

**In vitro Evidence for a Direct Anti-Fibrotic Role of the Immunosuppressive Drug
Mycophenolate Mofetil**

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Abbreviations :

mycophenolate mofetyl = MMF	fetal calf serum = FCS
mycophenolic acid = MPA	sodium dedocyl sulfate = SDS
systemic sclerosis = SSc	polymerase chain reaction = PCR
graft-versus-host disease = GVHD	chloridric acid = HCl
matrix metalloproteinase = MMP-1	
α -smooth muscle actin = α -SMA	
extracellular matrix = ECM	

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Abstract

The immunosuppressive drug mycophenolate mofetil (MMF) is used to prevent organ rejection after transplantation, and has shown some efficacy to prevent the fibrotic complications that occur during auto-immune diseases such as systemic sclerosis (SSc) or during graft-versus-host disease (GVHD). We tested the hypothesis that MMF may exert direct effects on fibroblast extracellular matrix remodeling. Incubation of human lung fibroblast cultures with MMF led to dose- and time-dependent reduction in the synthesis and expression of type I collagen. Inhibition of *COL1A1* and *COL1A2* mRNA steady-state levels occurred at the level of transcription, via repression of their promoters. In contrast, MMF significantly enhanced the expression and the synthesis of interstitial collagenase (MMP-1). MMF was also found to diminish the capacity of fibroblast to contract mechanically unloaded collagen lattices, and to reduce the synthesis of α -smooth muscle actin, a marker of the contractile myofibroblast phenotype. In addition, MMF diminished the fibroblasts motility. In conclusion, we provide novel mechanism by which MMF alters fibroblast functions important for wound healing and implicated in the development of tissue fibrosis, e.g. collagen production, extracellular matrix contraction, and cell migration. Such properties may contribute to the beneficial therapeutic effects of MMF against fibrotic lesions developing in SSc or during GVHD.

Introduction

The immunosuppressive xenobiotic drug mycophenolate mofetil (MMF) is currently used to prevent organ transplant rejections (Mele and Halloran, 2000; Ciancio et al., 2005; Danovitch, 2005; Shapiro et al., 2005). MMF efficacy has been mostly attributed to its inhibitory activity on lymphocyte functions, that make MMF an effective immunosuppressive drug. Mechanistically, it has been shown that mycophenolic acid (MPA), the bioactive compound of MMF, inhibits the activity of inosine monophosphate dehydrogenase, a rate-limiting enzyme for *de novo* synthesis of guanosine nucleotides (Allison and Eugui, 2000). By depleting the intracellular concentration of guanosine nucleotides, MPA acts as a powerful inhibitor of lymphocyte proliferation. While the principal therapeutic indication of MMF is transplantation, clinical and experimental observations suggest that it may also represent a plausible option for the treatment of immune diseases such as systemic sclerosis (SSc) (Stratton et al., 2001; Liossis et al., 2006) or graft-versus-host disease (GVHD) (Arai and Vogelsang, 2000; Zeiser et al., 2004). For example, in rodent models, oral administration of MMF alone or in combination with cyclosporin A inhibits the sclerotic manifestations of GVHD (Allison and Eugui, 2000). In humans, successful treatment of high risk chronic GVHD with MMF has also been reported on a limited number of patients (Allison and Eugui, 2000; Kim et al., 2004; Krejci et al., 2005; Lopez et al., 2005).

SSc and GVHD are heterogeneous immune diseases characterized by predominant T cell activation, production of autoantibodies, and cytokine release. These events contribute to fibroblast activation and lead to increased deposition of collagen at various organ sites, ultimately producing localized fibrosis, lastly leading to organ failure and death (Derk and Jimenez, 2003; Ruzek et al., 2004; Abraham and Varga, 2005; Hess, 2006; Verrecchia et al., 2006). The development of fibrosis also involves a series of distinct, yet complementary, pathophysiological events that include attraction of blood-born cells (e.g., leukocytes, platelets, activated lymphocytes), local alteration of the microvasculature (interrupting non-thrombogenic, permeability-regulating, and cell-adhesive functions of the microcirculation), and activation of resident mesenchymal cells (e.g. fibroblasts,

endothelial cells, pericytes) leading to excessive collagen deposition and ECM contraction (Uitto and Kouba, 2000; Verrecchia and Mauviel, 2002a; Verrecchia and Mauviel, 2002b; Leask and Abraham, 2004). Tissue fibrosis is generally considered to arise due to a failure of the normal wound healing response to terminate (Eckes et al., 2000; Gabbiani, 2003). During this process, fibroblasts proliferate, migrate into the wound and synthesize elevated levels of ECM proteins, particularly fibrillar collagens. Alternatively, reduced matrix metalloproteinase (MMP) expression, and subsequent inhibition of collagen degradation may also contribute to the fibrotic process (Uitto and Kouba, 2000). A specialized form of fibroblasts present in wounds, called myofibroblasts, expresses high levels of α -smooth muscle actin (α -SMA) and consequently contracts the ECM, thereby allowing wound closure (Gabbiani, 2003). Myofibroblasts are present in abundance within fibrotic lesions and notably contribute to excessive ECM deposition and scarring (Gabbiani, 2003). The aim of this work was to investigate whether MMF exerts direct effects on fibroblast functions. We demonstrate that MMF inhibits collagen gene expression, ECM contraction, and fibroblast migration suggesting that it may exert direct antifibrotic activities independent from its role as an immunosuppressive drug.

Methods

Cell cultures. Human lung fibroblasts (WI-26) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 mg/ml streptomycin-G and 0.25 mg/ml Fungizone™) in 5% CO₂ at 37°C. Mycophenolate mofetil was obtained from Roche Pharmaceuticals (Nutley, NJ).

