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Matrix Pathobiology

Immunomodulator FTY720 Induces Myofibroblast Differentiation via the Lysophospholipid Receptor $S1P_3$ and Smad3 Signaling

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The novel immunomodulator FTY720 is an effective immunosuppressive agent in experimental models of transplantation and autoimmunity and is currently undergoing phase III clinical trials for multiple sclerosis. Phosphorylated FTY720 is a structural analogue of sphingosine 1-phosphate (S1P) and therefore acts as a high-affinity agonist at four of the five G protein-coupled S1P receptors. It has been well established that there exists a crosstalk between S1P and transforming growth factor (TGF)-β signaling. Because TGF-β is the most prominent inductor of fibrosis and myofibroblasts are primarily responsible for excessive matrix protein formation, we examined whether FTY720, in analogy to TGF-β, induces differentiation of fibroblasts into myofibroblasts. Indeed, FTY720 provoked myofibroblast differentiation comparable with that of TGF-β. For biological efficacy, FTY720 required endogenous phosphorylation because inhibition of sphingosine kinase completely prevented FTY720 from inducing the differentiation process. Moreover, we identified the lysophospholipid receptor $S1P_3$ as the crucial receptor subtype for FTY720-induced myofibroblast differentiation because the effect was abolished in fibroblasts isolated from $S1P_3$ knockout mice. Finally, we determined that downstream of $S1P_3$ signaling Smad3 activation is essential for myofibroblast differentiation in response to FTY720. Thus, FTY720 may have adverse fibrotic effects related to its activity on $S1P_3$ signaling. *(Am J Pathol 2007, 170:281–292; DOI: 10.2353/ajpath.2007.060485)*

The novel immunomodulator FTY720, which is undergoing phase III clinical trials for the treatment of multiple sclerosis, exhibits a different mechanism of action compared with that of currently used immunosuppressive molecules.[1–4] The immunomodulator consistently and selectively suppresses the levels of lymphocytes in blood and lymphatic fluid by almost 90% and simultaneously elicits sequestration of recirculating B and T cells into secondary lymphoid organs.[2] Investigations of the mechanisms of immunosuppression revealed that FTY720 is phosphorylated *in vivo* by sphingosine kinase subtype 2 (SphK2) and that the resulting phosphate ester (FTY720-P) shares striking structural homology to the natural biological mediator sphingosine 1-phosphate (S1P). Thus, FTY720-P is a true agonist of nanomolar potency for the S1P receptors $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$, but not $S1P_2$.[3,5,6] Indeed, most actions of FTY720-mediated immunosuppression can be mimicked by non-hydrolyzable phosphonate analogues of FTY720, indicating that the phosphorylated FTY720 is the active principle.[6] It has been well established that lymphocyte circulation between blood and lymphatic tissue is regulated by the $S1P_1$ receptor subtype and its natural ligand S1P.[3] The importance of this receptor subtype can be shown in mice with a specific deletion of $S1P_1$ in hematopoietic cells because thymocytes selectively require $S1P_1$ for egress from the thymus, whereas both T and B cells necessitate this subtype for egress from secondary lymphoid organs.[3] Recent studies have indicated that FTY720, after phosphorylation, induces a prolonged

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down-regulation of the receptor subtypes $S1P_1$ on thymocytes and lymphocytes, depriving them from the obligatory S1P-mediated signal to egress from lymphoid organs.[7] Besides the action on lymphocyte recirculation, FTY720 maintains vascular integrity of endothelial cells by enhancing adherens junction assembly.[8]

Although sequestration of lymphocytes is solely mediated by $S1P_1$, unspecific S1P receptor agonists elicit a variety of physiological and pathological responses.[9] It has been shown that high plasma levels of S1P lead to adverse cardiovascular effects, including hypotension, bradycardia, and coronary artery vasospasm, which are mediated after activation of the receptor subtype $S1P_3$.[10] Thus, it is not astonishing that high concentrations of FTY720 may also induce $S1P_3$-mediated bradycardia.[4]

