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Inhibition of Collagen Type I Synthesis by Skin Fibroblasts of Graft Versus Host Disease and Scleroderma Patients: Effect of Halofuginone

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ABSTRACT. The effect of halofuginone (a plant alkaloid) on collagen α1(I) gene expression and collagen synthesis was evaluated in human skin fibroblasts from patients with chronic graft-*versus*-host disease (cGvHD) or scleroderma and from a normal individual. Halofuginone caused a dose-dependent inhibition in collagen α1(I) gene expression and collagen synthesis in all cultures tested, the cGvHD fibroblasts being the least sensitive. In normal and scleroderma fibroblasts, concentrations of halofuginone as low as 10^{-10} M and 10^{-9} M were sufficient to cause a significant reduction in collagen α1(I) gene expression and collagen synthesis, respectively. In addition, halofuginone also inhibited the transforming growth factor β-induced collagen synthesis. Three days after halofuginone removal, collagen gene expression returned to control levels. The reduction of collagen mRNA transcript levels by halofuginone appeared to be dependent on new protein synthesis because simultaneous treatment of fibroblasts with protein synthesis inhibitors prevents the suppressive effect of halofuginone on collagen α1(I) mRNA gene expression. The ability of extremely low concentrations of halofuginone to inhibit collagen α1(I) synthesis specifically and transiently at the transcriptional level suggests that this material may be an important tool for studying collagen α1(I) gene regulation and may be used as a novel and promising antifibrotic therapy. BIOCHEM PHARMACOL 52;7:1057–1063, 1996.

KEY WORDS. fibrosis; autoimmune diseases; fibroblasts; collagen α1(I); plant alkaloid; TGFβ

Progressive fibroproliferative diseases exhibit excessive production of connective tissue, which results in the destruction of normal tissue architecture and function. The crucial role of collagen in fibrosis has prompted attempts to develop therapies that inhibit its accumulation [1]. Such therapies may target the synthesis of the procollagen polypeptide chains or inhibit some specific posttranslational events that will lead either to reduced formation of extracellular collagen fibers or to an accumulation of fibers with altered properties. Only a few inhibitors of collagen synthesis are available, despite the importance of this protein in sustaining tissue integrity and its involvement in various disorders. For example, nonphysiological amino acids have been used as competitive inhibitors of the action of key enzymes of collagen synthesis [2–4], and retinoids have been shown to inhibit collagen synthesis in animal models of fibrosis [5]. Most of these inhibitors, however, also inhibit the synthesis of other proteins or affect cell proliferation because they are not specific to the collagen biosynthetic pathway.

We demonstrated that halofuginone, an alkaloid originally isolated from the plant *Dichroa febrifuga* and commonly used as a coccidiostat in chickens and turkeys, suppressed avian skin collagen synthesis *in vivo* [6]. In culture, halofuginone attenuated collagen α1(I) gene expression and collagen production by murine and avian skin fibroblasts without affecting cell proliferation or collagenase activity [7]. Halofuginone specifically inhibited type α1(I) but not type II [7] or type III [8] collagen gene expression.

SSc[‖] is an autoimmune disease of connective tissue, characterized by excessive deposition of collagen in the dermis and multiple internal organs [9–11]. Elevated levels of collagen were demonstrated in skin biopsies derived from SSc patients [12]. In most cases, fibroblasts cultured from scleroderma patients synthesized higher levels of collagen than did fibroblasts of normal individuals [13–15]. The increase

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‖ *Abbreviations:* cGvHD, chronic graft-*versus*-host disease; SSc, systemic sclerosis, scleroderma; TSK, tight skin mouse; DMEM, Dulbecco's modified Eagle's medium; CDP, collagenase digestible proteins; NCDP, noncollagenase digestible proteins; TGFβ, transforming growth factor β.