Cell viability assesment. Subconfluent WI-26 fibroblasts were cultured in 96 well cell culture cluster and treated with various concentrations of MMF for 12h, 24h and 48h in the presence of 1% FCS. Cell viability was assessed with an MTT assay, according to the manufacturer's protocol (Promega, Madison, WI).

Collagen matrix contraction. Fibroblasts were harvested from monolayer cultures with 0.25% trypsin and 1 mM EDTA. Trypsin was subsequently neutralized with fetal calf serum-containing medium. Collagen lattices in 60-mm dishes were prepared with 7 ml of a mixture containing 3.10^5 fibroblasts and 1 mg/ml native type I collagen (Biocoat™; BD Biosciences) in medium supplemented with 1% FCS. When needed, MMF was added to the mixture before polymerization of the collagen matrix. Polymerization of collagen matrices required 60 min at 37 °C. To initiate lattice contraction, freshly polymerized matrices were released from the underlying culture dish with a few gentle taps on the dish (Javelaud et al., 2003).

Scratch wound closure assays. Confluent cell monolayers were wounded by mechanical scraping with a pipette tip. Wound width was assessed at the time of scrapping to ensure that all wounds were the same width at the start of the experiments (see corresponding figures). Cell culture medium was then replaced with fresh medium, with or without MMF at various concentrations, and wound closure was recorded photographically over time using phase-contrast microscopy (Javelaud et al., 2003).

Transwell™ Motility Assays. Single cell suspensions were seeded onto the upper surface of the filters in medium containing 10% fetal calf serum and allowed to migrate through the membrane. After a 24h or 48h incubation period, cells on the upper surface of the filter were wiped off with a

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cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with DiffQuick™ (Dade Behring, Düringen, Switzerland), and counted by bright-field microscopy at 200 in six random fields.

Reverse transcription-PCR analysis. Total RNA was extracted according to the manufacturer's instructions using RNeasy mini-kit (Qiagen). DNase I treatment (25 units, 15 min) of total RNA was directly performed on the spin columns to eliminate genomic contamination of the RNA samples. One microgram of total RNA was used for first strand cDNA synthesis using a RT-for-PCR kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBRGreen PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 sec at 95°C, 45 sec at 60°C, 1,5 min at 68°C, and finally 15 sec at 95°C, 20 sec at 63°C and 15 sec at 95°C. For each mRNA, gene expression was corrected against *GAPDH* mRNA content in each sample. Primers used are shown in table 1.

Transient cell transfections and reporter assays. Transient cell transfections were performed with jetPEI™ according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). pRSV-β-galactosidase was cotransfected in every experiment to monitor transfection efficiencies. CAT activity was measured using (¹⁴C)chloramphenicol as substrate, followed by thin-layer chromatography, as described previously (Verrecchia et al., 2002) . Quantitations were made with a PhosphorImager (Amersham Biosciences, Uppsala, Sweden).

Plasmid constructs. -3500COL1A2/CAT (gift from Francesco Ramirez, Mt Sinai School of Medicine, New York, NY), and -2300COL1A1/CAT (gift from John Varga, Northwestern University Feinberg School of Medicine, Chicago, IL) have been previously described (Boast et al., 1990; Chen et al., 1998).

Western blot analyses. Total cell extracts (30 µg) in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) were denatured by heating at 95°C for 3 min before resolution by SDS-polyacrylamide gel electrophoresis. After electrophoresis,

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proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose filters (Amersham Biosciences), immunoblotted with anti-type I collagen (Southern Biotech, Birmingham, AL), anti α -smooth muscle actin (Sigma), or anti-actin (Sigma) antibodies, all at a dilution of 1:1000 in 1x phosphate-buffered saline/5% nonfat milk for 1h. After incubation, filters were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. Filters were then washed, developed according to chemiluminescence protocols (ECL, Amersham Biosciences), and revealed with a PhosphoImager (Amersham Biosciences).

Quantative determination of MMP-1 production. The MMP-1 production in cell supernates was determined using the Quantikine Human pro-MMP-1 Immunoassay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Results

MMF inhibits type I collagen gene expression.

To determine whether MMF had a direct effect on collagen production by fibroblasts, WI-26 human fibroblast cultures were incubated with various concentrations of MMF, and type I collagen production was estimated by Western Blot analysis. As shown in Figure 1, total type I collagen production was reduced by MMF in a time- and dose-dependent manner. A decrease of approximately 60% and 80% with a concentration of 10 μ M, and a decrease of 50% and 72% with a concentration of 1 μ M were respectively observed after 48h and 72h of treatment. Even the lowest concentration of MMF, 0.1 μ M, had a significant inhibitory activity after 48h of incubation, suppressing collagen production by as much as 20%. No toxicity of MMF and no change in cell proliferation, as assessed by MTT test, was observed during the course of these experiments, at any of the concentrations tested (not shown).

To determine whether the reduction of type I collagen production in response to MMF occurred via modulation of the corresponding genes, *COL1A1*, and *COL1A2* mRNA steady-state levels were estimated by quantitative RT-PCR. As shown in Figure 2, MMF (10 μ M) decreased *COL1A1* (panel A) and *COL1A2* (panel B) mRNA levels in a time-dependent manner, the extent of inhibition reaching 60% and 75%, respectively, after 48 h, and reaching 80-85% after 72h of treatment. Similar results were obtained in response to mycophenolic acid (MPA), the bioactive compound of MMF (data not shown). Next, to determine whether MMF repressed type I collagen gene expression at the transcriptional level, transient cell transfections were performed with the collagen promoter/gene reporter constructs -2300COL1A1/CAT, and -3500COL1A2/CAT. As shown in Figure 3, *COL1A1* (panel A) and *COL1A2* (panel B) gene promoter activities were respectively reduced by 63% and 50% in presence of MMF (10 μ M) after 48h of treatment, suggesting that the effect of MMF observed at the mRNA levels occur via transcriptional down-regulation of the corresponding genes.

