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Research Paper

FK506 Microparticles Mitigate Experimental Colitis with Minor Renal Calcineurin Suppression

Alf Lamprecht,[1,2,3,4] Hiromitsu Yamamoto,[1] Nathalie Ubrich,[2] Hirofumi Takeuchi,[1] Philippe Maincent,[2] and Yoshiaki Kawashima[1]

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Purpose. FK506 microparticles providing selective colonic drug delivery were tested for their efficiency in a local treatment to the inflamed gut tissue in inflammatory bowel disease (IBD). Because FK506 proved its distinct mitigating potential in the treatment of IBD, risking, however, severe adverse effects, a more selective delivery to the site of inflammation may further improve efficiency and tolerability.
Methods. A model colitis was induced to male Wistar rats by trinitrobenzenesulfonic acid. FK506 was entrapped into microspheres (MS) prepared with the pH-sensitive polymer Eudragit P-4135F in order to allow drug delivery to the colon. Clinical activity score, colon/body weight index, and myeloperoxidase activity were determined to assess the inflammation, and adverse effects of FK506 resulting from its systemic absorption were quantified as well.
Results. The clinical activity score and myeloperoxidase activity decreased after the administration of all FK506-containing formulations. The MS formulations proved to be as efficient in mitigating the experimental colitis as the subcutaneous drug solution (myeloperoxidase activity, MS: 9.64 ± 6.6 U/mg tissue; subcutaneous: 7.48 ± 6.96 U/mg) and to be superior to drug solution given by oral route (oral: 12.66 ± 5.46 U/mg; untreated colitis control: 21.88 ± 4.12 U/mg). The FK506 subcutaneous group exhibited increased levels of adverse effects, whereas the FK506-MS group proved its potential to retain the drug from systemic absorption as evidenced by reduced nephrotoxicity.
Conclusions. The development of this selective delivery system for FK506 should be given particular consideration in the treatment of IBD, as it allows therapy that profits from FK506's high immune suppressive effect with a simultaneously reduced nephrotoxicity.

KEY WORDS: colitis; colon delivery; inflammatory bowel disease; microspheres; tacrolimus.

INTRODUCTION

The general principle of a pharmacological treatment in inflammatory bowel disease (IBD) is to induce remission of outbreaks and to prevent outbreaks during remission. With this goal, standard pharmaceutical products are 5-aminosalicylic acid and glucocorticoid preparations with different delivery profiles in the gastrointestinal tract (1). In more severe cases, other types of drugs have been proposed for the therapy. Especially, azathioprine is currently used in Crohn disease with methotrexate as the second-line treatment (2,3). Unfortunately, this antimetabolite therapy possesses significant drawbacks including myelosuppression and increased risk of malignant transformation (4). The more recently proposed use of a monoclonal antibody against tumor necrosis factor-α in the treatment of Crohn's disease showed distinct success in the therapy due to its specificity, thus allowing a reduction of adverse effects. However, it was found that patients develop antibodies against the treatment with an increased risk of infusion reactions and a reduced duration of response to treatment (5). Therefore, the potential of low-molecular-weight immune suppressors might still be of interest, but surely requiring the design of specific drug delivery systems providing a local treatment. FK506 (tacrolimus), initially developed as an immune suppressive drug used to inhibit transplantation rejection, was successfully applied in IBD to treat refractory ulcerative colitis where corticoid treatment failed (6,7). After forming a complex with the cytosolic receptor FKBP-12, FK506 acts by inhibiting the activity of the enzyme calcineurin (8–10). The mechanism of this immunosuppresive effect is not selective to the inflamed tissue and is also responsible for the nephrotoxicity of FK506, which has been reported as a main adverse effect (11).

