Original article

Insulin-like growth factor I improves aspects of mycophenolate mofetil-impaired anastomotic healing in an experimental model

R. A. Inglin, G. Baumann, O. J. Wagner, D. Candinas and B. Egger

Department of Visceral and Transplantation Surgery, Inselspital, University of Berne, CH-3010 Berne, Switzerland
Correspondence to: Dr B. Egger (e-mail: bernhard-egger@bluewin.ch)

Background: **Patients taking immunosuppressants after transplantation may require intestinal surgery. Mycophenolate mofetil (MMF) has been found to impair the healing of colonic anastomoses in rats. This study examined whether insulin-like growth factor (IGF) I prevents MMF impairment of anastomotic healing.**

Methods: **Sixty-three rats were divided into three groups (MMF, MMF/IGF and control). Animals underwent a sigmoid colon anastomosis with a 6/0 suture, and were killed on days 2, 4 and 6 after surgery. Investigations included bursting pressure measurement, morphometric analysis, and assessment of mucosal proliferation by 5-bromo-2′-deoxyuridine and Ki67 immunohistochemistry of the anastomoses.**

Results: **The leak rate was three of 21, one of 20 and two of 20 in the MMF, MMF/IGF-I and control groups respectively. Anastomotic bursting pressures were significantly lower in the MMF group than in the control group on days 2 and 4, but there was no significant difference by day 6. Values in the MMF/IGF-I and control groups were similar. Colonic crypt depth was significantly reduced in MMF-treated animals on days 2 and 4, but this impairment was attenuated by IGF-I on day 4. Similarly, IGF-I reduced the negative impact of MMF on mucosal proliferation on days 2 and 6.**

Conclusion: **Exogenous IGF-I improves some aspects of MMF-impaired anastomotic healing.**

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Introduction

The number of patients treated with immunosuppressive therapy after solid organ transplantation has steadily risen over recent decades. Surgeons are increasingly being confronted with transplant patients who develop gastrointestinal disease and complications[1], and consequently require bowel resection and anastomosis. Immunosuppressive drugs are generally known to impair the healing and strength of wounds of the skin and anastomoses by inhibiting the inflammatory and cellular phase of healing. Such inhibition has been described for steroids[2], ciclosporin[3], tacrolimus[4], rapamycin[5] and mycophenolate mofetil (MMF)[6]. In humans, adverse events resulting from immunosuppression with MMF involving the gastrointestinal tract[7] include nausea, vomiting, abdominal pain and diarrhoea[8]. Additional studies have provided evidence for a possible direct toxic effect on intestinal epithelial cells[9]. Recent reports on impaired anastomotic healing in experimental models[6,10] underline the fact that MMF and/or its metabolites affect the integrity of the intestinal mucosa.

Insulin-like growth factor (IGF) I and its truncated form stimulate the proliferation of epithelial and non-epithelial cells, and promote intestinal wound healing[11,12]. IGF-I has been shown to promote and accelerate healing of bowel anastomoses in rats[13,14]. IGF-I is a single-chain polypeptide homologous to proinsulin. It contributes to cell regulation and stimulates protein synthesis via signalling through type 1 receptors, which are homologous to insulin receptors, and activates

phosphorylation cascades[15]. IGF-I receptors have been characterized throughout the gastrointestinal tract[16]. Six specific binding proteins, IGF-binding proteins (IGFBPs) 1–6, allow additional tissue compartment-specific control of IGF-I activity; IGFBP production favours storage, whereas cleavage leads to activation of cell growth[15].

IGF-I might be a useful agent for neutralizing the negative influences of MMF on anastomotic repair and stability. The aim of this study was to evaluate the effect of systemic IGF-I administration in rats that had been treated with MMF.

Methods

The Animal Care and Use Review Committee of the University of Berne approved this study in accordance with the standards set forth in the Animal Welfare Act and other Federal, State, and Local Statutes and Regulations related to animals.