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in collagen synthesis was the result of induction of collagen α1(I) gene expression, which was observed in skin biopsies of scleroderma patients *in vivo* [15] and by skin fibroblasts *in vitro* [16]. SSc has many clinical features in common with other connective tissue, autoimmune, and autoimmunelike diseases. cGvHD, a major complication occurring in patients after allogeneic bone marrow transplantation, is believed to be the result of an autoimmunelike process mediated by immunocompetent T cells [17]. Chronic GvHD often results in sclerodermoid-fibrotic skin lesions [18]. We demonstrated that halofuginone in a dose-dependent manner abrogated the increase in skin collagen, prevented the thickening of the dermis and the loss of the subdermal fat in two *in vivo* models of scleroderma: the murine cGvHD and the TSK [19].

The usefulness of antifibrotic drugs depends on their specificity and requires identification of the mechanism of their action. In this study we have evaluated the effect and mode of action of halofuginone on collagen synthesis and collagen α1(I) gene expression in skin fibroblasts derived from cGvHD and SSc patients.

MATERIALS AND METHODS

Reagents

[2,3,4,5 ^{3}H]L-proline (122 Ci/mmole) and [α-^{32}P]ATP (6000 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, England). Highly purified *Clostridium histolyticum* collagenase type VII free of nonspecific proteases, β-aminopropionitrile, TGFβ, DMEM, actinomycin D, cycloheximide, and trypsin-EDTA solution (0.25%–0.02%) were obtained from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Biochemical Industries (Bet-HaEmek, Israel). Halofuginone bromhydrate-[trans-7-bromo-6-chloro-3-((3-(3-hydroxy-2-piperidyl) acetonyl))-4(3H)-quinazolinone hydrobromide] was obtained from Roussel UCLAF (Paris, France). A 4.0-Kb XbaI-SalI fragment of the human proα1(I) collagen plasmid-COL1A1 [20] was used as a probe for Northern blot analysis.

Cells

Skin biopsies (3 mm) were taken from three different cGvHD patients (38–45 years old) and one SSc patient (diffuse cutaneous scleroderma) from areas that were macroscopically and histopathologically involved with the disease, which was from the upper back below the right scapula. The cGvHD diagnoses was confirmed by pathology. All three cGvHD patients had extensive cGvHD according to the Seattle grading criteria [21]. A normal skin biopsy was taken from a 42-year-old woman who had undergone plastic surgery. All biopsies were taken according to the guidelines established by the Hadassah University Hospital Experimentation Committee. Biopsies were placed as explants, dermis down, in a 35-mm tissue culture dish in 1 mL EM (enriched medium based on DMEM with 10% inactivated FCS, 2mM L-glutamine, 0.1% nonessential amino acids, 100 mg/mL penicillin, and 100 pg/mL streptomycin) and incubated at 37°C in a humidified atmosphere of 5% CO_2. Media were changed once a week until primary fibroblasts grew out of the explant and became confluent. Fibroblasts (matched for passage number) were seeded in EM in 35-mm^2 petri dishes or in wells of multiwell cluster tissue culture plates. Rat-1-transformed fibroblast cell line was cultured in DMEM containing 5% FCS [7]. All experiments were performed with cells that were approximately 80% confluent.

Evaluation of Collagen and Noncollagen Protein Synthesis

Cells were incubated with different concentrations of halofuginone in 0.5 mL glutamine-free DMEM containing 5% FCS, ascorbic acid (50 μg/mL), β-aminopropionitrile (50 μg/mL), and 2 μCi of [^{3}H]proline. At the end of incubation, the medium was decanted and incubated with or without collagenase for 18 hr, followed by TCA precipitation. The amount of radiolabeled collagen was estimated as the difference between total proline [^{3}H]-containing proteins and those left after collagenase digestion [22, 23].

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated by using the guanidinium-thiocyanate-phenol technique [24]. RNA was subjected to 1% agarose denaturing gel electrophoresis followed by blotting onto a nylon filter (GeneScreen Plus, New England Nuclear, Boston, MA, USA). cDNA probes were labeled by using the random primer procedure [25], with a commercial kit (Boehringer, Germany). Hybridization was performed overnight at 40°C in a solution containing 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 40% formamide, and 200 mg/mL denatured herring sperm DNA. Hybridization was followed by two 30-min washes in 2 × SSc (1 × SSc contains 0.15 M NaCl and 0.015 M sodium citrate)/1% SDS, and two 20-min washes in 1 × SSc/0.1% SDS. The filters were exposed to X-ray film (Agfa-Curix) −70°C by using intensifying screens. RNA was monitored by hybridization of the same blots with murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA.