A major therapeutic strategy consists in the oral administration of drug delivery systems releasing their entrapped active compounds selectively to the colon. For instance, enzymatically degradable carriers rely on the enzymatic activity of colonic bacteria similar to the mechanism of prodrugs. Others, among them most of the commercialized systems, are based on the change of the luminal pH during the gastrointestinal passage or perform a time-dependent drug release (12,13). Based on the assumption that a varying enzymatic

[1] Laboratory of Pharmaceutical Engineering, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585, Japan.
[2] Faculty of Medicine, Inserm Erit-M 0323, University of Nancy-1, Nancy, France.
[3] Department of Biopharmaceutics, Saarland University, Saarbrücken, Germany.
[4] To whom correspondence should be addressed. (e-mail: Alf.Lamprecht@pharma.uhp-nancy.fr)

ABBREVIATIONS: IBD, inflammatory bowel disease; MS, microspheres.

activity of the colonic bacteria impedes drug delivery more intensively than a possible change of the luminal pH, the latter strategy is preferred for the design of various dosage forms such as pellets, capsules, or tablets. However, their efficiency is decreased in many cases due to diarrhea, a major symptom of IBD that both accelerates the carrier elimination (14,15) and reduces the possible drug release time and absorption from the delivery system. As reported from previous work, especially drug carrier systems with a size larger than 200 μm are strongly subjected to the diarrhea symptoms, resulting in a decreased gastrointestinal transit time and leading therefore to a distinct risk of inefficiency (14,15). A size reduction of the drug carrier system might be an option in order to circumvent those problems, as it was proposed by using micro- and nanocarriers (16,17).

Here, the use of a pH-sensitive polymer is proposed for the design of microparticles in order to achieve colonic delivery. Compared to the other pH-sensitive polymers, Eudragit P-4135F dissolves at a pH around 7.2, allowing a further retention of drug release toward the colonic tissue (18,19). This report evaluates the mitigating potential of FK506 in IBD when delivered locally to the colon after oral administration by a microparticulate delivery system based on Eudragit P-4135F. The study focuses especially on the comparative analysis of the mitigating efficiency and the level of adverse effects. To determine the efficiency of the microspheres (MS) treatment, control rats received FK506 as a solution either by oral or subcutaneous route.

MATERIALS AND METHODS

Materials

Eudragit P-4135F was a kind gift from Röhm Pharma Polymers (Tokyo, Japan), and FK506 (tacrolimus) was received as a kind gift from Fujisawa Co. Ltd. (Osaka, Japan). Polyvinyl alcohol was purchased from Sigma (Deisenhofen, Germany). All other chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and were of analytical grade.

Methods

Preparation of Microspheres

The preparation of MS was based on an oil/water emulsification-solvent evaporation method. Eudragit P-4135F, 200 mg, was dissolved in 3 ml methylene chloride together with 50 mg of FK506. This solution was poured into 75 ml of 1% w/w polyvinyl alcohol and an oil/water emulsion was formed by extensive stirring with a three-blade propeller at 500 rpm. The system was kept under agitation until methylene chloride was evaporated. After decantation, the microspheres were filtered (HVLP filter, Millipore, pore size 0.45 μm), washed extensively with distilled water, and lyophilized overnight.

In Vitro Characterization of Microspheres

Microspheres were analyzed for their size distribution using a LDSA 2400A particle size analyzer (Tohnichi Computer Co. Ltd., Tokyo, Japan). For scanning electron microscopy (SEM), the particles were fixed on supports with carbon-glue, coated with gold using a gold sputter module in a high-vacuum evaporator, and then observed with the scanning electron microscope (JEOL JSM-T330A scanning microscope, Tokyo, Japan) at 10 kV.

Drug loading was determined by high-performance liquid chromatography (HPLC) after extraction from the particles as described elsewhere in detail (20). The *in vitro* drug release was analyzed by the use of a paddle apparatus (USP XXIII). Drug-loaded microparticles were suspended in 100 ml phosphate buffer of pH 1.2 containing 0.1% polysorbate 80 at 37°C. After 2 and 4 h, particles were filtered and resuspended in buffers of pH 6.8 or 7.4, respectively. Aliquots of the dissolution medium (300 μl) were withdrawn at predetermined time intervals and directly analyzed by HPLC.