Experimental protocol

MMF was dissolved in a solution containing benzyl alcohol, polysorbate 80, sodium chloride and sodium carboxymethylcellulose at a pH of 3·5. Human recombinant IGF-I was a gift from Chiron (Emeryville, California, USA). The purified and dissolved sterile product (7·5 mg/ml in a 0·9 per cent sodium chloride (w/v) solution) was transported on ice and stored at 4°C. Weight-adjusted dilution with 0·9 per cent sodium chloride was made just before injection.

Sixty-three male Sprague–Dawley rats weighing 230–260 g each (Biotechnology or Animal Breeding Division, Biological Research Laboratories, Füllinsdorf, Switzerland) were used in these studies. The animals had free access to a standard laboratory diet and water, but were fasted for 12 h before surgery. They were divided into three groups, each containing 21 rats. The MMF group received 25 mg per kg bodyweight MMF and vehicle (5 ml 0·9 per cent sodium chloride solution), the MMF/IGF-I group received 25 mg per kg bodyweight MMF and 1 mg per kg bodyweight IGF-I, and the control group was treated with the vehicle solutions of both MMF and IGF-I (5 ml each). MMF treatment was initiated 3 days before and IGF-I on the day before surgery. Treatment was administered intraperitoneally once daily until death. Animals were killed on days 2, 4 and 6 after operation.

An intraperitoneal MMF dose of 25 mg per kg bodyweight per day is equivalent to an intravenous dose of 2 g MMF per day in humans weighing 80 kg[6]. An intravenous dose of 2 g/day is a safe and acceptable alternative method of MMF administration in the immediate period after transplantation. Even after oral administration, the bioavailability of mycophenolic acid in humans is nearly 100 per cent, and pharmacokinetic measures such as area under the curve are very similar in humans and rats. It has also been demonstrated in an experimental model that 1 mg per kg bodyweight IGF-I administered intraperitoneally is an appropriate dose for improving and accelerating healing of colonic anastomoses[14].

Operative technique

The operative technique has been described previously[6]. After laparotomy and dissection of the bowel, the anastomosis was performed by a single surgeon using microsurgical techniques in an inverted and interrupted fashion with 6/0 polydioxanone sutures (PDS II; Ethicon, Brussels, Belgium). The surgeon was blinded to the treatment of the animals. At the completion of the operation, pentazocine (1 mg per kg bodyweight) was administered subcutaneously on the first and second days after surgery for pain relief. Just after the operation 10 ml physiological sodium chloride was injected subcutaneously to substitute for perioperative fluid loss.

Bursting pressure measurement

Bursting pressure was evaluated by investigators blinded to the treatment group as described previously[6]. After dissection of the anastomotic site, the tissue was submerged in a saline bath and the bursting pressure was measured using a sphygmomanometer with an in-line pressure transducer, by increasing the intraluminal pressure in increments of 10 mmHg over 10 s at 10-s intervals. Bursting pressure was determined by noting leakage of air or gross rupture at the anastomotic site.

Histological assessment and morphometric analysis

Once bursting pressure had been recorded, the colonic specimen and anastomosis was opened longitudinally, fixed in 10 per cent zinc–formalin and embedded in paraffin. Transverse sections of the embedded tissue were stained with haematoxylin and eosin, and a histological assessment was performed. A computed measurement of the depth of the colonic crypts was made. Fifteen random measurements were performed per animal at least 2 mm away from the anastomotic site where the glands were perpendicular to the underlying muscularis.