RESULTS

Inhibition of Collagen α1(I) Gene Expression by Halofuginone

Cultures of skin fibroblasts from one normal and one SSc patient (Fig. 1) and three cGvHD patients (Fig. 2) were prepared. After 24 hr incubation with different concentrations of halofuginone, total RNA was prepared and Northern blot analysis was performed by using a specific probe for human collagen α1(I). Collagen α1(I) gene expression was attenuated in response to halofuginone in all cultures

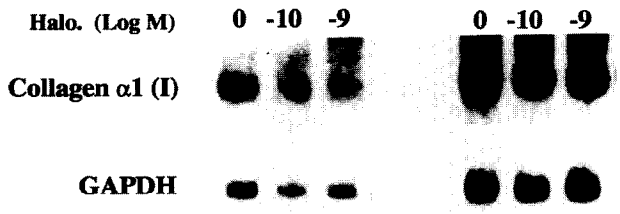

FIG. 1. Inhibition of collagen α1 (I) gene expression by halofuginone. Skin fibroblasts of a healthy individual (left panel) and an SSc patient (right panel) were incubated with different concentrations of halofuginone for 24 hr. At the end of the incubation, total RNA was prepared, and collagen α1 (I) gene expression was assessed by hybridization with the human proα1(I) collagen plasmid COL1A1. Each lane was loaded with 20 μg RNA, and the integrity and the amount of RNA was monitored by hybridizing the same blots with a GAPDH cDNA-specific probe.

tested. In the normal and the SSc skin fibroblasts, concentrations of halofuginone as low as 10^{-10} M were sufficient to cause a significant reduction in collagen α1(I) gene expression. Higher concentrations of halofuginone further reduced collagen α1(I) gene expression (Fig. 1). Cells derived from the cGvHD patients were less sensitive, and concentrations between 10^{-8} and 10^{-7} M of halofuginone were needed to demonstrate significant inhibition in collagen α1(I) gene expression (Fig. 2).

Inhibition of Collagen Synthesis by Halofuginone

Skin fibroblasts incubated for 24 hr in the presence of [^{3}H]proline exported radiolabeled proteins into the medium, of which 40% were collagenase digestible (CDP) and the remaining were, by definition, noncollagenase digestible proteins (NCDP). Halofuginone inhibited, in a dose-dependent manner, the radiolabeled proline incorporation into CDP by skin fibroblasts derived from the healthy individual and the scleroderma and cGvHD patients (Fig. 3). Fibroblasts from the healthy individual were the most sensitive to halofuginone compared with fibroblasts derived from either SSc or cGvHD patients. A decrease in [^{3}H]proline incorporation into CDP was observed at concentrations of halofuginone as low as 10^{-9} M. Fibroblasts from cGvHD patients were the least sensitive; concentrations of

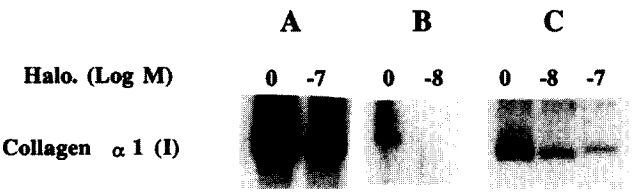

FIG. 2. Halofuginone inhibition of collagen α1(I) gene expression by skin fibroblasts of three cGvHD patients. Cells were incubated with different concentrations of halofuginone for 24 hr. At the end of the incubation, total RNA was prepared, and collagen α1(I) gene expression was assessed by hybridization with the human proα1(I) collagen plasmid COL1A1. Each lane was loaded with 20 μg RNA. A, B, and C represent three different patients.