MMF increases MMP-1 gene expression and protein synthesis

We first wanted to determine whether MMF had direct effect on MMP-1 synthesis. To address this point, MMP-1 protein production was measured using MMP-1 immunoassay approach. As shown in Figure 4A, MMF treatment of WI-26 fibroblasts led to increase in MMP-1 protein production, up to 2,6-fold and 4,6 fold respectively after 48h and 72h at the concentration of 10 μ M. We next determined whether MMF modulates MMP-1 and/or TIMP-1 gene expression. To address this point, the effects of MMF on MMP-1 and TIMP-1 mRNA steady-state levels were studied by quantitative RT-PCR. MMF treatment of WI-26 fibroblasts led to a time dependent increase in *MMP-1* mRNA levels, up to 5,3-fold after 72h at the concentration of 10 μ M (Fig. 4B) . On the other hand, TIMP-1 gene expression remained unaltered over the same incubation period (data not shown).

MMF reduces fibroblast capacity to contract free-floating collagen lattices.

We next wanted to determine whether MMF alters ECM contraction by fibroblasts, a phenomenon that mechanistically involves both ECM remodeling and cell motility (Grinnell, 1994). To this end, WI-26 were seeded in free-floating, mechanically unloaded, collagen gels, and incubated in the presence or absence of MMF. The kinetics of collagen lattice contraction was then recorded over a 5-day period. Results shown in Figure 5 indicate that MMF had a potent dose-dependent inhibitory effect on collagen lattice contraction by WI-26 fibroblasts. At day 5, MMF treatment at concentrations ranging from 0,1 μ M to 10 μ M resulted in significant greater lattice diameter relative to control, indicating inhibition of fibroblast contractile activity: up to 50% increase in lattice diameter relative to control was observed at 5 days following treatment with 10 μ M MMF. In addition, MPA (10 μ M) mimics the effect of MMF (data not shown).

MMF reduces α -smooth muscle actin gene expression

α -SMA expression is the hallmark of the myofibroblast phenotype, and is thought to contribute to their contractile activity (Skalli et al., 1986; Desmouliere et al., 2005). To determine whether MMF had a direct effect on α -SMA expression, WI-26 fibroblast cultures were incubated with MMF for various time periods, following which α -SMA production was measured by Western Blot analysis.

As shown in Figure 6A, α -SMA production was reduced by MMF in a time dependent manner. A decrease of 22%, 30% and 69% with a MMF concentration of 10 μ M was respectively observed after 2, 3, and 5 days of treatment. To determine whether the reduction of α -SMA production by MMF occurred via modulation of the corresponding gene, we measured α -SMA mRNA steady-state levels following MMF treatment by quantitative RT-PCR. As shown in Figure 6B, MMF at a concentration of 10 μ M decreased α -SMA mRNA levels in a time-dependent manner. A decrease of 34%, 60% and 83% with a MMF concentration of 10 μ M was respectively observed after 2 days, 3 days and 5 days of treatment.

MMF inhibits fibroblast motility

The effects of MMF on WI-26 fibroblasts migration were assessed firstly in a scrape wounding assay. As shown in Figure 7A, photomicrographs taken 3h, 8h and 24h after wounding show delayed wound closure by WI-26 fibroblast cultures treated with MMF at concentrations ranging from 0,1 μ M to 10 μ M compared with untreated control cultures. Quantitation of the wound closure over time revealed a significant inhibitory effect of MMF on fibroblast motility (Fig. 7B), even the lowest concentration of MMF, 0.1 μ M, after 24h of incubation. Secondly, we utilized another motility assay based on the Transwell™ system, which is able to measure the cell migratory potential through nylon membrane pores. As shown in Figure 7C, in presence of 10 μ M MMF WI-26 fibroblasts exhibited a much lower capacity to migrate through the Transwell™ membrane than in absence of MMF. These data, in full compliance with those obtained using the *in vitro* wound closure assay, allow us to conclude that MMF alter fibroblast motility.

Discussion

In this study, we unveil direct inhibitory activity of the immunosuppressive drugs MMF on several fibroblast functions that are exacerbated during fibrotic processes. Specifically, we show that MMF transcriptionally inhibits type I collagen expression, enhanced the expression of MMP-1, and alters both the migratory and contractile functions of fibroblasts. These results suggest that MMF may have direct anti-fibrotic properties in addition to its well-defined immunosuppressive effects.

MMF is a potent suppressor of T and B cell proliferation, but may also be cytostatic for monocytes, vascular smooth muscle cells and fibroblasts (Gregory et al., 1993; Allison and Eugui, 2000). It should be noted that under our experimental conditions, (e.g., at confluency in medium containing 1% FCS), fibroblasts are contact-inhibited and do not proliferate, and MMF has no cytotoxic effect while exerting significant downregulation of collagen gene expression and contraction. Most importantly, the MMF concentrations used for this in vitro study were calculated in order to achieve similar concentrations as those currently recommended for plasma levels in clinical practice among patients treated by MMF. The daily dose recommended for these patients is 2 g orally in order to reach plasma levels varying from 1 mg/l (trough levels) to 5 mg/l at 3 hours which corresponded to concentrations of 0.1 to 10 μ M (Manzia et al., 2005).

