Inhibition of collagen gene expression in systemic sclerosis dermal fibroblasts by mithramycin

N Sandorfi, N Louneva, E Hitraya, G Hajnoczky, B Saitta, S A Jimenez


Background: The anti-tumour antibiotic mithramycin is also a potent inhibitor of fibrosis after glaucoma surgery. This drug displays high affinity binding to GC-rich sequences in DNA, including those present in the promoter of the gene encoding the α1 chain of type I collagen (COL1A1).

Objective: To evaluate the effects of mithramycin on COL1A1 expression in systemic sclerosis fibroblasts.

Methods: Confluent cultures of dermal fibroblasts from patients with recent onset diffuse systemic sclerosis were treated with mithramycin in vitro. Cell viability and protein expression were examined by fluorescence and confocal imaging. Type I collagen production was analysed by confocal imaging and metabolic labelling. COL1A1 messenger RNA levels and stability were assessed by northern hybridisation, and COL1A1 transcription was examined by transient transfections.

Results: Treatment of systemic sclerosis fibroblasts with mithramycin (10–100 nmol/l) did not cause significant cytotoxicity. Type I collagen biosynthesis decreased by 33–40% and 50–70% in cells cultured with mithramycin at 10 nmol/l and 100 nmol/l, respectively. Mithramycin at 50 nmol/l decreased COL1A1 mRNA levels by 40–60%. The effects of mithramycin on collagen gene expression were mediated by transcriptional and post-transcriptional mechanisms as shown by the reduction of COL1A1 promoter activity and by a decrease in the stability of these transcripts, respectively.

Conclusions: Mithramycin causes potent inhibition of collagen production and gene expression in systemic sclerosis dermal fibroblasts in vitro in the absence of cytotoxic effects. These results suggest that this drug may be an effective treatment for the fibrotic process which is the hallmark of systemic sclerosis.
dermal fibroblasts, causing profound inhibition of the production of the corresponding protein. These effects appear to be mediated by both transcriptional and post-transcriptional mechanisms and suggest that the drug is a potentially effective therapeutic agent for the severe fibrotic process in systemic sclerosis.

MATERIALS AND METHODS

Cell cultures
Fibroblasts were obtained from skin biopsy specimens from the leading edge of the affected forearm (volar surface) of patients with diffuse systemic sclerosis of recent onset, as described previously. None of the patients had been treated previously with immunosuppressant drugs or with drugs known to affect collagen metabolism. The cells were cultured at 37°C in a 5% CO₂ humidified atmosphere in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% vitamins, 2 mM glutamine, and antibiotics (Cellgro, Mediatech, Inc, Herndon, VA). When the cells reached confluence the cell cultures were supplemented with 50 μg/ml ascorbic acid for 24 hours before initiation of the experiments to optimise their level of collagen production. Fibroblasts were used for experiments between passages 4 and 8. Mithramycin (Sigma, St Louis, MO) was dissolved in distilled water and the solution stored at 4°C until use. Initially, six systemic sclerosis and four normal cell lines were screened employing two concentrations of mithramycin (10⁻³ and 10⁻⁷ mol/l). The results obtained were similar for all cell lines. From these cell lines we examined four systemic sclerosis cell lines by confocal microscopy studies. From these four cell lines we selected the two systemic sclerosis cell lines which exhibited the highest levels of type I collagen production for more detailed study in order to examine the effects of the drug on pathological fibroblasts, which were clearly collagen overproducers.

Cell viability assessment
Equal numbers of systemic sclerosis fibroblasts were plated in 35 mm dishes and cultured to confluence in 10% MEM as described above. The cultures were then treated with various concentrations of mithramycin for 48 hours. Cell counting was performed in duplicate using a fluorescence microscope. To visualise the nuclei, the cells were labelled with Hoechst 33422 (1 μg/ml). Phase contrast and Hoechst fluorescence images were acquired as described previously. An equal number of areas (10 fields, each 0.16 mm²) were examined in each dish. To determine cell viability, the morphology of the cells and the presence of nuclear condensation were evaluated, as described previously.