Most recently, we have shown that there also exists a crosstalk between S1P receptors and the signaling cascade of transforming growth factor (TGF)-β in a variety of cells.[11–13] In general, TGF-β signals through transmembrane receptor serine/threonine kinases to activate signaling intermediates, called Smad proteins, which then translocate into the nucleus and act as transcription factors.[14] In different cell types, it has been shown that S1P augments Smad3 phosphorylation, one of the two homologous proteins, which signals from TGF-β/activin, and that the abrogation of Smad3 prevents S1P-mediated effects, indicating a surprising but essential role of Smad3 in the signaling cascade of the lysophospholipid.[11,12]

Besides its immunomodulatory effect, TGF-β possesses multiple biological actions, which contribute to the capacity that it plays in many fibrotic diseases.[15] A key role is the TGF-β-mediated differentiation of fibroblasts into myofibroblasts because these cells are primarily responsible for excessive matrix protein formation.[16] Because of a crosstalk between S1P and TGF-β signaling, it was therefore of interest whether FTY720 may also influence myofibroblast differentiation. Indeed, here we show that FTY720 strongly increases myofibroblast differentiation as a result of interaction of the phosphorylated analogue with the $S1P_3$ receptor subtype and the subsequent activation of Smad3.

Materials and Methods

Materials

N,N-Dimethylsphingosine (DMS), pertussis toxin (PTX), and protein G agarose were purchased from Calbiochem (Bad Soden, Germany). FuGene was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal anti-Smad1,2,3, rabbit polyclonal anti-$S1P_1$, goat polyclonal anti-$S1P_3$, goat polyclonal anti-$S1P_4$, goat polyclonal anti-$S1P_5$ antibodies, goat polyclonal anti-TGF-β antibodies, normal goat IgG, and anti-goat IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LumiGlo reagent, peroxide, and anti-rabbit and anti-mouse IgG horseradish peroxidase were obtained from New England Biolabs (Beverly, MA). FTY720 was purchased from Calbiochem. Bovine serum albumin, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and L-glutamine solution were from Seromed Biochrom (Berlin, Germany). Aprotinin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide, Dulbecco's modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid (EDTA), leupeptin, Mowiol, murine monoclonal anti-α-smooth muscle actin (α-SMA) antibodies, fluorescein isothiocyanate (FITC)-linked anti-mouse antibodies, penicillin, pepstatin, sodium dodecyl sulfate (SDS), TGF-β1, sodium fluoride, sodium orthovanadate, streptomycin, Tris, Triton X-100, trypsin, and Tween 20 were purchased from Sigma (Deisenhofen, Germany). OptiMEM was from Invitrogen (Karlsruhe, Germany). Oligonucleotides were synthesized at Tib Molbiol (Berlin, Germany). Polyvinylidene difluoride membranes were purchased from Millipore (Schwalbach, Germany).

Synthesis of FTY720-P

FTY720-P (phosphoric acid mono[(*R/S*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl]ester) was synthesized from FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) as recently described.[17] In brief, FTY720 was protected as an oxazolidinone by the addition of benzyl chloroformate. Then, phosphorylation of the free hydroxyl group was performed by the addition of 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphepintriphenyl phosphite and a subsequent oxidation to obtain (*R/S*)-4-[2-(4-octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3λ^5-benzo[*e*][1,3,2]dioxaphosphepin-3-yloxymethyl)oxazolidin-2-one. The phosphate-protecting group was removed by hydrogenation, and the oxazolidinone was cleaved with lithium hydroxide yielding to FTY720-P. Identity was checked by electrospray ionization time-of-flight mass spectrometry using an Agilent 6210 TOF LC/MS (Waldbronn, Germany). Electrospray ionization-mass spectrometry, *m/z*: 386 $(M - H)^-$. Moreover, purity of FTY720-P was measured by high-performance liquid chromatography (HPLC).[18] Therefore, synthesized FTY720-P or standard FTY720 was dissolved in 275 μl of methanol/0.07 mol/L K_2HPO_4 (9:1). A derivatization mixture of 10 mg of o-phthaldialdehyde, 200 μl of ethanol, 10 μl of 2-mercaptoethanol, and 10 ml of 3% boric acid was prepared and adjusted to pH 10.5 with KOH. Twenty-five μl of the derivatization mixture was added to the resolved FTY720 or FTY720-P for 15 minutes at room temperature. The derivatives were analyzed by a Merck Hitachi LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany). Separation was done with a gradient of methanol and 0.07 mol/L K_2HPO_4. Resulting profiles were evaluated using the Merck system manager software, indicating no detectable amount of FTY720 in the synthesized FTY720-P.

