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).

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.

Systemic sclerosis is a disease of unknown aetiology that is characterised by the excessive deposition of collagen and other extracellular matrix proteins in skin and several internal organs, microvascular fibroproliferative lesions, and humoral and cellular immunological abnormalities. The precise mechanism(s) involved in the tissue fibrosis are unknown, although this process is clearly responsible for most of the clinical manifestations of the disease. Indeed, the severity of systemic sclerosis symptoms and mortality are determined by the extent and degree of tissue fibrosis. Extensive recent efforts have been devoted to a study of the mechanisms involved in the pathological increase of collagen gene expression in systemic sclerosis. Although these have not been entirely elucidated, it has been shown that fibroblasts from affected skin in culture produce excessive amounts of various collagens and display increased transcription rates of the corresponding genes.

The modulation of collagen transcript stability may also be altered in systemic sclerosis as shown by the increased stability of collagen mRNA in systemic sclerosis dermal fibroblasts cultured in tridimensional lattices. The most abundant extracellular matrix protein in tissues affected by systemic sclerosis is type I collagen. Type I collagen is assembled from two α1 chains and one α2 chain, which are encoded in COL1A1 and COL1A2, respectively. The transcriptional activity of the two genes is coordinated under most physiological and pathological conditions. The transcriptional regulation of these genes results from tightly regulated interactions between transcription factors and regulatory elements contained within the promoters and first introns of the collagen genes. It has been suggested that in the case of COL1A1 expression one of the most important regulatory transcription factors belongs to the Sp1 family of DNA binding proteins. It has been demonstrated that Sp1 has an important role in the pathogenesis of fibrosis, and increased Sp1 binding activity and phosphorylation have been observed in fibroblasts cultured from skin affected by systemic sclerosis. Therefore, interference with Sp1 binding to its cognate elements in the COL1A1 gene may allow modulation of its increased transcriptional activity. One of the possible ways of accomplishing this is by competing for the Sp1-specific GC-rich binding site in COL1A1. Previous studies have shown that drugs which can bind to GC-rich regions of DNA such as mithramycin, doxorubicin, and mitoxantrone may inhibit formation of the Sp1-DNA complex.

The expression of collagen genes can also be regulated at the post-transcriptional level because changes in the stability of their corresponding mRNA also contribute to its turnover and steady state levels. Indeed, it has been shown that cortisol and other glucocorticoids regulate α1(I) and α2(I) procollagen steady state mRNA levels by decreasing the stability of their corresponding transcripts. Furthermore, interferon γ, one of the most potent inhibitors of collagen production, in addition to its transcriptional effects reduces the half life of α1(I) and α3(III) procollagen mRNA.

Given the important role of Sp1 in the regulation of type I collagen gene expression and its potential participation in the abnormally increased expression of collagen genes in systemic sclerosis, this study aimed at examining the effect of mithramycin, a potent inhibitor of Sp1 binding to DNA, on COL1A1 gene expression and transcriptional activity in fibroblasts from patients with systemic sclerosis. We also examined the effects of the drug on the stability of COL1A1 transcripts. The results show that mithramycin is a potent inhibitor of COL1A1 gene expression in systemic sclerosis.

Abbreviations: CAT, chloramphenicol acetyl transferase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimal essential medium; SDS, sodium dodecyl sulphate

Sergio Jimenez, MD, Division of Rheumatology, Department of Medicine, Thomas Jefferson University Hospital, 233 S 10th Street, Room 509 BLSB, Philadelphia PA 19107-5041; USA; sergio.jimenez@jefferson.edu