During wound healing, fibroblasts undergo a transition to the myofibroblast phenotype, that corresponds to the commencement of connective-tissue compaction and the contraction of the wound (Singer and Clark, 1999; Desmouliere et al., 2005). Such phenotype is also found in pathological fibrotic situations, as they occur for example during complications of organ transplantation, when excessive matrix deposition and excessive scarring takes place within the graft, leading to organ failure (Uitto and Kouba, 2000).

In our study, MMF was shown to reduce fibroblast capacity to contract free-floating collagen lattices, accompanied with a reduction in α -SMA expression. These results are in accordance with a previous report showing that MMF reduce α -SMA production by fibroblasts in nephrectomized rats, thus preventing fibrosis (Badid et al., 2000). α -SMA participates importantly in force

production by myofibroblasts both *in vitro* (Hinz et al., 2001a) and *in vivo* (Hinz et al., 2001b), contributing to scar formation, tissue contraction, scar hypertrophy, and potentially, fibrosis. Furthermore, myofibroblasts not only promote ECM contraction but also synthesize elevated levels of ECM components (Desmouliere et al., 2005). Thus, the persistence of myofibroblasts within a fibrotic lesion leads to excessive scarring with the functional impairment of the affected organ(s). The ability of MMF to decrease α -SMA expression and subsequent matrix contraction by fibroblast is thus likely to confer direct anti-fibrotic activities to this immunosuppressive drug. Recently, we demonstrated that rapamycin, an other immunosuppressive drug, post-transcriptionally alters fibrillar collagen synthesis by repressing their mRNA stability (Poulalhon et al., 2006), therefore utilizing mechanisms that are clearly distinct from those of MMF. Also, rapamycin did not affect the migratory potential of fibroblast or their capacity to contract unloaded collagen lattices (unpublished data). Thus, our studies suggest that both immunosuppressive drugs directly affect fibroblast functions, but with distinct molecular mechanisms that are independent from their immunosuppressive activity.

In conclusion, we have identified MMF as a molecule that may affect fibroblast functions important for wound healing and implicated in tissue scarring and fibrosis. These results suggest that MMF represents a novel therapeutic option for the treatment of fibrotic complications of autoimmune diseases and graft rejection.

References

- Abraham DJ and Varga J (2005) Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol* **26**:587-595.
- Allison AC and Eugui EM (2000) Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* **47**:85-118.
- Arai S and Vogelsang GB (2000) Management of graft-versus-host disease. *Blood Rev* **14**:190-204.
- Badid C, Vincent M, McGregor B, Melin M, Hadj-Aissa A, Veysseyre C, Hartmann DJ, Desmouliere A and Laville M (2000) Mycophenolate mofetil reduces myofibroblast infiltration and collagen III deposition in rat remnant kidney. *Kidney Int* **58**:51-61.
- Boast S, Su MW, Ramirez F, Sanchez M and Avvedimento EV (1990) Functional analysis of cis-acting DNA sequences controlling transcription of the human type I collagen genes. *J Biol Chem* **265**:13351-13356.
- Chen SJ, Artlett CM, Jimenez SA and Varga J (1998) Modulation of human alpha1(I) procollagen gene activity by interaction with Sp1 and Sp3 transcription factors in vitro. *Gene* **215**:101-110.
- Ciancio G, Miller J and Gonwa TA (2005) Review of major clinical trials with mycophenolate mofetil in renal transplantation. *Transplantation* **80**:S191-200.
- Danovitch GM (2005) Mycophenolate mofetil: a decade of clinical experience. *Transplantation* **80**:S272-274.

Derk CT and Jimenez SA (2003) Systemic sclerosis: current views of its pathogenesis. *Autoimmun Rev* **2**:181-191.

Desmouliere A, Chaponnier C and Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* **13**:7-12.

Eckes B, Zigrino P, Kessler D, Holtkotter O, Shephard P, Mauch C and Krieg T (2000) Fibroblast-matrix interactions in wound healing and fibrosis. *Matrix Biol* **19**:325-332.

Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* **200**:500-503.

Gregory CR, Pratt RE, Huie P, Shorthouse R, Dzau VJ, Billingham ME and Morris RE (1993) Effects of treatment with cyclosporine, FK 506, rapamycin, mycophenolic acid, or deoxyspergualin on vascular muscle proliferation in vitro and in vivo. *Transplant Proc* **25**:770-771.

Grinnell F (1994) Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* **124**:401-404.

Hess AD (2006) Modulation of graft-versus-host disease: role of regulatory T lymphocytes. *Biol Blood Marrow Transplant* **12**:13-21.

Hinz B, Celetta G, Tomasek JJ, Gabbiani G and Chaponnier C (2001a) Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* **12**:2730-2741.

Hinz B, Mastrangelo D, Iselin CE, Chaponnier C and Gabbiani G (2001b) Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol* **159**:1009-1020.

Javelaud D, Laboureau J, Gabison E, Verrecchia F and Mauviel A (2003) Disruption of basal JNK activity differentially affects key fibroblast functions important for wound healing. *J Biol Chem* **278**:24624-24628.

Kim JG, Sohn SK, Kim DH, Lee NY, Suh JS, Lee KS and Lee KB (2004) Different efficacy of mycophenolate mofetil as salvage treatment for acute and chronic GVHD after allogeneic stem cell transplant. *Eur J Haematol* **73**:56-61.

Krejci M, Doubek M, Buchler T, Brychtova Y, Vorlicek J and Mayer J (2005) Mycophenolate mofetil for the treatment of acute and chronic steroid-refractory graft-versus-host disease. *Ann Hematol* **84**:681-685.

Leask A and Abraham DJ (2004) TGF-beta signaling and the fibrotic response. *Faseb J* **18**:816-827.