Preparation of Human Fibroblasts

To isolate human fibroblasts, juvenile foreskin from surgery was incubated at 37°C for 2.5 hours in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinization was terminated by the addition of DMEM containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS) and centrifuged at 250 × *g* for 5 minutes. The pellet was resuspended in DMEM containing 7.5% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Fibroblasts were pooled from several donors and cultured at 37°C in 5% CO_2. Only cells of the third to sixth passage were used for the experiments.

Preparation of Wild-Type and Smad3$^{(-/-)}$ Fibroblasts

Murine fibroblasts, isolated from polymerase chain reaction-genotyped wild-type and Smad3 knockout newborn mice, were kindly provided from Dr. Anita Roberts (National Institutes of Health, National Cancer Institute, Bethesda, MD).[19] Cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. For detection of wild-type and Smad3$^{(-/-)}$ fibroblasts the primer sequences 5′-CCACTTCATTGCCATATGCCCTG-3′ (located 5′ to the deletion) and 5′-CCCGAACAGTTGGATTCACACA-3′ (located within the deletion) were used. Smad3$^{(-/-)}$ fibroblasts were identified using the primer located to the 5′ deletion and a primer specific for the pLoxpneo cassette (5′-CCAGACTGCCTTGGGAAAAGC-3′).

Preparation of Wild-Type and S1P$_3^{(-/-)}$ Fibroblasts

Wild-type and $S1P_3$ knockout mice were generated by Dr. Jerold Chun as recently described.[20] To isolate murine fibroblasts, skin was incubated at 37°C for 2.5 hours in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinization was completed by the addition of DMEM containing 10% FBS. Isolated fibroblasts were washed with PBS and centrifuged at 250 × *g* for 5 minutes. The pellet was resuspended in DMEM containing 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and cultured at 37°C in 5% CO_2. Cells were genotyped by polymerase chain reaction. The following primers were used: 5′-CACAGCAAGCAGACCTCCAGA-3′, 5′-TGGTGTGCGGCTGTCTAGTCAA-3′, and 5′-ATCGATACCGTCGATCGACCT-3′.

Real-Time Polymerase Chain Reaction (PCR)

Real-time PCR assays were performed using the SYBR Green PCR Master Mix on ABI Prism 7900HT sequence detection system according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Amplification was performed in 10-μl reactions (primer concentration, 250 nmol/L, 1× SYBR Green Master Mix) containing 2 μl of cDNA (equivalent to 10 ng of total RNA) in 40 cycles of 95°C, 15 seconds, 60°C, 1 minute. Primers were purchased at SuperArray Bioscience Corporation (Frederick, MD). Total RNA of three different sets of fibroblasts were used to analyze receptor expression. Data normalization was performed using GADPH as reference gene. Relative mRNA expression was quantified using the comparative C_T method according to the ABI manual.

Cell Viability Assay

Cell viability was measured by the MTT dye reduction assay. Cells, seeded into 24-well plates for 24 hours, were incubated with FTY720 or FTY720-P for 24 hours at 37°C in 5% CO_2. After the addition of 100 μl of MTT solution (5 mg/ml) per well, the plates were incubated for another 4 hours. The supernatants were removed, and the formazan crystals were solubilized in 1 ml of dimethyl sulfoxide. The optical density was determined at 540 nm using a scanning microplate spectrophotometer (Multiscan Plus; Labsystems, Helsinki, Finland).

Immunofluorescence Microscopy of α-SMA

Fibroblasts were seeded into 12-well plates, each containing a glass coverslip, and cultured for 24 hours in DMEM containing 2 nmol/L L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 7.5% FBS. Then they were serum-deprived for 48 hours in DMEM supplemented with 2 nmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Quiescent fibroblasts were stimulated with the indicated substances for 72 hours. Cells were washed with ice-cold PBS and fixed in methanol at 4°C for 2 minutes. Cells were treated with blocking buffer (1% bovine serum albumin in PBS) for 30 minutes followed by incubation with murine anti-α-SMA antibodies (1:50 diluted in blocking buffer). Coverslips were washed three times with blocking buffer and incubated with FITC-linked anti-mouse antibodies (1:125 diluted in blocking buffer). After 30 minutes, fibroblasts were washed three times with blocking buffer and fixed by a Mowiol mounting medium. Staining was examined using the Olympus BX41 fluorescence microscope (Hamburg, Germany) and documented by the digital camera Nikon DXM1200 (Düsseldorf, Germany). Appropriate emission filter settings and controls were included for bleed-through effects.

