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Reduction in dermal fibrosis in the tight-skin (Tsk) mouse after local application of halofuginone

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Abstract

The effect of dermal application of halofuginone—an inhibitor of collagen type I synthesis—on skin collagen and collagen α1(I) gene expression in an animal model of scleroderma and chronic graft versus host disease (cGvHD) was evaluated. Halofuginone-containing cream was applied on the tight-skin mouse (Tsk) and skin biopsies were taken for collagen staining by sirius red and for collagen α1(I) gene expression by *in situ* hybridization. In addition, cell proliferation was evaluated by immunostaining for proliferation cell nuclear antigen (PCNA) alone or in combination with collagen α1(I) probe. The number of mast cells was assessed by toluidine blue. Dermal application of halofuginone (0.01%) for 60 days was as good as systemic administration (1 μg/mouse/day) in reducing collagen α1(I) gene expression in skin biopsy and almost as good in reducing skin width. Halofuginone was stable and effective only at acidic pH. The effect of halofuginone (0.03%) was time-dependent. After 40 days of daily treatment, a significant reduction in the collagen α1(I) gene expression was observed and further decrease was observed after 60 days. The reduction in collagen α1(I) gene expression and the reduction in the proliferation of dermal fibroblasts probably occur in the same subset of cells. No effect of halofuginone on the proliferation of keratinocytes or on mast cell number was observed. These results suggest that target-oriented application of halofuginone may become a novel therapy for fibrotic disorders in general and for scleroderma in particular.

Keywords: Extracellular matrix; Collagen type I; Fibrosis; Scleroderma

1. Introduction

SSc and cGvHD, which occur in 25–40% of patients following allogeneic transplantation, are multistage autoimmune connective tissue disorders characterized by deposition of collagen and other connective tissue components in the skin and visceral organs. At present, no adequate therapy exists for these diseases, and in many instances they progress inexorably, eventually causing significant patient morbidity and death. Although the mechanisms responsible for the excessive connective tissue deposition in SSc and cGvHD are unknown, many of the clinical manifestations are due to the progressive fibrosis of affected organs [1,2]. Collagen type I has been identified as the major ECM protein synthesized *in vitro* by fibroblasts derived from the skin of SSc patients [3] and *in vivo* by those in skin biopsies [4]. Direct transcriptional activation of the collagen α1(I) gene was observed in skin fibroblasts derived from SSc patients [5] which probably occur in a subset of the fibroblast population [6]. Increased synthesis of other matrix proteins such as glycosaminoglycans has also been demonstrated [7]. No animal model exhibits all aspects of SSc and cGvHD, but the cutaneous hyperplasia with histopathological and biochemical alterations in skin similar to those found in human SSc occur in the Tsk mouse [8]. The mouse Tsk mutation has been localized to the fibrillin-1 gene on chromosome 2 [9]. The fibrillin-1 gene in the Tsk mice contains a tandem internal duplication that grossly alters the structure and function of the encoded protein, a key component of the ECM microfibrils. The mutation is transmitted

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Abbreviations: Tsk, the tight-skin mouse; cGvHD, chronic graft versus host disease; PCNA, proliferation cell nuclear antigen; SSc, systemic sclerosis; and ECM, extracellular matrix.

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as an autosomal dominant trait: the homozygous die *in utero* by 8–10 days of gestation, while the heterozygote Tsk/+ mice develop coetaneous hyperplasia and connective tissue abnormalities in skin, heart, and lungs [10,11]. The increase in connective tissue deposition was associated mainly with an increase in collagen type I [8,12], although increases in collagen types III [8], VI [13], and in glycosaminoglycans [14] were also observed.

Halofuginone is a well-known inhibitor of collagen type I synthesis [15]; it has been found to inhibit the gene expression of collagen type α1(I) but not of type II [16] or type III [17]. In culture, halofuginone has been found to attenuate collagen α1(I) gene expression and collagen production by murine, avian, and human skin fibroblasts, derived either from SSc and cGvHD patients [18]. In animal models of fibrosis, systemic administration of halofuginone prevented the increase in collagen synthesis and collagen α1(I) gene expression. The model included mice afflicted with cGvHD [19], rats with liver fibrosis caused by dimethylnitrosamine [20], rats with pulmonary fibrosis after bleomycin treatment [21], and rats developing adhesion following surgery [22,23]. In the Tsk mice, systemic administration of halofuginone caused a decrease in the collagen content and dermis width [19]. Obviously, a local administration of the drug is more target-oriented, and may be more efficacious with less toxicity. As a proof of principal, we reported that topical treatment of a single cGvHD patient with halofuginone caused a transient attenuation of collagen α1(I) gene expression [24]. In the present study, we used the Tsk mice as a further step towards developing a dermal therapy for SSc and cGvHD patients.