Lioussis SN, Bounas A and Andonopoulos AP (2006) Mycophenolate mofetil as first-line treatment improves clinically evident early scleroderma lung disease. *Rheumatology (Oxford)* **45**:1005-1008.

Lopez F, Parker P, Nademanee A, Rodriguez R, Al-Kadhimi Z, Bhatia R, Cohen S, Falk P, Fung H, Kirschbaum M, Krishnan A, Kogut N, Molina A, Nakamura R, O'Donnell M, Popplewell L, Pullarkat V, Rosenthal J, Sahebi F, Smith E, Snyder D, Somlo G, Spielberger R, Stein A,

- Sweetman R, Zain J and Forman S (2005) Efficacy of mycophenolate mofetil in the treatment of chronic graft-versus-host disease. *Biol Blood Marrow Transplant* **11**:307-313.
- Manzia TM, De Liguori Carino N, Orlando G, Toti L, De Luca L, D'Andria D, Cardillo A, Anselmo A, Casciani CU and Tisone G (2005) Use of mycophenolate mofetil in liver transplantation: a literature review. *Transplant Proc* **37**:2616-2617.
- Mele TS and Halloran PF (2000) The use of mycophenolate mofetil in transplant recipients. *Immunopharmacology* **47**:215-245.
- Poulalhon N, Farge D, Roos N, Tacheau C, Neuzillet C, Michel L, Mauviel A and Verrecchia F (2006) Modulation of Collagen and MMP-1 Gene Expression in Fibroblasts by the Immunosuppressive Drug Rapamycin: A DIRECT ROLE AS AN ANTIFIBROTIC AGENT? *J Biol Chem* **281**:33045-33052.
- Ruzek MC, Jha S, Ledbetter S, Richards SM and Garman RD (2004) A modified model of graft-versus-host-induced systemic sclerosis (scleroderma) exhibits all major aspects of the human disease. *Arthritis Rheum* **50**:1319-1331.
- Shapiro R, Young JB, Milford EL, Trotter JF, Bustami RT and Leichtman AB (2005) Immunosuppression: evolution in practice and trends, 1993-2003. *Am J Transplant* **5**:874-886.
- Singer AJ and Clark RA (1999) Cutaneous wound healing. *N Engl J Med* **341**:738-746.

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Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillesen D and Gabbiani G (1986) A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* **103**:2787-2796.

Stratton RJ, Wilson H and Black CM (2001) Pilot study of anti-thymocyte globulin plus mycophenolate mofetil in recent-onset diffuse scleroderma. *Rheumatology (Oxford)* **40**:84-88.

Uitto J and Kouba D (2000) Cytokine modulation of extracellular matrix gene expression: relevance to fibrotic skin diseases. *J Dermatol Sci* **24 Suppl 1**:S60-69.

Verrecchia F and Mauviel A (2002a) Control of connective tissue gene expression by TGF beta: role of Smad proteins in fibrosis. *Curr Rheumatol Rep* **4**:143-149.

Verrecchia F and Mauviel A (2002b) Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Invest Dermatol* **118**:211-215.

Verrecchia F, Mauviel A and Farge D (2006) Transforming growth factor-beta signaling through the Smad proteins: Role in systemic sclerosis. *Autoimmun Rev* **5**:563-569.

Verrecchia F, Wagner EF and Mauviel A (2002) Distinct involvement of the Jun-N-terminal kinase and NF-kappaB pathways in the repression of the human COL1A2 gene by TNF-alpha. *EMBO Rep* **3**:1069-1074.

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Zeiser R, Marks R, Bertz H and Finke J (2004) Immunopathogenesis of acute graft-versus-host disease: implications for novel preventive and therapeutic strategies. *Ann Hematol* **83**:551-565.

Footnotes

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Legends for Figures

Fig.1. *MMF decreases type I collagen production*

Confluent fibroblast cultures were treated with various concentrations of MMF (10 μ M, 1 μ M or 0,1 μ M) for 24, 48 and 72h, as indicated. After incubations, type I collagen production was detected by Western Blot analysis of whole cell lysates (upper panels). Specificity of the modulation was confirmed with an anti-actin antibody. The ratio of type I collagen to actin is plotted from the values of one representative experiment of four experiments (24h lower panel, 48h middle panel, and 72h right panel).

Fig.2. *MMF decreases COL1A1 and COL1A2 mRNA levels*

Confluent fibroblast cultures were treated with MMF (10 μ M) for 12h, 24h, 48h or 72h, following which total RNA was extracted and *COL1A1* (A) and *COL1A2* (B) mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean \pm S.D. of at least three independent experiments performed, each with duplicate samples.

Fig.3. *MMF decreases COL1A1 and COL1A2 transcription*

Fibroblast cultures were transfected with -2300COL1A1/CAT (A) and -3500COL1A2/CAT (B) collagen promoter/reporter constructs. 12h after transfections, MMF (10 μ M) was added and incubations continued for another 48h. Autoradiograms from CAT assays are from a representative experiment, each for the three experiments being performed with duplicate samples (upper panels). Quantitation of CAT activity in each samples using a phosphorimager is plotted as % of control for each promoter. Values are mean \pm SD of three experiments performed with duplicate samples (lower panels).

Fig. 4. *MMF increases MMP-1 both at mRNA and protein levels.*

(A) Subconfluent fibroblasts were treated with MMF (10 μ M) or not for various times (24h, 48h and 72h). After incubation cell culture supernates were collected and MMP-1 protein levels were estimated by immunoassay. Bars indicate mean \pm S.D. of two independent experiments performed, each with sixuplicate samples. MMP-1 protein levels were corrected for protein levels in untreated fibroblast cultures at each time-point.