Immunoprecipitation

Human fibroblasts were seeded in six-well plates and cultured for 24 hours in DMEM containing 7.5% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and then medium was replaced by HEPES buffer (1 mol/L) for 2 hours. Cells were treated with TGF-β (2 ng/ml) or FTY720 (1 μmol/L) for 30 minutes. Fibroblasts were rinsed twice with ice-cold PBS and harvested in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS), containing pro-

tease inhibitors (1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) and phosphatase inhibitors (1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and 40 mmol/L β-glycerophosphate). Lysates were centrifuged at 14,000 × *g* for 30 minutes. One hundred μg of lysate protein was immunoprecipitated overnight at 4°C with 0.2 μg of anti-Smad1,2,3 antibodies or 0.2 μg of normal goat IgG (IgG control), followed by a precipitation with 10 μl of protein G plus agarose at 4°C for 90 minutes. After four washes with complete RIPA buffer, the immunoprecipitates were eluted by boiling for 5 minutes in 60 μl of SDS sample buffer (100 mmol/L Tris/HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 200 mmol/L dithiothreitol).

Immunoblotting

For Western blot analysis, immunoprecipitates (20 μl) or cell lysates (10 to 20 μg of protein) were separated by SDS/polyacrylamide gel electrophoresis. Gels were blotted overnight onto polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS)-Tween 20 (0.1%) overnight at 4°C, membranes were incubated with the indicated specific primary antibodies for 0.5 or 2 hours at room temperature. The blots were washed three times in TBS-Tween 20 followed by incubation with the secondary horseradish peroxidase-conjugated antibodies for 1 hour at room temperature. Immunocomplexes were detected using an enhanced chemiluminescence detection method. Densitometry of films was performed using the Syngene GeneGenius (Cambridge, UK).

Anti-Sense Oligonucleotides

Anti-sense oligonucleotides (ASOs) were designed to surround the translational initiation site, a place empirically known to be most effective for inhibition of gene expression. The following specific ASOs as well as same-length control oligonucleotides (with the same nucleotides but randomly scrambled sequence) were synthesized: Smad3 ASO: 5′-GCAGGATGGACGACAT-3′, control oligonucleotides 5′-GTGGACAGCTAGAGAC-3′; SphK2 ASO: 5′-CAGGGGAAGAGGCAGGTCAGACA-3′, control oligonucleotides: 5′-TGCAAGCTCACCAACCCCACATA-3′; $S1P_1$: ASO 5′-GACGCTGGTGGGCCCCAT-3′, control oligonucleotides: 5′-ATGGGGCCCACCAGCGTC-3′; $S1P_3$: ASO 5′-CGGGAGGGCAGTTGCCAT-3′, control oligonucleotides: 5′-ATGGCAACTGCCCTCCCG-3′; $S1P_4$: ASO 5′-GAAGGCCAGCAGGATCATCAGCAC-3′, control oligonucleotides: 5′-ACCTAGCCAACCCTCCATGAAGGC-3′; $S1P_5$: ASO 5′-CAACATGCCACAAAGGCCAGGAG-3′, control oligonucleotides: 5′-GCAACAACATAACGGGCCAGCAG-3′. Cells were seeded in six-well plates and cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin for 12 hours. Control oligonucleotides and ASOs were solubilized in OptiMEM and FuGENE (1 μg of DNA/2 μl) to achieve a final concentration of 500 nmol/L of oligonucleotides. Then the solution was added to fibroblasts for 72 hours. Abrogation of protein expression was verified by immunoblotting.