2. Materials and methods

2.1. Materials

Sirius red F3B was obtained from BDH Laboratory Supplies. A rat collagen α1(I) 1600-bp probe was a gift from B.E. Kream, University of Connecticut, CT, USA. Halofuginone bromhydrate was obtained from Collgard Biopharmaceuticals Ltd. PCNA antibodies were from Dako A/S.

2.2. Animals and treatments

All animal experiments were carried out according to the guidelines of the Hebrew University-Hadassah institutional committee for care and use of laboratory animals. Mice were kept in the Hadassah University Hospital animal germ-free facility with free access to water and food. Male Tsk mice aged 5–8 weeks from Harlan, USA were used in all experiments. The protocol included daily topical application of halofuginone cream on the back skin. Vehicle without halofuginone was applied on control mice. The oily phase contained white beeswax (125 g), soft white paraffin (125 g), and liquid paraffin (500 g), and the aqueous phase contained borax (10 g) and distilled water. The animals were routinely shaved prior to halofuginone application and no effect was observed on hair re-growth. Halofuginone, when given systemically, was injected intraperitoneally at a concentration of 1 μg/mouse/day.

2.3. Preparation of sections and in situ hybridization

Skin biopsies were collected into phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS at 4°. Serial 5-μm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. For hybridization, the sections were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions, rinsed in distilled water (5 min), and incubated in 2 $\times$ SSC at 70° for 30 min. The sections were then rinsed in distilled water and treated with pronase (0.125 mg/mL in 50 mM Tris–HCl, 5 mM ethylenediamine tetra acetic acid (EDTA), pH 7.5 for 10 min. After digestion, the slides were rinsed with distilled water, post-fixed in 10% formalin in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded ethanol solutions, and air-dried for several hours. Before hybridization, the 1600-bp rat collagen α1(I) insert was cut out from the original plasmid (pUC18) and inserted into pSafyre. The sections were then hybridized with digoxigenin-labelled collagen α1(I) probe [20]. For blocking, slides were incubated with 3% goat serum in TBS for one hour after which they were incubated with goat polyclonal digoxigenin antibodies conjugated to alkaline phosphatase in 1% serum for an additional hour. Color was developed with alkaline phosphatase substrate NBT (4-nitro blue tetrazolium chlorid) and BCIP (5-bromo-4-cloro-3-indolyl-phosphate) in the presence of levamizole. No hybridization was observed with the sense probe.

2.4. Immunostaining for PCNA

To assess cell proliferation, sections were immunostained with PCNA antibodies diluted 1:50 with 3% goat serum for 1 hr following 45 min of incubation with horseradish peroxidase-conjugated goat antibodies to mouse IgG, and peroxidase activity was revealed by using DAB as chromogen. Negative controls without primary anti-PCNA antibody did not show any nuclear staining. Digitized maps of the *in situ* hybridization for collagen α1(I) and PCNA staining were analyzed by means of NIH image software. A binary presentation was derived and used in determination of the stain density [25]. The results are the mean of 5 sections from the same area size from each biopsy. For double staining, skin sections were first hybridized with the collagen α1(I) probe without the alkaline phosphatase substrates. In the second step, the sections were immunostained with the PCNA antibodies and then the alkaline phosphatase substrates were added until purple color was achieved.