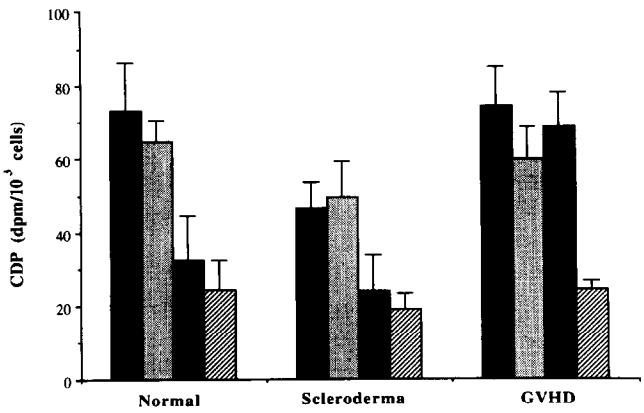

FIG. 3. Halofuginone inhibition of [^{3}H]proline incorporation to CDP exported by skin fibroblasts. Cells from a healthy subject and from cGvHD and SSc patients were incubated for 24 hr in the presence of [^{3}H]proline and different concentrations of halofuginone. At the end of the incubation, the media were collected and [^{3}H]proline-containing CDP was evaluated. The results are expressed as the means ± SE of four different wells. From left to right: light gray bar, control, no addition; thick-striped bar, 10^{-9}M; thin-striped bar, 10^{-8}M; dark gray bar, 10^{-7}M of halofuginone.

10^{-7} M were needed to inhibit significantly [^{3}H]proline incorporation into CDP (Fig. 3).

TGFβ has been suggested to play a role in regulating collagen production in scleroderma [26]. Incubation of skin fibroblasts derived from the healthy individual with TGFβ for 24 hr caused a dose-dependent induction of [^{3}H]proline incorporation to CDP: 150% of control with 3 ng/mL TGFβ. Halofuginone at a concentration of 10^{-8} M inhibited basal [^{3}H]proline incorporation into CDP by 44%. When incubated together, halofuginone inhibited TGFβ-induced collagen synthesis by 45% and 41% in the presence of 1 and 3 ng/mL TGFβ, respectively (Table 1).

Transient Inhibition of Collagen Synthesis by halofuginone

Experiments were performed to evaluate the time needed for fibroblasts to recover after halofuginone removal. Skin

TABLE 1. Inhibition of [^{3}H]proline incorporation to CDP in TGFβ-induced cells by halofuginone

Treatment	CDP (dpm × 10^5 cells)
Control	265 ± 10^b
TGFβ 1 ng/ml	289 ± 13^b
TGFβ 3 ng/ml	404 ± 9^a
Halofuginone 10^{-8} M	116 ± 4^d
TGFβ 1 ng/ml + halofuginone 10^{-8} M	131 ± 4^d
TGFβ 3 ng/ml + halofuginone 10^{-8} M	167 ± 5^c

Fibroblasts derived from a normal individual were incubated in the presence of [^{3}H]proline and TGFβ (1 and 3 ng/mL) or halofuginone (10^{-8} M), or a combination of the two. After 24 hr, the media were collected and the radiolabeled CDP was determined. The results are expressed as the means ± SE of four wells. Means without a common superscript differ significantly ($P < 0.05$) according to Duncan's multiple range test.

fibroblasts from a cGvHD patient were incubated with or without 10^{-7} M halofuginone for 24 hr, after which the media were replaced with fresh media without halofuginone (Fig. 4). The inhibitory effect of halofuginone on [^{3}H]proline incorporation into CDP by skin fibroblasts persists at the same level 24 hr after removal of halofuginone from the medium. In the second experiment, skin fibroblasts derived from the same patient were incubated with or without 10^{-8} M halofuginone for different time periods up to 72 hr. Incubation of the cells with halofuginone for 72 hr caused a marked reduction in collagen α1(I) gene expression (Fig. 5, lane 2). Cells incubated for 24 hr in the presence of halofuginone and for an additional 48 hr without halofuginone recovered and exhibited the same collagen α1(I) gene expression as the untreated cells (Fig. 5, lane 3). Cells incubated with halofuginone for 24 hr, after which collagen α1(I) gene expression was evaluated immediately or 24 hr later, did not recover and exhibited low levels of α1(I) collagen gene expression (Fig. 5, lanes 4, 5). These results suggest that more than 24 hr are needed for the cells to recover after halofuginone removal.