Animal Treatment

Experiments were carried out in compliance with the regulations of the committees of the Gifu Pharmaceutical University (Gifu, Japan) in line with the Japanese legislation on animal experiments. The model colitis was induced to male Wistar rats (average weight 230g; 10 weeks; n = 6 to 8 per group) by the following procedure: after light narcotizing with ether, the rats were catheterized 8 cm intrarectally, and 500 μl of trinitrobenzenesulfonic acid in ethanol was applied (dose was 140 mg/kg body weight in ethanol, 50% solution). For 2 days, the rats were housed without treatment to maintain the development of a full inflammatory bowel disease model. Starting from day 5, the different groups received FK506 as subcutaneous injection (FK506sc) or orally, either as solution (FK506oral; solubilized with 0.1% polysorbate 80) or as MS formulation (FK506-MS). FK506 solution or MS suspension (0.5 ml) were administered once daily for 7 continuous days (FK506 solution: 1 mg/kg; suspension of FK506-MS: equivalent dose containing). Colitis controls received saline instead of free drug or drug-containing particles. Where FK506 was administered to healthy animals, a subcutaneous injection was given. Animals were sacrificed 24 h after the last drug/particle administration and colons were resected.

Clinical Activity Score System, Colon/Body Weight Index, and Myeloperoxidase Activity

Colitis activity was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding as previously described elsewhere (17,21).

Resected colon tissue samples were opened longitudinally and rinsed with iced phosphate buffer to remove luminal content and weighed. The colon/body weight index was calculated as a quotient of the colon wet weight compared to the total body weight of each rat.

Histologic assessment was carried out by light microscopy of colon tissue samples embedded by a standard paraffin technique. The degree of inflammation was graded using the criteria described by Gonzalez *et al.* (22), and the score represented the sum of eight individual variables graded 0–3 depending on the severity of the changes (0 = no change; 1 = mild; 2 = moderate; 3 = severe). The variables evaluated were erosion, ulceration, necrosis, hemorrhage, edema, and inflammatory cell infiltration.

The measurement of the myeloperoxidase activity was performed to quantify the severity of the colitis. It is a reliable index of inflammation caused by infiltration of activated neu-

trophils into the inflamed tissue. Activities were analyzed according to Krawisz *et al.* (23).

Ca^{2+} Amount in the Urine, Creatinine and Blood Urea Nitrogen Levels

FK506 was administered to rats daily over 5 weeks by either subcutaneous or oral route (FK506 solution or MS). The Ca^{2+} amounts in urine and weight loss were assessed weekly. After 5 weeks, blood samples were taken and kidneys were resected. Ca^{2+} amount in the urine was analyzed by a Wako Ca^{2+} kit (Tokyo, Japan) according to the supplier's instructions. Creatinine and blood urea nitrogen (BUN) levels were determined with the respective kits from Biomérieux (Marcy-l'Etoile, France) according to the supplier's instructions.

Calcineurin Activity in Kidney Tissue

Tissue samples were minced in 1 ml of hexadecyltrimethylammonium bromide buffer (0.5% w/w) on ice and homogenized. The homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 10,000 rpm for 3 min. The supernatant was analyzed for the calcineurin activity by a Biomol Green calcineurin assay kit (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) according to the supplier's instructions.

Statistical Analysis

The results were expressed as mean values ± SD. For the analysis of statistical significance, the Kruskal-Wallis test was applied followed by Dunn's test for all pairwise comparison, except when normality and equal variance were passed it was followed by the Tukey test. In all cases, $p < 0.05$ was considered to be significant.