Accepted 14 May 2005
Published Online First 18 May 2005

See end of article for authors’ affiliations

Correspondence to: Dr S A Jimenez, Thomas Jefferson University, Division of Rheumatology, 233 S 10th Street, Room 509 BLSB, Philadelphia PA 19107-5041; USA; sergio.jimenez@jefferson.edu
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, Hendron, 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 (10⁻⁵ and 10⁻⁶ 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 synthesis 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-aminohippurinitrile and L-[U-¹⁴C]proline (ICN Biomed, specific activity: 247 mCi/mmol) were added and the incubations were continued for 24 hours. β-Aminopropionitrile 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 fluorochemistry 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. Equivalent 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-labelled 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. 14 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.
Mithramycin (50 nmol/l) decreased the steady state mRNA level of α1(I) collagen by 40% compared with the untreated control cells. These differences were statistically significant (fig 3A). The inhibitory effects of mithramycin on collagen production were confirmed by SDS gel electrophoresis of media proteins, which showed a dose related decrease in newly synthesised collagen determined in pooled media plus cell lysates by a specific collagenase digestion assay decreased by 30% after 10 nmol/l mithramycin treatment and by 53% after administration of 100 nmol/l mithramycin compared with untreated control cells. These differences were statistically significant (fig 3A). The inhibitory effects of mithramycin on collagen production were confirmed by SDS gel electrophoresis of media proteins, which showed a dose related decrease in newly synthesised collagenous proteins after treatment with the drug (fig 3B).

**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 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 x11(1) 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.

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. Another study found that mithramycin (100 nmol/l) inhibited COL1A1 activity by 60% in embryonic lung fibroblasts in culture. However, the results of these studies were recently retracted. 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. The proximal promoter is relatively GC 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. The transcriptional activity of Sp1 can be modulated at the

---

**Figure 5** COL1A1 promoter activity after mithramycin treatment. Transient transfection of systemic sclerosis fibroblasts with COL1A1 promoter-CAT constructs followed by mithramycin (50–100 nmol/l) was performed as described in “Materials and methods”. (A) The results with −84 bp, −174 bp, and −804 bp constructs are shown. The efficiency of transfection was corrected by assays of β-galactosidase activity measured by an enzymatic assay. These experiments were repeated three times, each in duplicate. p Values obtained comparing the transcriptional activity of each of the constructs in cells treated with mithramycin compared with the transcriptional activity of the same constructs in the untreated cells were significant (p < 0.001 for each of the constructs).

**Figure 6** mRNA stability after mithramycin treatment. The effects of mithramycin on COL1A1 mRNA stability were examined as described in “Materials and methods”. (A) Confluent cultures of systemic sclerosis dermal fibroblasts were either kept in culture medium containing 10% FBS and ascorbic acid (50 μg/ml) (lanes 1–7) or were incubated for 4 hours with mithramycin (50 nmol/l; lanes 8–14) before addition of α-amanitin (1 μg/ml). Total RNA was prepared from cells at various intervals after α-amanitin addition and examined by northern analysis. Northern analysis was performed on samples prepared at the time of α-amanitin addition (time point 0); lanes 1, 2, 8, 9; at 6 hours after α-amanitin administration: lanes 3, 4, 10, 11; at 18 hours after α-amanitin treatment: lanes 5, 6, 12, 13; and at 24 hours after α-amanitin: lanes 7 and 14. (B) COL1A1 mRNA stability after mithramycin treatment assessed by densitometric analysis. Values shown were corrected to GAPDH and represent the average of duplicate cultures of different cell lines. The 24 hour value was not included in the graph because it was not performed in duplicate.
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 1 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 α1(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 controls. 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.

ACKNOWLEDGEMENTS

Supported by NIH grant AR19161 (to SAJ). Dr Nora Sandorfi was supported by NIH training grant AR07583.

Authors’ affiliations

N Sandorfi, N Louneva, E Hitraya, B Saitta, S A Jimenez, Division of Rheumatology, Department of Medicine, Thomas Jefferson University, Philadelphia PA 19107, USA

G Hajnoczky, Department of Pathology, Anatomy and Cell Biology of Thomas Jefferson University, Philadelphia PA 19107, USA

Dr B Saitta’s current address: Coriell Institute for Medical Research/UMDNJ, Molecular Biology Laboratory, 403 Haddon Avenue, Camden, NJ 08103, USA

Dr E Hitraya’s current address: Genentech, Biotherapeutics Unit, 1 DNA Way, South San Francisco, CA 94080, USA

REFERENCES


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

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

Ann Rheum Dis 2005 64: 1685-1691 originally published online May 18, 2005
doi: 10.1136/ard.2005.037515

Updated information and services can be found at:
http://ard.bmj.com/content/64/12/1685

These include:

References
This article cites 17 articles, 5 of which you can access for free at:
http://ard.bmj.com/content/64/12/1685#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Connective tissue disease (4253)
Ophthalmology (128)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/