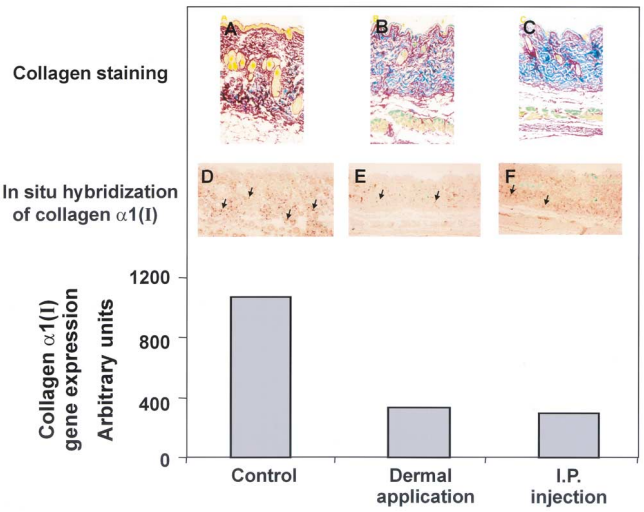

Fig. 1. Effects of halofuginone given locally or systemically on collagen synthesis in Tsk mice. Skin biopsies were taken from control mice, mice treated with halofuginone locally (0.01% daily), and mice treated with halofuginone systemically by i.p. injection (1 μg/mouse once a day). After 60 days, skin biopsies were stained for collagen (upper panel) and hybridized with the collagen α1(I) probe (middle panel). The lower panel is the quantitative image analysis of the *in situ* hybridization (mean of two mice/group). Note the increase in collagen degradation (represented by blue color), decrease in the dermis width, and decrease in the expression of the collagen α1(I) gene expression due to halofuginone treatment.

2.5. Staining for collagen and mast cells

Differential staining of collagenous and non-collagenous proteins was performed with 0.1% sirius red with 0.1% fast green as a counter-stain, in saturated picric acid. By this procedure collagen is stained red [20]. Mast cells were identified by toluidine blue, and cells containing metachromatic granules were counted in 10 random grids.

2.6. Halofuginone analysis

Halofuginone was determined by high pressure liquid chromatography (HPLC, HP 1050) with HPCHEM software using a C8 column. Halofuginone was eluted with acetonitrile and 0.1 M sodium citrate buffer pH 3.0 at a 30:70 v/v and detected by UV at 254 nm. The retention time for halofuginone under these conditions was 8.8 min.

3. Results

3.1. Effect of local versus systemic administration of halofuginone on skin collagen synthesis

Tsk mice were treated locally with a vehicle cream as a negative control, with a cream containing 0.01% halofuginone, or injected intraperitoneally with halofuginone (1 μg/mouse once a day). After 60 days of treatment, skin biopsies were taken for evaluation of collagen α1(I) gene expression by *in situ* hybridization and stained for collagen by sirius red (Fig. 1). The duration of the experiment was based on our previous study, demonstrating the efficacy of systemic administration of halofuginone [19]. In all the control mice, high levels of the collagen α1(I) gene expression were observed, which is characteristic of the Tsk phenotype. The skin biopsies of the Tsk mice either treated with 0.01% ointment or injected intraperitoneally, with halofuginone, exhibited much lower expression of the collagen α1(I) gene. Although skin width and skin collagen staining were reduced with both routes of administration, somewhat better results were observed with the systemic treatment (Fig. 1).

3.2. Effect of pH of halofuginone cream on skin collagen synthesis

Stability studies of halofuginone in buffer solutions and in cream revealed that halofuginone is stable at pH 5.5, but increases in both pH and temperature caused halofuginone degradation. After 7 days in cream at pH 5.5, no degradation of halofuginone was observed at temperatures of 4 and 30° while at pH 9.5, degradations of 7% and 40%, respectively, were observed. The effect of pH on the biological activity of halofuginone was verified in *in vivo* studies. Tsk mice were treated daily with 0.03% halofuginone cream at pH 9.0 or 5.5 for 57 days. No differences were observed in the expression of the collagen α1(I) gene, skin collagen staining, or skin width between the control animals and those treated with halofuginone at pH 9.0 (Fig. 2). At pH 5.5, halofuginone caused a 60% decrease in the collagen α1(I) gene expression, accompanied by decreases in skin collagen staining and skin width.