Halofuginone Mode of Action

Two approaches were taken to elucidate possible regulation of the collagen α1(I) gene by halofuginone. The first is DNA methylation of the collagen α1(I) gene, which was correlated with inhibition of the collagen gene expression [27], and the second is indirect effect of halofuginone on collagen α1(I) gene expression.

Cells were incubated with 5×10^{-6} M 5-azacytidine (5-azaC), a potent inhibitor of DNA methyltransferase, or

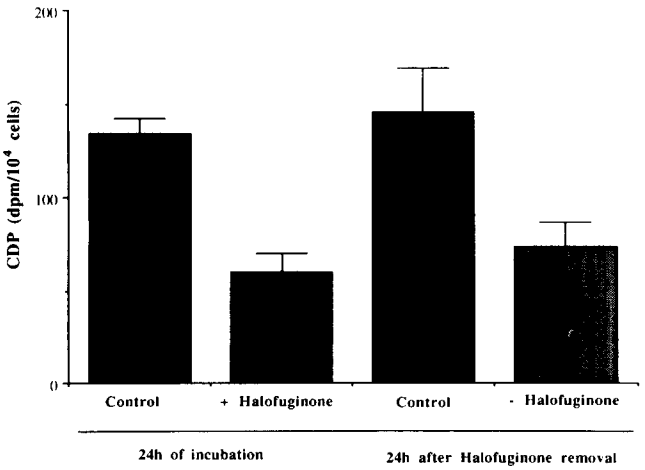

Fig. 4. Effect of halofuginone removal on [^{3}H]proline incorporation to CDP exported by skin fibroblasts of a cGvHD patient. Cells were incubated with or without 10^{-7} M halofuginone for 24 hr, after which the media were collected for determination of [^{3}H]proline-containing CDP. In parallel wells, the media were replaced by fresh media without halofuginone for an additional 24 hr and then [^{3}H]proline incorporation to CDP was determined. The results are the means ± SE of four different wells.

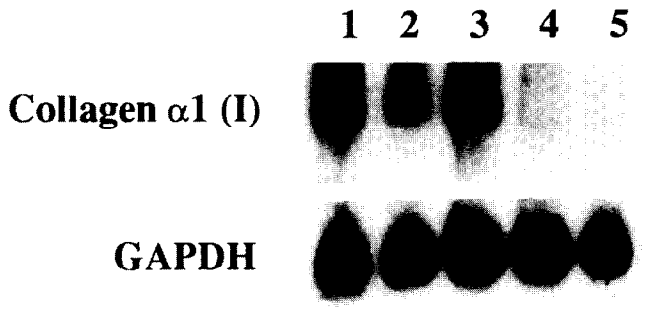

FIG. 5. Effect of halofuginone removal on collagen α1(I) gene expression by skin fibroblasts of a cGvHD patient. Cells were incubated for 72 hr with or without 10^{-8} M halofuginone. Lane 1: Control cells incubated for 72 hr without halofuginone. Lane 2: Cells incubated for 72 hr with halofuginone. Lane 3: Cells incubated for the first 24 hr with halofuginone and an additional 48 hr without the drug. Lane 4: Cells incubated for 24 hr without halofuginone followed by 24 hr with the drug, after which the media were replaced with fresh media without the drug for an additional 24 hr. Lane 5: Halofuginone was added only for the last 24 hr. At the end of the incubation, RNA was prepared, and collagen α1(I) gene expression was assessed by hybridization with the 4.0 Kb XbaI-SalI fragment of the human proα1(I) collagen plasmid COL1A1. Each lane was loaded with 20 μg RNA, and the integrity and amount of the RNA were monitored by hybridizing the same blots with a GAPDH cDNA-specific probe.

halofuginone (10^{-9} M), or a combination of the two. After 24 hr, [^{3}H]proline incorporation to CDP and NCDP was determined. 5-azaC by itself did not affect [^{3}H]proline incorporation to collagen and noncollagen proteins and was unable to restore the halofuginone-dependent inhibition of collagen synthesis (data not shown).