(B) Subconfluent fibroblast cultures were treated with MMF (10 μ M, black bars) or not (white bars) for various times (24h, 48h and 72h). MMP-1 mRNA levels were estimated by quantitative RT-PCR. Bars indicate mean \pm S.D. of two independent experiments performed, each with duplicate samples. mRNA levels were corrected for expression levels in untreated fibroblast cultures at each time-point.

Fig. 5. *MMF inhibits fibroblast ability to contract free-floating collagen lattices*

Contraction of free-floating collagen lattices seeded with WI-26 fibroblasts was carried out for the times shown (1 day to 5 days), in the absence or presence of increasing concentrations of MMF (0,1 μ M, 1 μ M and 10 μ M). (A) Photographs of one representative experiment. (B) Graphic representation of the mean \pm SD of three experiments : ✕, untreated fibroblasts ; ■, MMF at 10 μ M ; ▲ MMF at 1 μ M ; ● MMF at 0,1 μ M.

Fig.6. *MMF reduces α -SMA expression*

(A) Confluent fibroblast cultures were treated with various concentrations of MMF (10 μ M) for 1 day, 2 days, 3 days and 5 days as indicated. After incubations, α -SMA production was detected by Western Blot analysis of whole cell lysates. Specificity of the modulation was confirmed with an anti-actin antibody. The ratio of α -SMA to actin is plotted from the values of one representative experiment of two experiments (lower panel).

(B) Confluent fibroblast cultures were treated with MMF (10 μ M) for 1 day, 2 days, 3 days and 5 days as indicated. After incubations, α -SMA mRNA steady-state levels were determined by

quantitative RT-PCR. Bars indicate mean \pm S.D. of three independent experiments performed, each with duplicate samples.

Fig.7. MMF reduces fibroblast motility

(A) Confluent WI-26 fibroblast cultures were preincubated for 24h with increasing concentrations of MMF (0,1 μ M, 1 μ M and 10 μ M) before mechanical wounding with a pipet tip. Wound closure was monitored optically by phase contrast microscopy and photographed at 0h, 8h and 24h. (A) Photographs of one representative experiment. (B) Graphic representation of the mean of three experiments: ✕, untreated fibroblasts ; ■, MMF at 10 μ M ; ▲ MMF at 1 μ M ; ● MMF at 0,1 μ M. For each sample, the value is corrected against the width of the wound at the start of the experiment.

(C) The migration of WI-26 fibroblasts was determined in a Transwell™ assay system after incubation with 10 μ M MMF during 24h or 48h. Results are the mean S.D. of triplicate samples in a representative experiment.

Name	Forward sequence	Reverse sequence
COL1A1	5'-GGGCAAGACAGTGATTGAATA-3'	5'-ACGTCGAAGCCGAATTCCT-3'
COL1A2	5'-TCTCTACTGGCGAAACCTGTA-3'	5'-TCCTAGCCAGACGTGTTTCTT-3'
MMP-1	5'-CCCCAAAAGCGTGTGACAGTA-3'	5'-GGTAGAAGGGATTTGTGCG-3'
TIMP-1	5'-GCTGACATCCGGTTCGTCTAC-3'	5'-GTTGTGGGACCTGTGGAAGTA-3'
α -SMA	5'-CCCCATCTATGAGGGCTATGC-3'	5'-CGGCCAGCCAGATCC-3'
GAPDH	5'-GCTCCTCCTGTTCGACAGTCA-3'	5'-ACCTTCCATGGTGTCTGA-3'