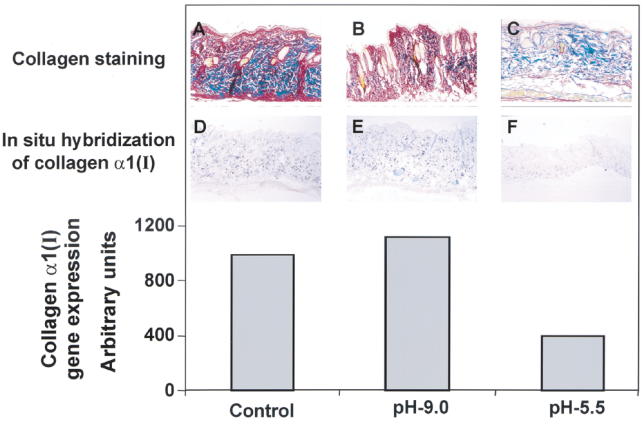

Fig. 2. Effect of pH of halofuginone cream on skin collagen synthesis. Halofuginone was applied on the skin of the Tsk mice at pH of 9.0 or 5.5, both at a concentration of 0.03%. After 57 days, skin biopsies were stained for collagen (upper panel) and hybridized with the collagen α1(I) probe (middle panel). The lower panel is the quantitative image analysis of the *in situ* hybridization (mean of two mice/group). Note the decrease in the collagen staining and the decrease in the expression of the collagen α1(I) gene after applying the halofuginone at pH 5.5 compared with halofuginone at pH 9.0 or with the controls treated with carrier alone.

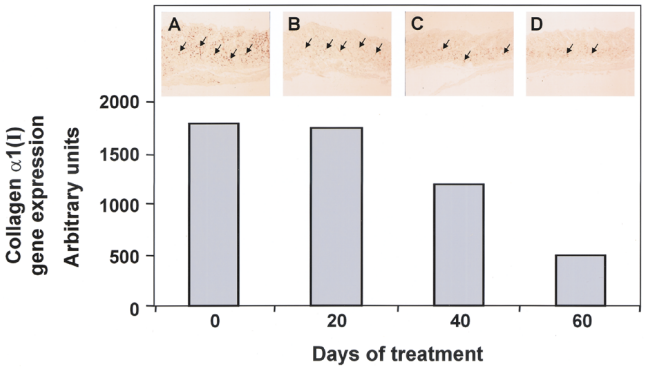

Fig. 3. The effect of halofuginone on collagen type I gene expression is time-dependent. Halofuginone was applied daily on the Tsk mice skin for various intervals. Skin biopsies were hybridized with the collagen α1(I) probe (upper panel). The lower panel shows the quantitative image analysis of the *in situ* hybridization (mean of two mice/group).

3.3. Effect of duration of halofuginone treatment on skin collagen synthesis

Tsk mice were treated daily with 0.03% halofuginone cream for various intervals. Skin biopsies taken after 20 days of halofuginone treatment exhibited the same levels of collagen α1(I) gene expression as the control treated with vehicle only (Fig. 3). After 40 days of daily treatment, a 35% reduction in the collagen α1(I) gene expression was observed, and a further reduction to 25% of the control level was observed after 60 days of treatment.

3.4. Effect of halofuginone on cell proliferation and mast cell number

In the control untreated Tsk mice, PCNA-stained nuclei were observed in the epidermal keratinocytes only at the basal and first suprabasal layers in epithelial cells of hair follicles, in epithelial cells of glandular appendages, and dermal fibroblasts (Fig. 4A). No accumulation of PCNA-stained fibroblasts was detected in any particular area of the dermis and the PCNA-positive fibroblasts were spread from the upper to the lower dermis. No changes in the distribution of numbers of PCNA-positive cells among the various locations were observed after 20 days of halofuginone treatment (Fig. 4B). After 40 days of treatment, fewer PCNA-staining nuclei were observed in the dermal fibroblasts (Fig. 4C) and after an additional 20 days, only a few fibroblasts at the lower part of the dermis were stained positive for PCNA (Fig. 4D). The PCNA staining of the epidermal keratinocytes and epithelial cells in hair follicles was not affected by the halofuginone treatment. All the PCNA-positive fibroblasts were also collagen type I-producing cells, as demonstrated by simultaneously immunostaining for PCNA and hybridizing with a specific collagen α1(I) probe (Fig. 5). The number of infiltrating mast cells was unaltered after halofuginone treatment at all the time points examined (data not shown).