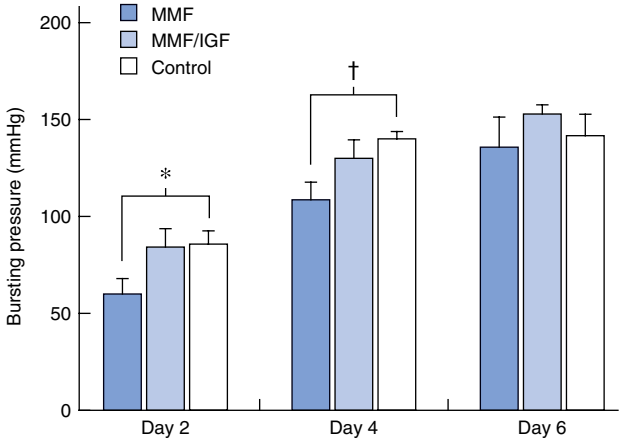

Fig. 1 Bursting pressure of left-sided colonic anastomoses in mycophenolate mofetil (MMF), MMF/insulin-like growth factor (IGF) I and control groups. Values are mean(s.e.m.). *$P = 0{\cdot}044$, †$P = 0{\cdot}011$ (two-tailed Mann–Whitney U test)

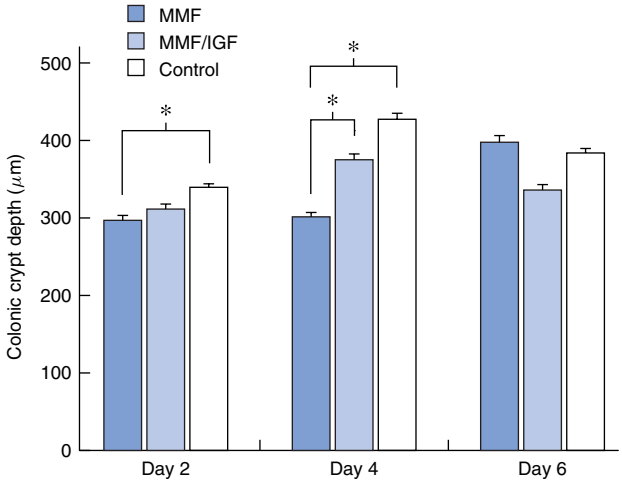

Fig. 2 Colonic crypt depth in mycophenolate mofetil (MMF), MMF/insulin-like growth factor (IGF) I and control groups. Values are mean(s.e.m.). *$P < 0{\cdot}001$ (two-tailed Mann–Whitney U test)

Assessment of proliferating cells

All rats killed on days 2 and 6 received an intraperitoneal injection of 50 mg/kg 5-bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich, Steinheim, Germany) 120 min before death. Paraffin-embedded sections of standard thickness were deparaffinized and BrdU immunohistochemistry was performed as described previously[14,17]. The total number of proliferating cells per crypt was counted, at a magnification of ×100 in ten transverse cuts of colonic crypts, by two independent investigators who were blinded to the treatment group. Results were expressed as the mean number of BrdU-positive cells per crypt.

Proliferating cells were also assessed using Ki67 antibody. Immunostaining was performed by an avidin–biotin–peroxidase complex technique using the standard procedure for VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, California, USA)[18]. In brief, deparaffinized sections were heated in a pressure cooker placed in a microwave oven (10 min at 89°C, 800 W) for antigen retrieval. A GenPoint™ Catalyzed Signal Amplification (CSA) System (DakoCytomation, Glostrup, Denmark) was applied. Sections were incubated overnight with primary mouse monoclonal antirat Ki67 antibody (MIB-5; DakoCytomation) at 1 : 100 dilution in phosphate-buffered saline in a humidified chamber at room temperature, and then incubated with biotinylated secondary antibody (rabbit antimouse IgG, 1 : 200 in Tris hydrochloride buffer) for 60 min. Sections were incubated with streptavidin–biotin complex (CSA; Dako), an additional amplification reagent (CSA; Dako) and streptavidin–horseradish peroxidase, each for 15 min. Finally, they were stained for approximately 5 min in a diaminobenzidine substrate chromogen solution (CSA; Dako), counterstained with haematoxylin, dehydrated and mounted (Aquatex; Boehringer-Mannheim, Mannheim, Germany).

The ratio of Ki67-labelled cells/total number of cells per crypt was calculated after counting cells at 100× magnification in ten longitudinal cuts of colonic crypts by two independent investigators who were blinded to the treatment groups. Results were expressed as the mean ratio.

