation on α1(I) procollagen protein levels in quiescent human lung fibroblasts was examined. Fibroblasts incubated in amino acid deficient medium decreased α1(I) procollagen protein levels without affecting fibronectin levels or total protein levels, indicating a selective effect on α1(I) procollagen. Other studies have shown that amino acid deprivation decreases the rate of protein synthesis [Vaughan et al., 1971; Venrooij et al., 1972]. However, in our studies fibroblasts were rendered quiescent by reducing the serum content of the medium and total protein levels were not affected by amino acid deprivation.

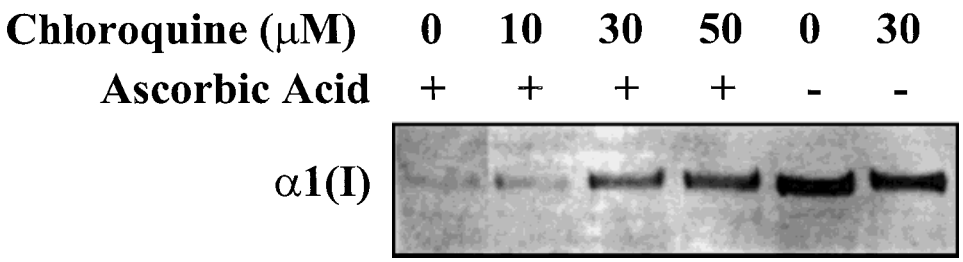

Fig. 8. Effect of chloroquine on α1(I) procollagen protein levels. Quiescent lung fibroblast cultures were incubated in amino acid deficient medium for 48 h. The medium was replaced with amino acid deficient medium containing chloroquine at the indicated concentrations in the presence or absence of ascorbic acid (250 μM) for 24 h. Total protein was extracted from the cell layer and α1(I) procollagen protein levels were assessed by Western blot analysis.

Quiescent fibroblasts incubated in amino acid deficient medium decrease α1(I) procollagen mRNA levels by mechanisms involving decreases in both the rate of transcription and the stability of the transcript [Krupsky et al., 1997]. In these studies, amino acid deprivation resulted in a more pronounced decrease in α1(I) procollagen protein levels than inα1(I) procollagen mRNA levels, suggesting post-transcriptional events were responsible for the further decrease inα1(I) procollagen protein levels. This was supported by the observation that the addition of TGF-β to fibroblasts incubated in amino acid deficient medium increased α1(I) procollagen mRNA levels, but had no effect on α1(I) procollagen protein levels.

Cystine availability regulates α1(I) procollagen mRNA levels in quiescent human lung fibroblasts [Rishikof et al., 1998a]. Cystine also regulated α1(I) procollagen protein levels. A threshold cystine concentration of 0.02 mM was necessary to detect α1(I) procollagen protein levels. Human lung fibroblasts transport cystine almost exclusively by the amino acid transport system x_c^- in which the influx of cystine is coupled to the efflux of glutamate [Bannai and Kitamura, 1980 1982; Bannai, 1986]. Hyperoxia induces system x_c^- transport of cystine in fibroblasts [Bannai et al., 1989], whereas prostaglandin E_2 (PGE_2) inhibits system x_c^- transport [Rishikof et al., 1998b]. PGE_2 also decreases α1(I) procollagen mRNA and protein levels [Goldstein and Polgar, 1982; Fine et al., 1992]. Following uptake, cystine is rapidly reduced to cysteine in the intracellular compartment [States et al., 1974; Oshima et al., 1976]. Intracellular cysteine is necessary for the biosynthesis of proteins, glutathione, and several active metabolites and may be necessary for the proper folding of α1(I) procollagen protein.

In the absence of ascorbic acid,α1(I) procollagen protein levels increased in quiescent fibroblasts incubated in either amino acid deficient or complete medium. These results are also consistent with post-transcriptional events since the α1(I) procollagen mRNA levels were not affected by ascorbic acid. Ascorbic acid is a cofactor for the enzymatic activity of prolyl hydroxylase in the formation of hydroxyproline in procollagen. Hydroxyproline residues stabilize the triple helical structure of procollagen. The absence of ascorbic acid results in the accumulation of underhydroxylated, nonhelical procollagen in the ER [Rowe and Schwarz, 1983; Schwarz, 1985].

The increased α1(I) procollagen protein levels in quiescent fibroblasts incubated in the absence of ascorbic acid are likely a result of the inhibition of translocation through the ER due to improper folding. Improperly folded eukaryotic secretory proteins are usually unable to pass through the secretory pathway and are either retained or degraded intracellularly [Lippincott-Schwartz et al., 1988; Lodish, 1988; Rose and Doms, 1988]. These findings suggest that following amino acid deprivation, the translational mechanism for α1(I) procollagen is intact and post-translational events are responsible for the decreased α1(I) procollagen protein levels.

In the presence of ascorbic acid, intracellular α1(I) procollagen protein levels in amino acid deprived quiescent fibroblasts increased when treated with chloroquine. Chloroquine is a lysosomotropic agent that raises the pH in lysosomes and inactivates lysosomal hydrolases [Wibo and Poole, 1974; Amenta et al., 1978]. Chloroquine inhibits the degradation of intracellular proteins [Wibo and Poole, 1974; Amenta et al., 1978; Berg et al., 1984] including newly

synthesized α1(I) procollagen protein that is improperly folded [Berg et al., 1980]. In the absence of ascorbic acid, intracellular α1(I) procollagen protein levels in amino acid deprived fibroblasts were not affected when treated with chloroquine. This was likely due to the inhibition of translocation of procollagen through the ER and the Golgi into lysosomes for degradation. These data suggest that following amino acid deprivation, newly synthesized α1(I) procollagen protein was degraded intracellularly primarily by a process that involved lysosomal proteinases.

Brefeldin A and monensin cause retention of secretory proteins in the ER and the Golgi, respectively [Mollenhauer et al., 1990; Klausner et al., 1992; Andersson and Warburton, 1995]. Their use in HFL-1 fibroblasts demonstrate that type I collagen is not degraded in these organelles [Andersson and Warburton, 1995]. In IMR-90 fibroblasts incubated in amino acid deficient or complete medium in the absence of ascorbic acid, α1(I) procollagen protein was retained in the ER, but without apparent degradation. In amino acid deficient medium in the presence of ascorbic acid, α1(I) procollagen protein was translocated through the ER and the Golgi into lysosomes where it was degraded.

Prior to secretion, 15 to 30% of newly synthesized collagen is degraded intracellularly [Rennard et al., 1982; Bienkowski, 1984a,b]. The mechanism of basal intracellular collagen degradation is incompletely understood, however, it is thought to occur in nonlysosomal compartments [Berg et al., 1980; Barile et al., 1990]. In cells induced to synthesize improperly folded collagen using proline analogs, intracellular collagen degradation can be increased [Berg et al., 1980]. The increase in intracellular degradation above the basal level occurs in lysosomes [Berg et al., 1980]. The results presented here suggest that following amino acid deprivation of quiescent fibroblasts, newly synthesized type I collagen is improperly folded and undergoes intracellular degradation in lysosomes.

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