Table 1: Primer sequences used for quantitative RT-PCR

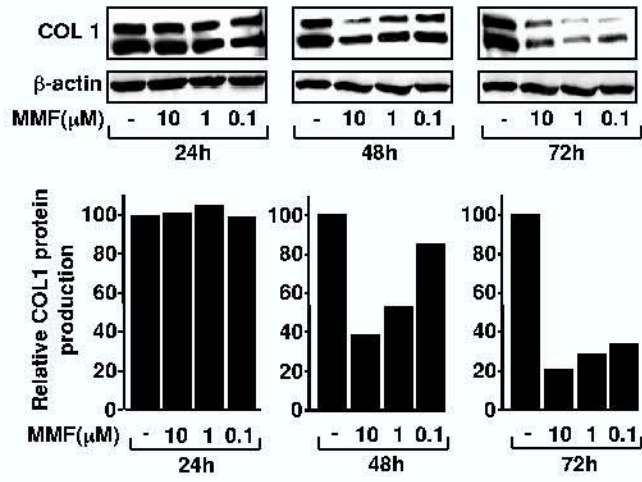


Figure 1

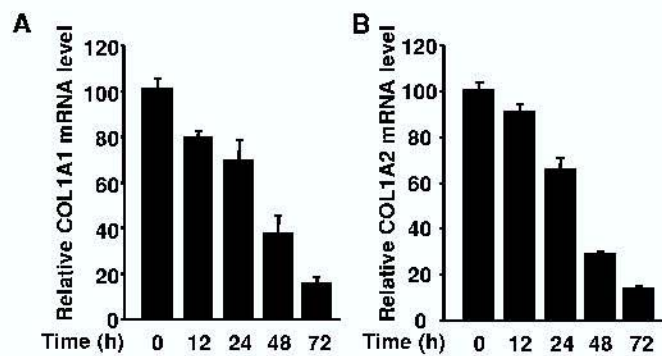


Figure 2

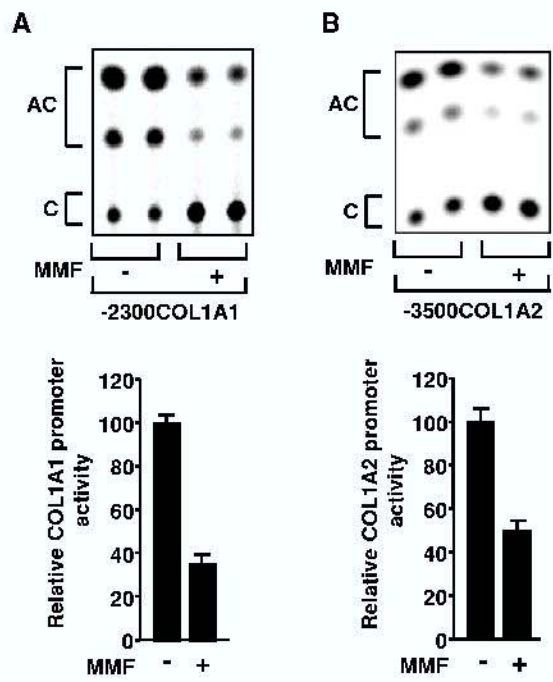


Figure 3

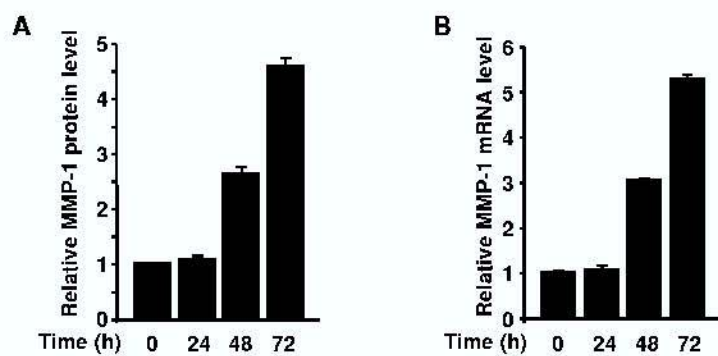