RESULTS

A representative example of the FK506-MS is given in Fig. 1. They appeared spherical with a particle diameter at around 150 μm entrapping the drug inside the polymeric matrix. Further MS characteristics such as particle size, encapsulation efficiency, and drug load are shown in Table I. Figure 2 illustrates the *in vitro* drug release profiles obtained from the incubation of MS formulations in phosphate buffer systems with changing pH over time. In general, the drug release occurred in strong dependency of the buffer pH in which the MS were suspended. FK506 was retained efficiently inside the microspheres when tested in *in vitro* buffer systems at pH 1.2 and 6.8, as around 95% of the initial drug load was still present inside the MS after 4 h of incubation. On the contrary, a comparatively fast release was observed at pH 7.4, which delivered nearly 100% of the incorporated drug within 30 min.

Table I. General Characteristics of the FK506 Microspheres[a]

Diameter (μm)	145.2 ± 10.9
Drug load (%)	10.8 ± 1.1
Encapsulation rate (%)	52.8 ± 4.9

[a] n = 3; Data are shown as mean ± SD.

In order to evaluate the therapeutic value of FK506-MS, the effect of the carrier system was studied on a preexisting colitis. After inducing the experimental colitis, the clinical score increased rapidly and consistently for the next 3 days for all groups (Fig. 3).

During the whole treatment period, FK506 lowered the clinical activity so that all drug-receiving groups showed decreasing index values after a lag time of 24 to 48 h. The difference between drug-treated groups and colitis controls became significant on day 7 for the group treated with MS while for the other treated groups statistically significant differences were not observed.

Histologic sections of the colon demonstrated significant influences by the various treatments (Fig. 4). With FK506-MS treatment, mucosal and submucosal tissue was found partially intact, whereas in colitis control a complete disintegration of the mucosa occurred. Also, swelling of the submucosa was reduced in FK506-treated groups although not reaching the level of healthy control.

Similar to observations from the clinical activity index, the drug-treated groups showed decreased values in the co-

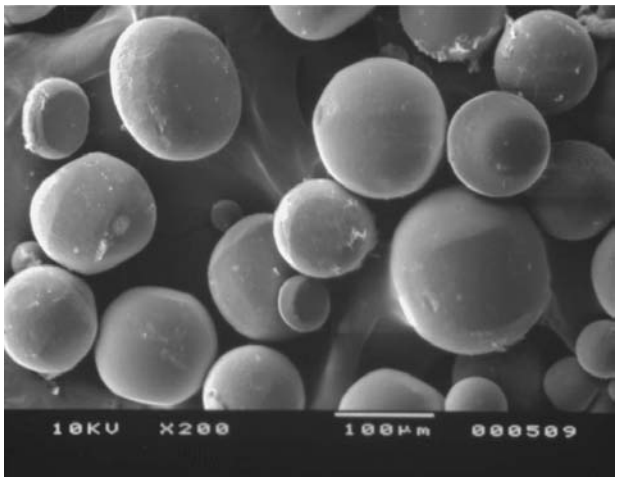

Fig. 1. Scanning electron micrograph of pH-sensitive Eudragit P-4135F microspheres.

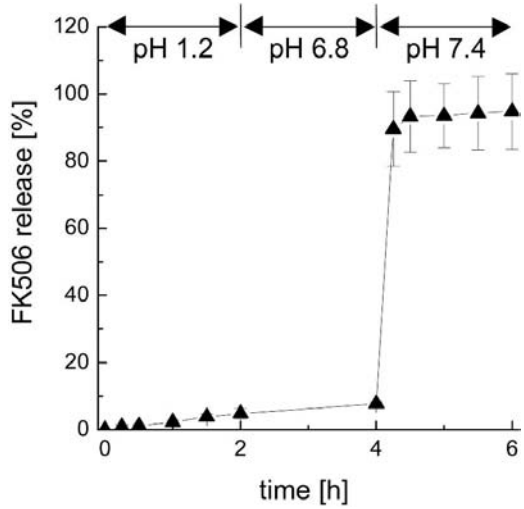

Fig. 2. Cumulated FK506 release vs. time of an Eudragit P-4135F microsphere formulation in phosphate buffer systems of pH 1.2, 6.8, and 7.4 replaced after 2 h, respectively (n = 3). Data are shown as mean ± SD.

lon/body weight ratio compared with the untreated colitis control group (Fig. 5A). However, only levels after the FK506-MS treatment were found to be significantly lower ($p < 0.05$) compared with the colitis control. The histologic damage score revealed higher treatment efficiency by FK506sc and FK506-MS compared to FK506oral where results were not statistically different from untreated colitis controls. Myeloperoxidase activity in samples from the inflamed colonic tissue revealed similar therapeutic effects for FK506-MS and FK506sc treated groups but a less efficient treatment by oral FK506 solution (Fig. 5C).