To determine whether halofuginone affects collagen α1(I) gene directly, fibroblasts were incubated with halofuginone alone or with combination of protein synthesis inhibitors (Fig. 6). Halofuginone at a concentration of 10^{-8} M inhibited collagen α1(I) mRNA levels as compared with the untreated cells (Fig. 6, lanes 2,6,10). Actinomycine D at concentration of 5 μg/mL, which did not affect collagen α1(I) mRNA levels (Fig. 6, lanes 3,7), abolished the halofuginone-dependent collagen α1(I) gene expression after 3 and 6 hr of incubation (Fig. 6, lanes 4,8). Similar results were observed with 10 μg/mL of cycloheximide (Fig. 6, lanes 9–12).

DISCUSSION

We reported that halofuginone inhibited collagen synthesis in avian skin [6], in cGvHD and the TSK *in vivo* [19], and by avian and murine fibroblasts in culture [7]. Specific inhibition of collagen α1(I) by halofuginone was demonstrated by using cells that in culture express more than a single type of collagen such as avian chondrocytes (types I and II) [7] and smooth muscle cells (types I and III) [8]. To assess the potential role of halofuginone as antifibrotic therapy, we evaluated its effect on [^{3}H]proline incorpora-

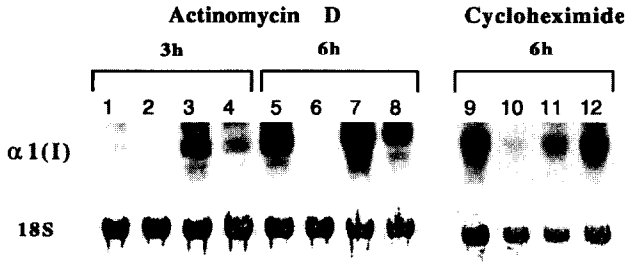

FIG. 6. Effect of actinomycine D and cycloheximide on halofuginone-dependent inhibition of collagen α1(I) gene expression. Rat-1 cells were cultured in petri dishes with 10 mL of DMEM with 5% FCS. When the cultures reached 75% confluency, the media was replaced with fresh DMEM containing halofuginone, actinomycine D, cycloheximide, and combinations of halofuginone with each of the protein synthesis inhibitors. At the end of the incubation (3 or 6 hr), RNA was prepared, and collagen α1(I) gene expression was assessed by hybridization with the human proα1(I) collagen probe. Each lane was loaded with 20 μg RNA, and the integrity and amount of the RNA were monitored by methylene blue staining of 18S RNA. Lanes 1, 5, 9: Controls: no additive. Lanes 2, 6, 10: 10^{-8}M halofuginone. Lanes 3 and 7: 5 μg/mL actinomycine D. Lane 11: 10 μg/mL cycloheximide. Lanes 4 and 8: 10^{-8}M halofuginone and 5 μg/mL actinomycine D. Lane 12: 10^{-8}M halofuginone and 10 μg/mL cycloheximide.

tion into collagen and collagen α1(I) gene expression in human skin fibroblasts derived from a healthy individual and from cGvHD and SSc patients. In these cultures, no overproduction of collagen was observed (Fig. 3). This result may be the result of heterogeneity of the cultured fibroblast subpopulations [16, 28] or of a reduction in the increase in collagen synthesis to the level of nonaffected controls during subculturing [29]. At this stage, because of the limited number of patients used in this study and the variation in collagen synthesis by different cultures, a comparison of sensitivity against halofuginone between cells derived from different patients is impractical. Nevertheless, halofuginone inhibited in a dose-dependent manner collagen α1(I) gene expression (Figs. 1, 2) and [^{3}H]proline incorporation into collagen (Fig. 3) in all cultures tested. In SSc, the increase in collagen production is thought to be the result of the TGFβ released from lymphocytes, monocytes, and platelets present in the initial inflammatory phase of the disease, which stimulates the fibroblasts to produce increased amounts of collagen [26]. The production of TGFβ by SSc fibroblast as a mechanism for sustaining the initial stimulus in an autocrine manner is, however, controversial [30]. Halofuginone, in addition to its effect on the basal level of collagen synthesis, also inhibited the TGFβ-induced collagen synthesis (Table 1).