Figure 4

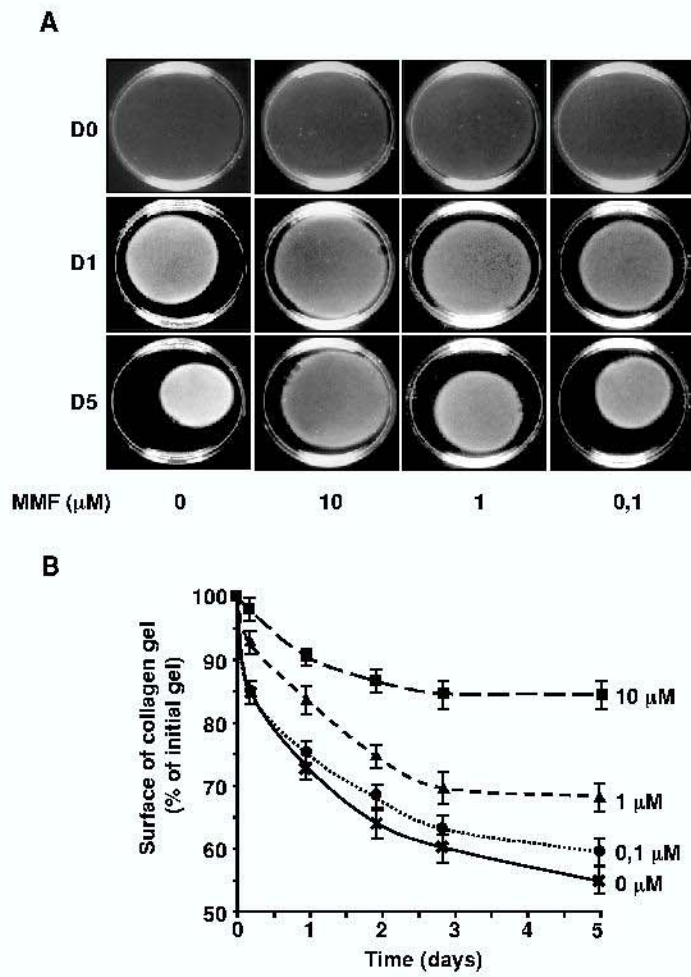


Figure 5

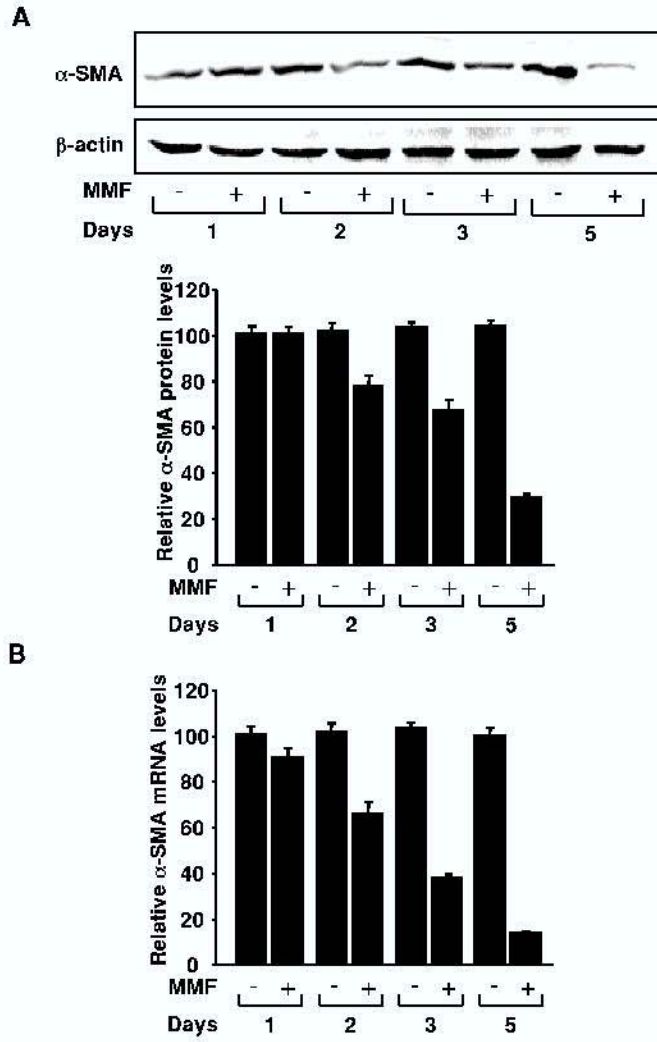


Figure 6

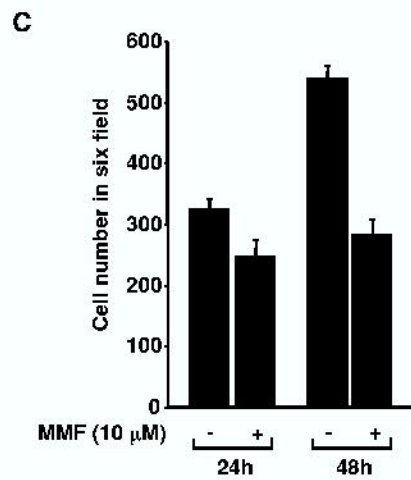
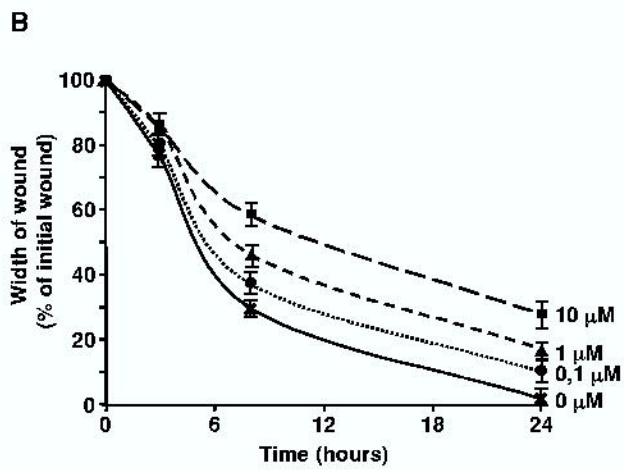
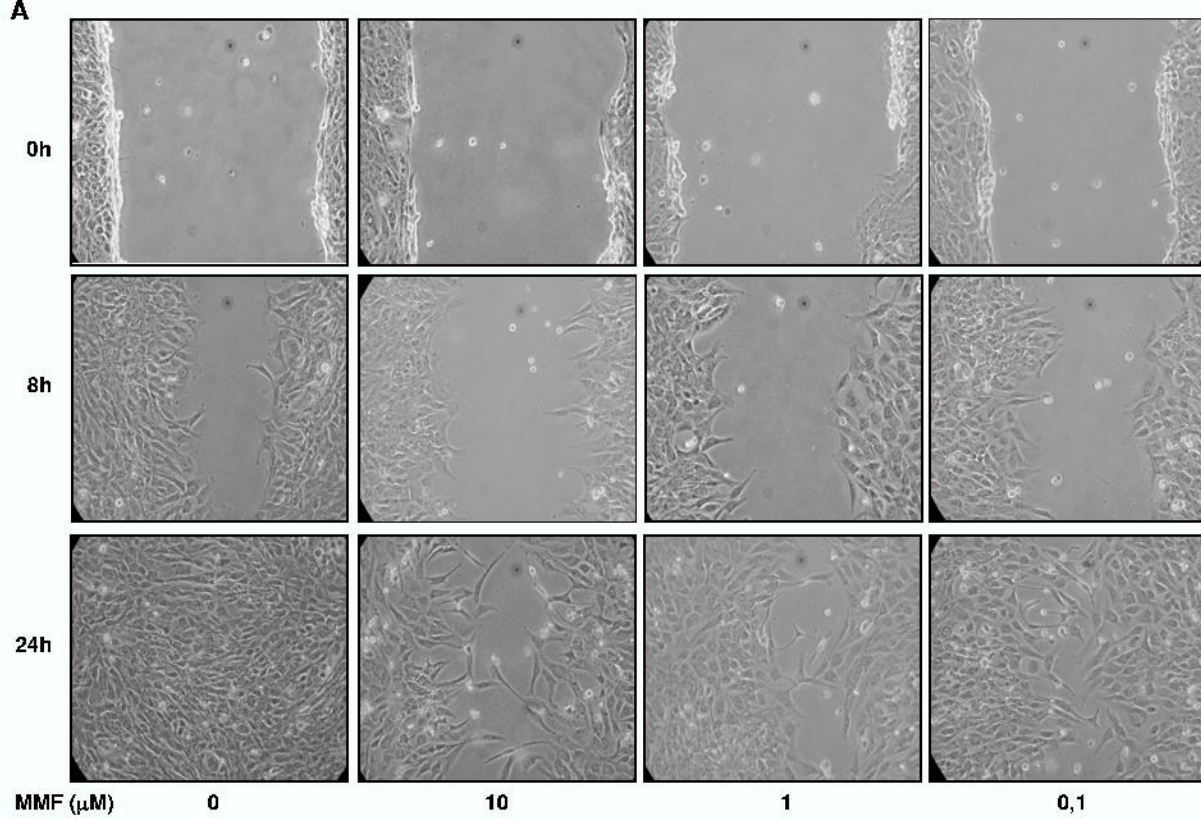


Figure 7