Metabolic labelling for the estimation of collagen biosynthesis
Systemic sclerosis fibroblasts were grown to confluence in Eagle’s MEM supplemented with 10% FBS, 1% vitamins, 2 mM glutamine, antibiotics, and 50 μg/ml ascorbic acid, and then they were treated with various concentrations of mithramycin. After 5 hours of incubation, 100 μg/ml β-aminoproprionitrile and L-[U-¹⁴C]proline (ICN Biomed, specific activity: 247 mCi/mmol) were added and the incubations were continued for 24 hours. β-Aminoproprionitrile was used to prevent collagen cross linking and to allow newly synthesised molecules to remain in the culture medium. Aliquots of medium were dialysed to remove unincorporated radioactive proline. Total incorporation of L-[U-¹⁴C]proline into macromolecules was measured in a scintillation spectrometer. Aliquots of medium and cell layers were pooled and used for determination of proteins sensitive to collagenase, employing a bacterial collagenase digestion assay, as described previously. Two separate experiments each in duplicate were performed on two different cell lines. The labelled proteins in the medium were also analysed by sodium dodecyl sulphate (SDS)-polyacrylamide slab gel electrophoresis in 7.5% SDS-polyacrylamide gels under reducing conditions, as described previously. Samples were electrophoresed for 5 hours at 100 V constant voltage. After electrophoresis, the gels were processed for fluorography and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).

Confocal imaging of collagen in control and mithramycin treated systemic sclerosis dermal fibroblasts
Systemic sclerosis fibroblasts (four different cell lines in five separate experiments) were plated on poly-L-lysine treated coverslips in 35 mm dishes at a density of 35 000 cells/dish and cultured for 24 hours before mithramycin treatment. Various concentrations of mithramycin were added and the cells were cultured for 48 hours and then prepared for analysis. The fibroblasts were fixed with 3% paraformaldehyde and then permeabilised with digitonin. After blocking non-specific binding with 1.5% goat serum and 1% bovine serum albumin for 1 hour, the cells were incubated with a 1:200 dilution of an affinity purified anti-type I collagen polyclonal antibody which exhibits no cross reactivity to types II, III, IV, V, and VI collagens (Rockland, PA). A fluorescein conjugated secondary antibody was applied along with rhodamine labelled phalloidin to stain F-actin, the polymeric form of actin. The coverslips were mounted on slides using antifade reagent (SlowFade Light Antifade Kit, Molecular Probes, Eugene, OR). Fluorescence was visualised using a laser scanning confocal microscope system (BioRad Radiance 2000 confocal system coupled to an Olympus IX70 microscope, 40X oil objective, 1.35NA) equipped for dual excitation and emission (KrAr laser illumination at 488 nm for FITC and 568 nm for rhodamine). Fluorescence of the FITC conjugated antirabbit IgG was visualised as green colour and that of the rhodamine labelled phalloidin as red colour. To measure the collagen immunostaining, 10 fields (3–10 cells/field) were taken from each sample. Data analysis was performed using custom-made software. A mask was drawn over every whole cell area and the average fluorescent intensity was calculated for each cell. Then the mean fluorescence of the cells was calculated and was normalised to the control.

Analysis of steady state mRNA levels by northern blot hybridisation
Fibroblasts in confluent culture were either maintained under control conditions or were treated with various concentrations of mithramycin (5, 10, 50, and 100 nmol/l) for 48 hours, and total RNA extracted as described previously. Equal aliquots of the isolated total RNA were electrophoresed on formaldehyde–1% agarose gels and then transferred onto nitrocellulose membranes. The filters were hybridised to ³²P-radiolabelled human complementary DNA (cDNA) for COL1A1. Equivalent loading and transfer were evaluated by hybridisations with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Results were quantified to determine the relative amounts of mRNA in duplicate samples using densitometry.

Transient transfections of systemic sclerosis fibroblasts with COL1A1 promoter-chloramphenicol acetyl transferase (CAT) constructs
Fibroblasts were grown to 70–80% confluence in 35 mm dishes. The cells were transfected with a total of 2.5 μg of various COL1A1 promoter constructs fused to the CAT
reporter gene and 0.2 μg of a vector containing *E coli* β-galactosidase cDNA (pCMV β-galactosidase) using Fugene-6 kit (Roche Molecular Biochemicals, Indianapolis, IN). Expression of β-galactosidase was used to correct for transfection efficiency. The constructs tested are progressive 5' deletions of the human COL1A1 promoter each cloned upstream of the CAT reporter gene. All constructs end at nucleotide +42 bp to assure the proper reading frame and their 5' ends were at −804 bp, −174 bp, and −84 bp. Four hours later the fibroblasts were either maintained in control conditions or treated with various concentrations of mithramycin for an additional 48 hours. Cytoplasmic extracts were examined to determine CAT activity using [14C]chloramphenicol as substrate. Acetylated and non-acetylated forms of radiolabelled chloramphenicol were separated by thin layer chromatography and visualised by autoradiography.