The variation of the severity of the adverse effect, namely the nephrotoxicity, was determined for the different drug formulations. Relative changes in animal weight, the urine volume, and the excreted calcium were described with respect to the untreated healthy control group (Fig. 6). Both, FK506oral and FK506-MS treatments did not influence the rat body weight, whereas a significant relative weight loss was determined for the FK506sc group. Regarding tubular function data, fractional excretion of calcium was higher in the FK506sc-treated group compared to FK506-MS. On the other hand, the relative urine excretion increased with all treatments, FK506oral was the highest, which is seemingly based on the water loss by the diarrhea of the FK506sc group.

BUN levels were increased after subcutaneous administration of FK506 (Fig. 7A) similar to values of the serum creatinine of the FK506sc group (Fig. 7B). FK506-MS treatment generally appeared to inhibit the increase of BUN and serum creatinine levels, as differences to the untreated control were not statistically significant. Endogenous creatinine clearance significantly decreased in FK506sc-treated rats (Fig. 7C). Treatment with FK506oral tended also to decrease creatinine clearance but not significantly, and FK506-MS showed levels of creatinine clearance similar to the control group.

Calcineurin expression was decreased in the kidney tissue samples of all FK506-treated groups compared to the control group receiving saline instead (Fig. 7D). Distinctly reduced calcineurin expression was detected after the administration of free FK506 by subcutaneous and oral routes, respectively. Calcineurin levels in the FK506-MS group were not significantly different from values in the control group. In general, the results showed a decreasing effect of the FK506 treatment on the kidney calcineurin expression in the order FK506sc > FK506oral > FK506-MS > control, suggesting significant improvements by FK506-MS compared to the other FK506 treatments.