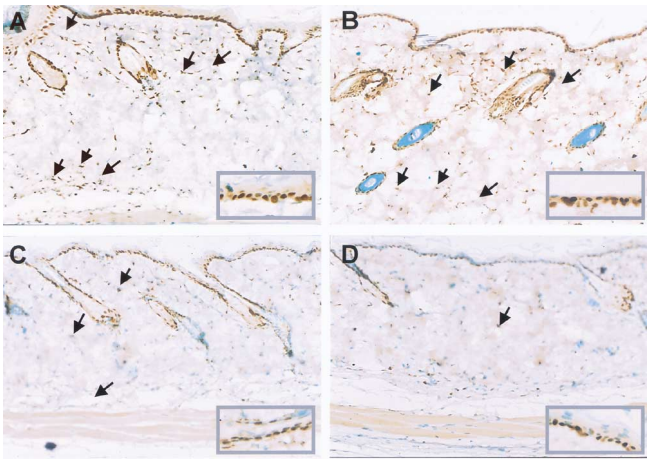

Fig. 4. Effect of halofuginone on cell proliferation. Skin biopsies taken from control Tsk mice (A); mice treated with halofuginone cream daily for 20 (B), 40 (C), and 60 (D) days were immunostained with anti-PCNA antibodies. The inset in each panel is a high-magnification image of the keratinocyte layer (×100). Note that halofuginone treatment caused reduction in the number of PCNA-positive cells in the dermis without affecting the proliferation levels of the keratynocytes.

4. Discussion

The aim of this study was to assess the efficacy of dermal application of halofuginone as a therapeutic modality for SSc, cGvHD, and other connective tissue abnormalities in which excessive collagen type I deposition is the hallmark of the disease. The mutant phenotype of the Tsk mice, which includes thickened skin and visceral fibrosis [9], has made the Tsk/+ mouse a favourite model for such a study. Dermal application of halofuginone was as efficient as systemic administration in reducing skin collagen α1(I) gene expression, although some beneficial effect of the systemic application on dermal width was observed (Fig. 1). Halofuginone was found to be stable at acidic pH and its

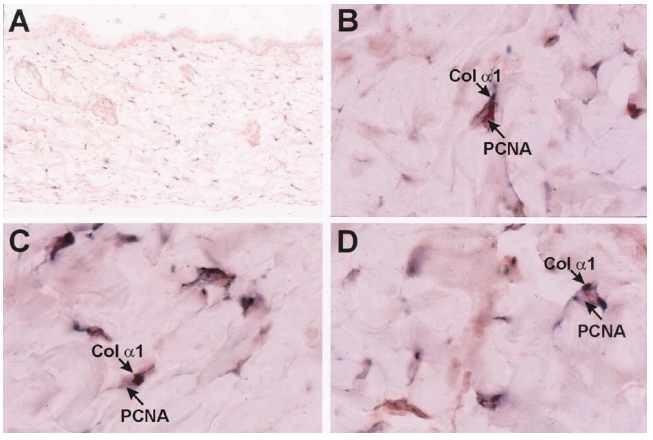

Fig. 5. Skin biopsies from Tsk mice were stained with PCNA antibodies and hybridized with the collagen α1(I) probe simultaneously. Example of few representative sections exhibiting positive staining for fibroblast proliferation and collagen type I synthesis.

degradation in solution and in cream was pH- and temperature-dependent. The acidic-based halofuginone cream when applied on the skin of the Tsk mice inhibited the collagen α1(I) gene expression, while no effect was observed with halofuginone cream at pH of 9.0 (Fig. 2). These results suggest that the observed inhibitory effect on collagen type I synthesis is due to halofuginone and not to any of its degradation product(s).