**Determination of mRNA stability**

Confluent fibroblasts were cultured in the presence or absence of 50 nmol/l mithramycin. The cultures received 1 μg/ml of the specific RNA polymerase II inhibitor α-amanitin (Sigma) 4 hours after the addition of mithramycin to arrest further gene transcription. Total RNA was then extracted at 0, 6, 18, and 24 hours after the addition of α-amanitin and processed by northern hybridisation. The experiment was performed in duplicate except for the 24 hour sample.

**RESULTS**

**Effects of mithramycin on cell viability**

The results of experiments employing various concentrations of mithramycin (1 nmol/l to 1 μmol/l) applied for 48 hours to confluent systemic sclerosis fibroblast cultures on cell viability showed that mithramycin at 1 μmol/l decreased the number of cells by over 80% (mean (SD) 82.5 (7.5%; n = 4), whereas 10 nmol/l, 50 nmol/l, or 100 nmol/l did not cause any detectable change in cell viability (fig 1). Concentrations of 50 nmol/l and 100 nmol/l did not cause any detectable cytotoxicity (data not shown). Phase contrast image analysis showed that treatment with mithramycin at 1 μmol/l altered the shape of the cells which shrunk and lost their cytoplasm, although the nuclear staining with Hoechst did not indicate an apoptotic process. No change in cell shape was seen using mithramycin at 10 nmol/l.

**Evaluation of type I collagen accumulation by confocal imaging after mithramycin treatment**

In immunocytochemistry experiments, evaluation of the mithramycin effect on type I collagen accumulation at the single cell level by confluent cultures of systemic sclerosis...
Figure 2  Evaluation of type I collagen production in systemic sclerosis dermal fibroblast monolayer cultures by confocal imaging after mithramycin treatment. Type I collagen present in individual cells of systemic sclerosis fibroblast cultures was examined by immunomicroscopy and confocal microscopy imaging as described in “Materials and methods” in five separate experiments using four cell lines. (A) Fluorescence of the FITC conjugated antirabbit IgG labelling type I collagen is shown in green and that of rhodamine labelled phalloidin labelling actin in red in one illustrative experiment. (B) Quantitative evaluation of the mithramycin effect on type I collagen production at the individual cell level showed that 48 hours of treatment with mithramycin at 10 nmol/l and 100 nmol/l decreased the protein level by about 33% and 50%, respectively. The results show the averages (SD) from five separate experiments with four different cell lines.
Collagen biosynthesis was decreased by 30% using mithramycin at 10 nmol/l and by 53% using mithramycin at 100 nmol/l. The results of one experiment are shown. (B) SDS gel electrophoresis of labelled proteins from the medium showed similar results with a 30% decrease in collagen biosynthesis was determined employing a specific collagenase assay in pooled media plus cell lysate samples as described in “Materials and methods”. Treatment with mithramycin (1, 10, or 100 nmol/l) showed that decrease in type I collagen by about 21%, 33%, and 50%, respectively (figs 2A and B). The differences between cells treated with the three concentrations of mithramycin and untreated cells were statistically significant (fig 2B). However, no significant change was obtained in the F-actin level in the cells treated with mithramycin (not shown).

**Evaluation of type 1 collagen biosynthesis by metabolic labelling after mithramycin treatment**

Metabolic labelling with L-[U-14C]proline of confluent fibroblast cultures incubated under control conditions or in the presence of mithramycin (3, 10, and 100 nmol/l) decreased the type I collagen levels by about 21%, 33%, and 50%, respectively (figs 2A and B). The differences between cells treated with the three concentrations of mithramycin and untreated cells were statistically significant (fig 2B). However, no significant change was obtained in the F-actin level in the cells treated with mithramycin (not shown).

**Analysis of steady state mRNA levels by northern blot hybridisation**

Northern hybridisation of total RNA from untreated and mithramycin treated cultures was used to determine whether the results seen at the collagen biosynthesis and confocal microscopy analyses were accompanied by parallel changes in the steady state levels of the corresponding COL1A1 transcripts. Two different cell lines were examined. As described in “Materials and methods” the intensity of bands corresponding to COL1A1 mRNA was corrected by the intensity of the band corresponding to GAPDH mRNA to normalise for differences in loading and transfer during the procedure. The inset in fig 4 shows a northern hybridisation analysis of results obtained with one cell line, and the bar graph shows the individual values and the averages of results obtained with both cell lines after correction for the levels of GAPDH mRNA. The mithramycin concentrations used were 5, 10, 50, and 100 nmol/l. A dose related decrease in COL1A1 transcripts upon treatment with mithramycin was seen. Mithramycin (50 nmol/l) decreased the steady state COL1A1 mRNA levels by 40 and 60% in the two cell lines examined when normalised to the expression of GAPDH (fig 4).