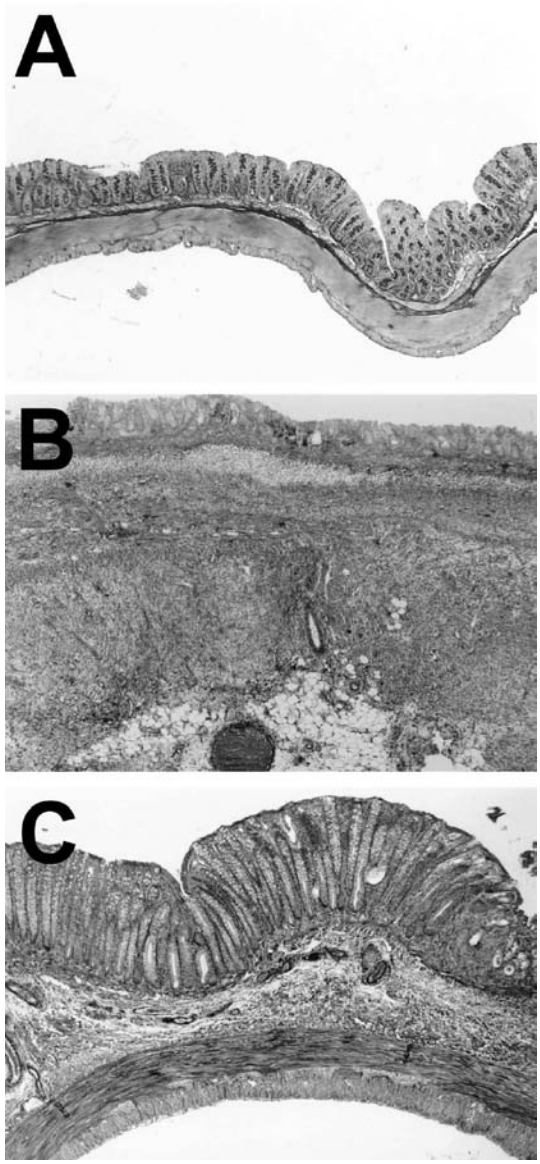

Fig. 4. Examples for histologic colon sections of healthy (A), untreated colitis (B), and FK506-MS treated tissue (C) in rats after treatment period on day 13 (×30 magnification).

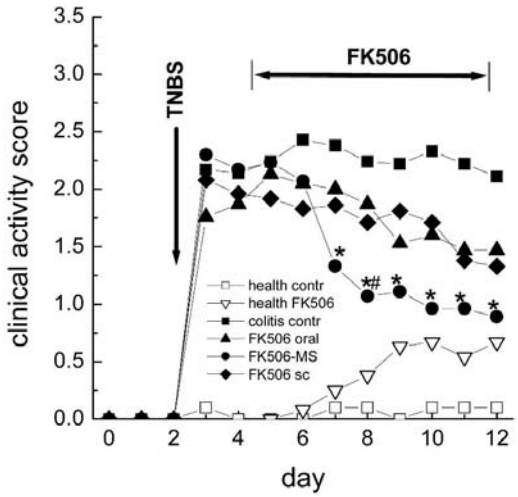

Fig. 3. Clinical activity score during the whole experimental period always determined for $n = 6$ animals. *$p < 0.05$ compared with colitis control rats given saline. #$p < 0.05$ compared with FK506oral.

DISCUSSION

FK506 demonstrated to be a very potent drug in the treatment of severe cases of IBD in animal studies as well as in clinical trials (7,8,24–26). However, the risk of distinct adverse effects after systemic absorption hinders the drug from becoming a standard therapeutic approach in the therapy of this disease pattern. Daily administration of FK506 for a 5-week period (as done in this study) induced renal dysfunction. FK506-induced functional nephrotoxicity consists of a

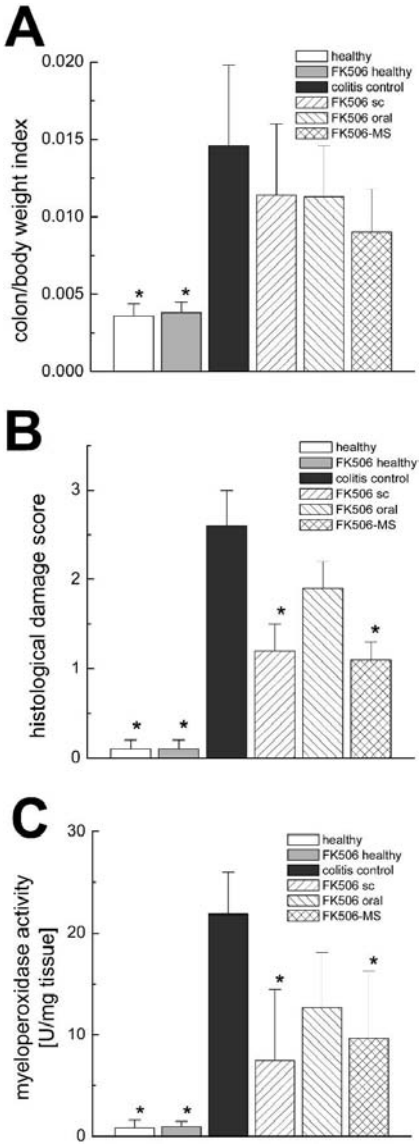

Fig. 5. Determination of colon/body weight ratio (A), a histologic damage score (B), and the myeloperoxidase activity (C) after final drug administration for n = 6 animals. *p < 0.05 compared with colitis control rats given saline. Data are shown as mean ± SD.