Measurement of TGF-β Secretion

Fibroblasts (1×10^5 cells/ml) were stimulated with FTY720 (1 μmol/L) throughout a time period of 24 hours. Then levels of TGF-β in the supernatant were quantified by selective enzyme-linked immunosorbent assay kits following the instructions of the manufacturer (Amersham Pharmacia Biotech, Freiburg, Germany). For measurement of latent complexes of TGF-β, activation was accomplished by acid treatment. Therefore, 0.5 ml of cell culture supernatants were treated with 0.1 ml of 1 mol/L HCl, incubated for 10 minutes, and then neutralized with 0.1 ml of 1.2 mol/L NaOH/0.5 mol/L HEPES. Samples were analyzed by a Fluostar Optima ELISA reader from BMG Labtech (Offenburg, Germany). The detection limit was 4 pg/ml.

Results

FTY720 Induces Transformation of Fibroblasts into Myofibroblasts

The expression of α-SMA is one of the most prominent features of myofibroblasts, which have a phenotype intermediate between smooth muscle cells and fibroblasts. TGF-β has been indicated as the crucial cytokine to induce transformation of fibroblasts into myofibroblasts.[16] Most recently, we figured out that there exists a crosstalk between TGF-β and S1P receptors and that S1P mimics biological effects of TGF-β in dendritic cells.[11] Based on the structural similarity between S1P and the phosphorylated FTY720, we investigated whether the immunomodulator FTY720 might also influence myofibroblast differentiation. Therefore, α-SMA expression in response to FTY720 was measured by Western blotting and immunofluorescence microscopy in primary human fibroblasts. For positive control experiments, fibroblasts were treated with TGF-β, and immunofluorescence indicated a pronounced α-SMA formation confirming that TGF-β stimulates the fibroblasts to differentiate into myofibroblasts (Figure 1). Most interestingly, treatment of primary cells with FTY720 also resulted in a distinct expression of α-SMA (Figure 1). Immunofluorescence of α-SMA in response to FTY720 showed a distinct appearance of numerous bundles of actin microfilaments comparable with TGF-β, whereas the cell shape was slightly elongated. The number of fibroblasts expressing α-SMA was drastically increased in a dose-dependent manner (Figure 1). A significant increase was detected at a concentration of 0.1 μmol/L FTY720, whereas a maximal effect occurred at 1 μmol/L inducing a similarly strong expression of α-SMA as the most effective dose of TGF-β (2 ng/ml) (Figure 1). It should be noted that higher concentrations of FTY720 did not further increase α-SMA formation attributable to a toxic