**COL1A1 promoter activity after mithramycin treatment**

Transient transfection of systemic sclerosis fibroblasts with COL1A1 promoter-CAT constructs followed by mithramycin (50–100 nmol/l) treatment showed that mithramycin had an inhibitory effect on the transcriptional activity of the three COL1A1 promoter constructs, which was mediated primarily by sequences contained in the region spanning −84 bp (fig 5). Administration of mithramycin at 50 nmol/l resulted in a
in approximately 30% inhibition of the promoter activity (not shown), whereas a concentration of 100 nmol/l mithramycin caused a promoter activity decrease by about 60% in cells that were transfected with the −84 bp COL1A1 construct.

**Messenger RNA stability after mithramycin treatment**

Confluent cultures of systemic sclerosis dermal fibroblasts were maintained in culture medium containing 10% fetal bovine serum and ascorbic acid or were incubated for 4 hours with mithramycin (50 nmol/l) before α-amanitin addition. Total RNA was prepared from cells at the time of α-amanitin addition (time 0) and at 6, 18, and 24 hours after its addition and the samples were examined by northern analysis. COL1A1 mRNA stability experiments showed that the stability of the x1(I) collagen transcripts decreased by 40% at 18 hours compared with untreated cells (figs 6A and B).

**DISCUSSION**

Mithramycin, a DNA binding anti-tumour antibiotic, has been used in the treatment of certain malignant testicular tumours and of hypercalcaemia and hypercalciuria associated with advanced neoplasms. It has also been used to prevent the fibrotic reaction after glaucoma surgery. It has been established that mithramycin has a high binding affinity to GC-rich DNA sequences that are commonly found in the Sp1 transcription factor binding site of the promoter region of numerous genes such as c-myc, H-ras, dihydrofolate reductase, c-Ki-ras, COL1A1, and COL1A2.7 11–10

In one study, mithramycin inhibited myointimal proliferation after balloon injury of the rat carotid artery in vivo. This action occurred through inhibition of transcription of the c-myc proto-oncogene.20 Another study found that mithramycin (100 nmol/l) inhibited COL1A1 activity by 60% in embryonic lung fibroblasts in culture.21 However, the results of these studies were recently retracted.22 Our previous transfection experiments have shown that the proximal COL1A1 promoter region extending to −174 bp upstream from the transcription initiation point resulted in maximal fibroblast-specific gene expression in systemic sclerosis fibroblasts.23 The proximal promoter is relatively G+C rich and contains two potential binding sites for Sp1. Functional assays indicated that Sp1 interacts with specific elements within the proximal promoter of the human COL1A1, and this interaction increases transcription from the promoter.7 The transcriptional activity of Sp1 can be modulated at the
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post-translational level by glycosylation or phosphorylation. Modulation of the level, or of the transcriptional activity of Sp1, may have an important role in collagen gene expression regulation and probably also in the overproduction of collagen.

Our experiments showed that mithramycin (10–100 nmol/l) in confluent cell culture did not cause cytotoxicity effects, although higher mithramycin concentrations resulted in cell death, which did not appear to occur through apoptosis. Type I collagen production decreased by 33–40% in cells cultured with 10 nmol/l, and by 50–70% in fibroblasts treated with 100 nmol/l mithramycin. Mithramycin at 50 nmol/l also decreased the steady state mRNA levels of type(I) collagen by 40–60% without affecting the mRNA levels of a control gene (GAPDH). The results of the experiments on the promoter activity of the COL1A1 gene indicated that the mithramycin effects on collagen gene expression were exerted through a region of the proximal COL1A1 promoter spanning only −84 bp upstream of the initiation of transcription site. This region contains a GC region which is probably an Sp1 binding site. Although mithramycin caused a reduction of COL1A1 promoter activity, its effects on collagen gene expression also appeared to be exerted at a post-transcriptional level. Indeed, we found that COL1A1 mRNA stability in cultures treated with mithramycin at 50 nmol/l was decreased by 40% at 18 hours compared with untreated cells. The reduction of COL1A1 transcript stability induced by mithramycin in systemic sclerosis fibroblasts demonstrated here is of particular relevance because previous studies have shown increased COL1A1 mRNA stability in tridimensional lattice cultures of these cells.

In summary, mithramycin at concentrations that are achievable in vivo, employing currently recommended doses of the drug, causes potent in vitro inhibition of collagen production by systemic sclerosis fibroblasts, and these effects appear to be due to a reduction of COL1A1 gene transcription as well as to a decrease in COL1A1 mRNA stability. Given these effects on collagen gene transcription and mRNA stability, we believe that mithramycin could prove to be a very effective treatment for systemic sclerosis and suggest that clinical trials should be conducted to examine its efficacy in patients affected by the disease.

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