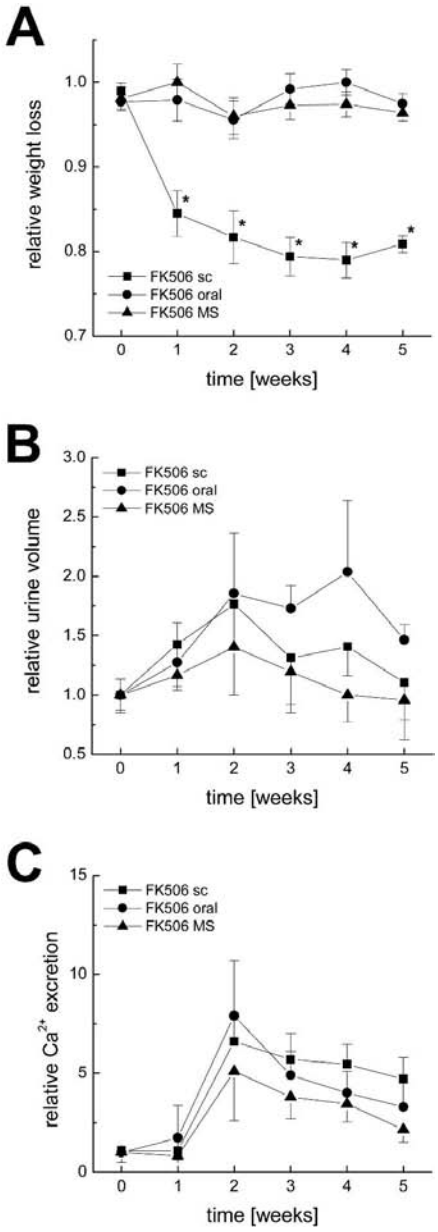

Fig. 6. relative weight loss (A), relative excreted urine volume (B), and relative calcium concentration in urine (C) all compared to untreated healthy animals during 5 weeks with daily administration of either FK506 solution (oral or subcutaneous) or FK506-MS for n = 6 animals. *p < 0.05 compared with FK506sc. Data are shown as mean ± SD.

dose-dependent reduction of renal blood flow and glomerular filtration rate. Thus, a local delivery of FK506 is of high interest, as it may allow reduced systemic availability of the drug, lowering the adverse effects.

The clinical course of the model colitis (characterized by the activity index), displays generally a therapeutic effect of the FK506 treatment similar to results from other animal studies (25,26). An improved therapeutic efficiency is determined from FK506-MS where the activity of the colitis was reduced compared to values from FK506 in solution, either injected or given orally. For both colon/body weight index and myeloperoxidase activity, the FK506-MS formulation reduced the inflammatory activity significantly, which was not the case with oral FK506 solution being the usual administration route in earlier trials. Besides, the presence of polysorbate 80 as solubilizer may influence results obtained after oral FK506 administration due to its interaction with efflux systems such as P-glycoprotein influencing the oral availability. Thus, values obtained from the oral FK506 solution here may demonstrate a higher therapeutic efficiency than expected from oral standard formulations.

FK506-MS clearly inhibited the elevation of BUN and serum creatinine levels and prevented the reduction of creatinine clearance in the treated rats compared to values from the FK506sc group. Moreover, measuring BUN, serum creatinine, and creatinine clearance revealed that FK506-MS levels did not differ from that of untreated control animals. Although this was also observed for the FK506oral group in some cases, it must be kept in mind that in terms of thera-