The effect of halofuginone on collagen type I synthesis is transient. One day after halofuginone removal, the cells still incorporated low levels of [^{3}H]proline into CDP (Fig. 4); 2 days after the cells still expressed low levels of collagen α1(I) RNA (Fig. 5). Only 3 days after halofuginone removal did the cells recover and collagen gene expression return to the control levels (Fig. 5).

The regulatory mechanisms responsible for the maintenance of normal procollagen mRNA expression have not been completely elucidated. One mechanism that has been correlated with inhibition of collagen gene expression is DNA methylation [27]. Loss of expression of collagen proα1(I) and proα2(I) genes is accompanied by hypermethylation of these genes in human SV40-transformed fibroblasts [31]. Methylation of the promoter and enhancer of proα1(I) collagen gene *in vitro* led to transcriptional inactivation [32]. Treatment of cells with 5-azaC, a potent inhibitor of DNA methyltransferase, restored transcriptional activity of the collagen genes. However, 5-azaC did not affect the halofuginone-dependent inhibition of collagen synthesis, suggesting that DNA methylation is not involved in halofuginone-dependent inhibition of collagen type I gene expression.

The many regulatory signals that can affect collagen gene transcription suggest that the collagen gene promoters are responsive to various *trans*-acting pathways. Several putative regulatory elements that may determine the transcriptional efficiency of the procollagen genes have been identified in their corresponding promoters [33, 34]. These putative regulatory elements are potential targets for the action of promoter-specific transcription effectors. Studies of the transcriptional regulation of different collagen genes in human and rodent cells demonstrated the presence of regulatory elements located immediately upstream of the transcription initiation site [20, 35–37]. Analysis of the murine α1(I) procollagen gene sequence encompassing −3700 to +1400 bp [38] revealed that a short segment of the promoter 220 bp upstream of the start of the transcription site was sufficient for expression of the gene, whereas farther upstream flanking sequences had negative effects on gene transcription. The reduction of collagen mRNA transcript levels by halofuginone appeared to be dependent on new protein synthesis because simultaneous treatment of fibroblasts with cycloheximide or actinomycin D blocks the suppressive effect of halofuginone on collagen α1(I) mRNA gene expression. The nature of the newly synthesized protein(s) and how it functions to decrease procollagen mRNA levels is unknown, but it could act as repressor a gene directly or indirectly by repressing transcription factors that activate procollagen promoter. Alternatively, newly synthesized proteins could decrease procollagen mRNA levels by decreasing mRNA stability or by enhancing mRNA decay.

Halofuginone, when fed to chicks immediately after hatching at concentrations that cause maximal reduction in skin collagen formation, did not alter the growth rate, suggesting that longitudinal bone growth was not affected [6]. Tissue-specific promoter sequences in transfected cells [37] and in cells from vascular smooth muscle [39] and osseous tissue [40] were identified in addition to the many promoter sequences that control fibroblasts collagen α1(I) gene. The existence of such specific promoter sequences within the collagen α1(I) gene may also explain the differ-

ence in sensitivity to halofuginone in transformed cells compared with fibroblasts in primary cultures [7] and the higher levels of halofuginone needed to demonstrate a significant effect on collagen α1(I) gene expression (Figs. 1, 2) and [^{3}H]proline incorporation to CDP (Fig. 3) by fibroblasts derived from the cGvHD patients compared with fibroblasts taken from a healthy subject.

In summary, we have demonstrated that halofuginone inhibits collagen synthesis by human skin fibroblasts and by cGvHD and SSc patient fibroblasts. The need for extremely low concentrations of halofuginone to inhibit specifically collagen type I synthesis at the transcriptional level regardless of animal species and the fact that halofuginone exerts its effect on skin collagen content at concentrations that do not affect cell proliferation *in vitro* [7] or animal growth *in vivo* [6, 19] suggest that halofuginone, on one hand, can be used as an important tool to understand the regulation of the collagen α1(I) gene and, on the other, may become a novel and promising antifibrotic therapy.

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