The fibrotic reaction is thought to involve the stimulative response of tissue cells, which results in increased proliferation as well as in ECM deposition. Various levels of transcription of the procollagen genes in fibroblasts in normal and fibrotic skin have been observed [26,27]. In SSc, the presence of an expanded subpopulation of fibroblasts containing high levels of procollagen mRNA has been detected [4,6,27]. A selective growth of the high-collagen-producing cells and selective transcriptional activation of some but not all fibroblasts has been suggested to be the cause of the fibrotic condition [28]. In the normal mouse, a high rate of fibroblast proliferation is linked to high-collagen gene expression. In the Tsk mouse, the onset of fibrosis coincides with the presence of a large number of high-collagen-expressing fibroblasts in a normal dermis [12]. Using PCNA antibodies and specific collagen α1(I) probe, we could demonstrate that the proliferative fibroblasts that are a subset of the fibroblasts within the dermis are those which are collagen type I-producing cells (Fig. 5). Topical application of halofuginone caused a simultaneous reduction in collagen synthesis by the fibroblasts (Fig. 3) which paralleled their proliferative ability (Fig. 4). The effect of halofuginone was restricted to the dermis fibroblasts without affecting other PCNA-positive cells such as epidermal keratinocytes. It is of interest that halofuginone inhibited the proliferation of fibroblasts, which are collagen-producing cells, but not of keratinocytes, which do not synthesise collagen. Although correlative, these results suggest that the effect of halofuginone on cell proliferation is secondary to the inhibition of collagen synthesis, and are in agreement with our previous observations: inhibition of ECM deposition and of bFGF-induced proliferation of arterial vascular smooth muscle cells but no anti-proliferative effect on vascular endothelial cells [29]; Halofuginone inhibited C6 glioma cell proliferation only after implantation in nude mice at the time the cells express the collagen α1(I) gene but not in culture when no collagen synthesis occurs [25]; and in liver, inhibition of proliferation of stellate cells which are the major collagen-producing cells in cirrhotic liver [30]. Another line of evidence suggesting that the effect of halofuginone on cell proliferation is due, at least in part, to the inhibition of collagen type I synthesis derives from the use of the Mov-13 fibroblasts. In the Mov-13 mouse, the production of type I collagen is disrupted by the insertion of a murine Moloney leukemia virus into the first intron of the α1(I) collagen gene [31]. In preliminary experiments, we found that the Mov-13 fibroblasts were less sensitive to inhibition in cell proliferation than normal fibroblasts (data not shown).

Transcriptional activation of ECM genes has been suggested to be more relevant to the pathogenesis of Tsk fibrosis than fibroblast apoptosis [32]. Clinical safety is a major concern when using an inhibitor of collagen synthesis systemically. The effect of halofuginone on collagen synthesis and cell proliferation was transient and not due to any toxic effect. Upon halofuginone removal, collagen gene expression [18] and cell proliferation [29] returned to control levels with no alteration in cell viability. The reduction in the collagen α1(I) gene expression in rats [22], rabbits [17], and in chickens raised on halofuginone-containing diets for 6 weeks from hatching [33] was achieved at concentrations at which no side effects were apparent. Recently, we reported that topical treatment of a cGvHD patient with halofuginone ameliorated the cutaneous manifestations without any adverse effect [24]. Moreover, collagen turnover in adult tissues is a very slow process and usually a low level of the collagen α1(I) gene is expressed. As a result of an insult, the collagen type I genes are turned on to form high levels of collagen at the injured site. Thus, by administering halofuginone systemically, it is actually targeted to the desire location. The association between mast cell number and degranulation in fibrosis in general [32], and in the Tsk mice and SSc in particular [33], was the basis for the hypothesis that the cutaneous lesions are secondary to, and dependent upon, mast cell proliferation and activation. On the other hand, the absence of mast cells had no measurable effect on the onset and early evolution of Tsk fibrosis, although some correlation with the severity of fibrosis has been observed [34]. Anti-transforming growth factor β (TGFβ) antibodies have caused reductions in both collagen synthesis and mast cell numbers [35,36]. In contrast to other agents such as cromolyn or ketotifen, the reduction in collagen α1(I) gene expression in the Tsk mice by halofuginone was not associated with reduction in the influx of mast cells in the present study. Although interactions between mast cells and fibroblasts probably occur, the effect of halofuginone is specific to the collagen biosynthetic pathway. At present, halofuginone is the only known inhibitor of collagen synthesis that is type-specific. The reduction of collagen mRNA transcript levels by halofuginone appears to be dependent on new protein synthesis, since simultaneous treatment of fibroblasts with cycloheximide or actinomycin D blocks the suppressive effect of halofuginone on collagen α1(I) mRNA gene expression [18]. The nature of the newly synthesized protein(s) and how they function to decrease procollagen mRNA levels is unknown, but they could act as repressor genes directly, or indirectly by repressing transcription factors that activate pro-collagen promoter. Alternatively, newly synthesized proteins could decrease pro-collagen mRNA levels by decreasing mRNA stability or by enhancing mRNA decay. The first intron of the α1(I) collagen gene is a putative site for halofuginone-dependent transcriptional regulation since

unlike fibroblasts, osteoblast and odontoblasts do not appear to utilize this site for transcriptional regulation [37]. Halofuginone was found to affect collagen α1(I) gene expression in soft tissues but not affect bone.

These results taken together suggest that dermal application of halofuginone may be a safe and promising therapy for Scc and cGvHD, since it targets the affected organ with no risk of systemic toxicity.

Acknowledgments

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