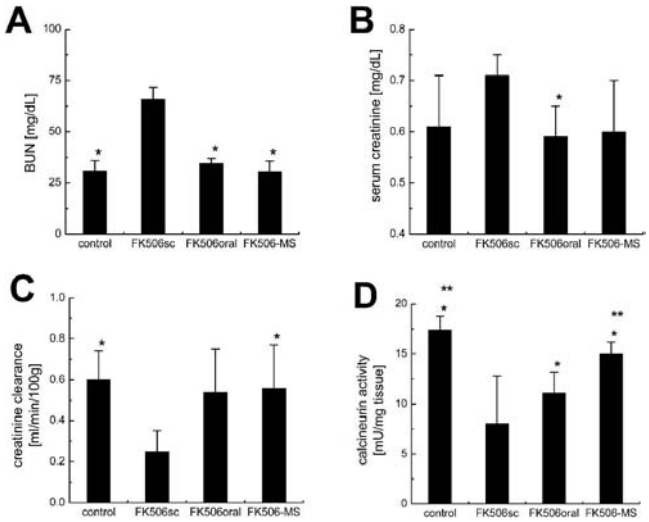

Fig. 7. Blood urea nitrogen (A), serum creatinine (B), creatinine clearance (C), and calcineurin activity (D) levels after 5 weeks with daily administration of either FK506 solution (oral or subcutaneous) or FK506-MS for n = 6 animals. *$p < 0.05$ compared with FK506sc. **$p < 0.05$ compared with FK506oral. Data are shown as mean ± SD.

peutic efficiency, the MS group was distinctly superior to the group receiving FK506 solution orally.

A general comparison of the different treatments elucidates the advantages of the developed system. Although the subcutaneous injection of FK506 can develop the full mitigating effect of the drug, also dramatically elevated adverse effects may be expected. When FK506 is administered orally as a solution, all absorbed drug is systemically available due to an early absorption during the passage of the upper parts of the gastrointestinal tract, where FK506 was found to be mainly absorbed in the duodenum, jejunum, and ileum (27). On the other hand, FK506 undergoes P-glycoprotein efflux and is a substrate to cytochrome P450 3A4, which causes a distinct metabolic degradation of the drug. Both are reasons for a generally lower oral bioavailability of FK506, responsible for a lower therapeutic effect but similarly a lower adverse effect level. FK506-MS can, however, retain the drug from early absorption during the passage in the upper intestine and may allow an intact passage until the colonic tissue. The residual absorption of the drug may be kept at relatively low levels by providing freely accessible drug only to the inflammation site and to sections of the healthy colonic tissue surrounding the inflamed tissue.

The administered solution as well as the tablet formulations tested in recent animal studies and clinical trials, respectively, aimed a pharmacological effect after systemic absorption. Thus, in all cases FK506 underwent a distinct metabolic drug loss during its transport across the intestinal barrier. Oppositely, when applying FK506-MS the drug is protected from mucosal metabolism (28) prior to local delivery. This avoids a substantial drug loss, maintaining the activity of the entrapped drug toward its local action at the inflammation site. Upon dissolution of the particle constituting polymer at the site of action, no further excipient is involved and FK506 is immediately available. These properties can solve simultaneously another general problem met in colonic delivery when diarrhea is not observed: the relatively low volume of available luminal fluid in the colon triggering the dissolution of the drug carrier. Complicated approaches developed to overcome the lack of fluid in the colon (29) are not needed when applying this MS formulation, due to the small diameter of the carrier, which guarantees the dissolution in small fluid quantities. This makes this microsize system superior to the standard tablet or capsule formulations.

Different from the prodrug strategies, the microparticle approach does not demand structural prerequisites for a chemical cross-linking with the carrying backbone structure. The preparation method of such microcarrier system can easily be adapted to most of the drugs.

In summary, FK506-MS selectively delivers the incorporated drug content to distal parts of the ileum and the colon. This drug delivery system allows the desired drug to be released in the inflamed tissue area with high efficiency. Our approach may allow the clinical use of FK506 for treatment of severe cases of IBD, as a reduction of adverse effects is possible due to the more locally focused effect of the drug. The development of such selective microparticles for FK506 delivery to the inflamed tissue should be given particular consideration in the treatment of IBD, as it allows therapy that profits from FK506's high immune-suppressive effect while simultaneously reducing the nephrotoxicity. Similarly, this strategy may be interesting for a large number of immunesuppressant drugs.

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