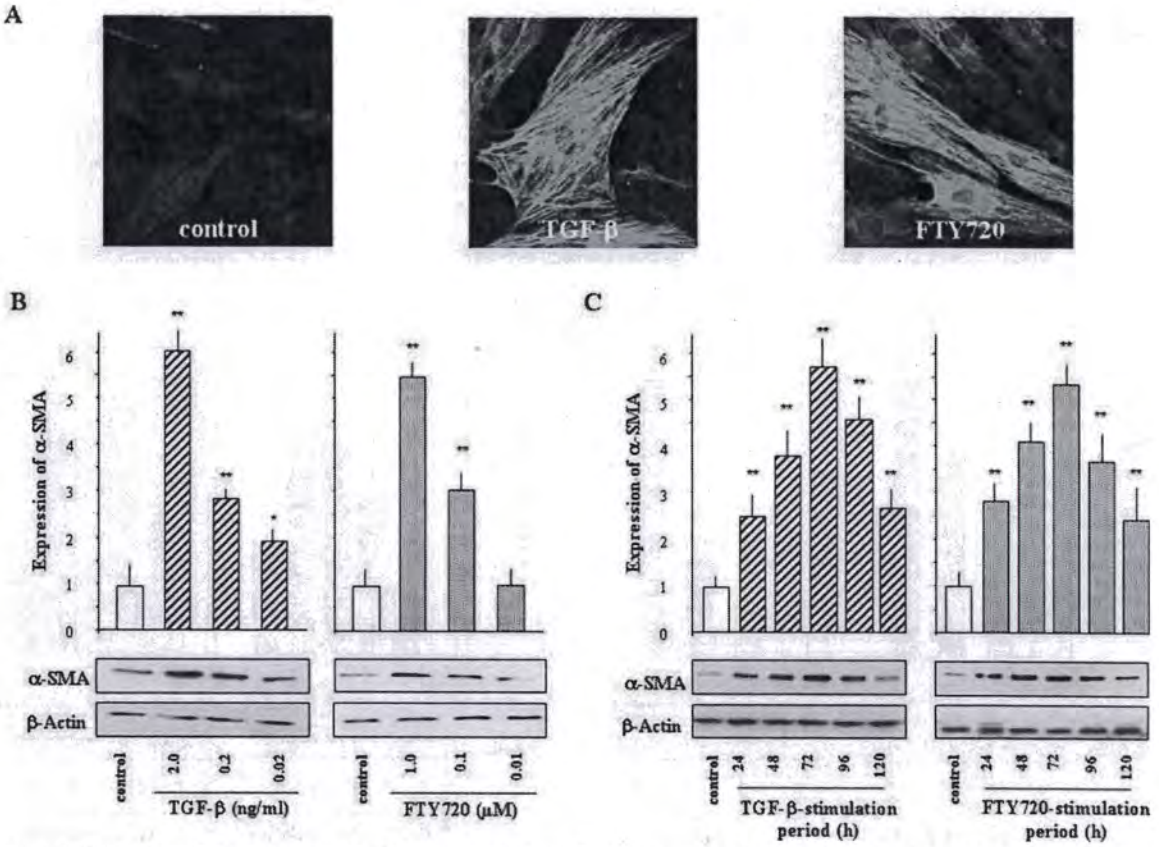

Figure 1. TGF-β and FTY720 induce myofibroblast differentiation. **A:** Human fibroblasts were stimulated with TGF-β (2 ng/ml) or FTY720 (1 μmol/L) for 72 hours followed by an immunofluorescence analysis of α-SMA. **B** and **C:** Cells were treated with the indicated concentrations of TGF-β or FTY720 for 72 hours (**B**) or with 2 ng/ml TGF-β or 1 μmol/L FTY720 for different stimulation periods (**C**). Then α-SMA was measured by Western blot analysis as described in Materials and Methods. All results were confirmed in three independent experiments. Densitometric analysis of α-SMA formation was performed after Western blot analysis. Values are normalized to β-actin levels and are expressed as an ×-fold increase of α-SMA formation compared with untreated cells ± SEM from at least three experiments. $^{*}P < 0.05$ and $^{**}P < 0.001$ indicate a statistically significant difference versus unstimulated control cells. Original magnifications, ×400.

effect of the immunosuppressive agent (data not shown). Furthermore, in analogy to TGF-β, a significant increase of α-SMA was first detected after a 24-hour treatment of fibroblasts with FTY720, whereas a maximal response was visible after 72 hours (Figure 1).

Phosphorylation of FTY720 Is Required for Myofibroblast Differentiation

Although FTY720 induces myofibroblast differentiation, a variety of studies indicate that phosphorylation of FTY720 by sphingosine kinase is necessary for its biological effects.[5,6] Therefore, we measured whether FTY720-P is also able to differentiate fibroblasts into myofibroblasts. Indeed, in Figure 2 it is presented that the phosphorylated product enhanced α-SMA formation comparable with FTY720. It is of interest that the kinetic of α-SMA formation induced by FTY720-P was very similar to FTY720 showing a maximal effect after an incubation period of 72 to 96 hours (Figure 2). These results suggest that fibroblasts induce a rapid phosphorylation of FTY720. Because FTY720-P shares structural homology to the natural biological mediator S1P, we examined whether the lysophospholipid S1P also induces myofibroblast differentiation. Actually, in Figure 2 it is shown that treatment of primary cells with S1P also resulted in the formation of α-SMA. A maximal effect occurred after an in-cubation period of 72 hours with 10 μmol/L S1P (Figure 2).

To examine whether the action of FTY720 is mediated by the phosphorylated product, conversion of FTY720 to FTY720-P was blocked by the sphingosine kinase inhibitor DMS. Indeed, when fibroblasts were treated with 5 μmol/L DMS, the ability of FTY720 to enhance α-SMA was almost completely diminished, whereas TGF-β-induced α-SMA formation was not affected in the presence of DMS (Figure 3, A and B). Moreover, several studies indicate that SphK2 is mainly responsible for the phosphorylation of FTY720. Therefore, we measured the ability of FTY720 to induce α-SMA expression after treatment with SphK2-ASO. In Figure 3C, it is shown that control oligonucleotides did not reduce α-SMA formation in response to FTY720. But when cells were treated with SphK2-ASO,

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