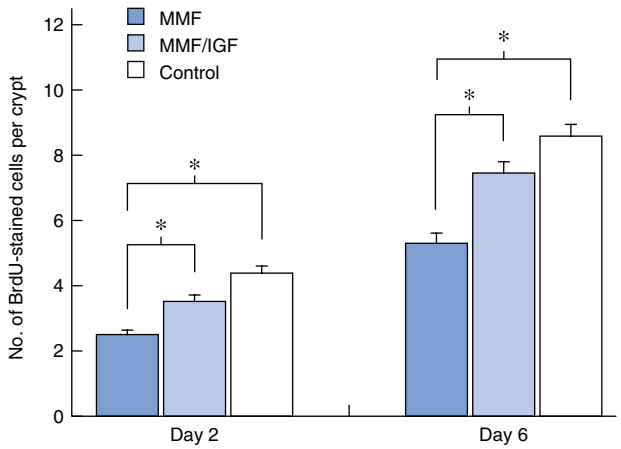

Fig. 3 Number of 5-bromo-2′-deoxyuridine (BrdU)-stained cells per crypt in mycophenolate mofetil (MMF), MMF/insulin-like growth factor (IGF) I and control groups. Values are mean(s.e.m.). *$P < 0{\cdot}001$ (two-tailed Mann–Whitney U test)

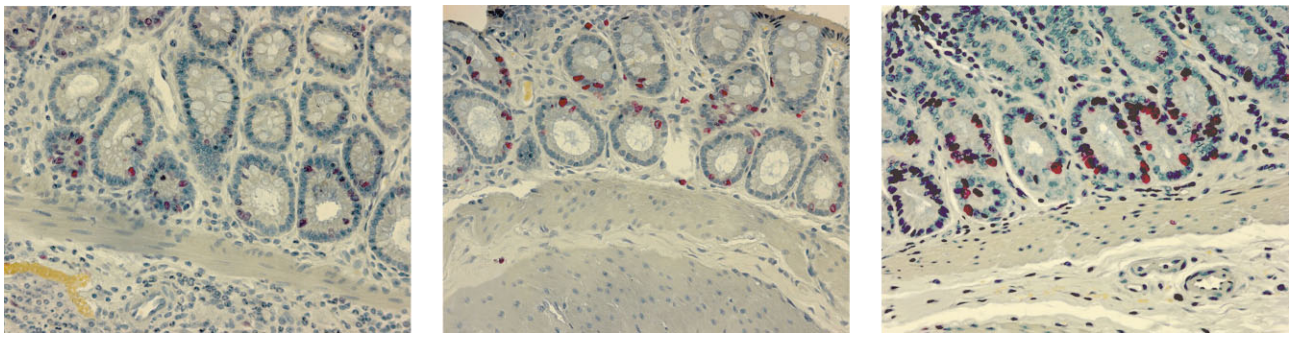

Fig. 4 Typical examples of 5-bromo-2′-deoxyuridine-stained cells in **a** mycophenolate mofetil (MMF), **b** MMF/insulin-like growth factor (IGF) I and **c** control groups (original magnification ×400)

Statistical analysis

Results are expressed as mean(s.e.m.). The presence of differences was assessed by analysis of variance (Kruskal–Wallis test), and significance between groups was calculated by the Mann–Whitney *U* test. Two-tailed $P < 0{\cdot}050$ was considered statistically significant. The NCSS/PASS Dawson edition, version 2.0.0.414 (Number Cruncher Statistical Systems, Kaysville, Utah, USA) was used for these calculations.

Results

Two animals, one in the control group and one in the MMF/IGF-I group, died during operation. All other animals tolerated the surgical intervention well and there were no further deaths during the study. There were no significant differences between the three groups in bodyweight just before death at each time point (data not shown).

Macroscopic and bursting pressure assessment

At necropsy, three contained leaks were noted in the MMF group (two on day 2, one on day 4), one in the MMF/IGF-I group (day 4) and two in the control group (day 6). However, all animals appeared well before death. All leaks were contained with either omentum or small bowel loops without evidence of peritonitis. As anticipated, anastomotic failure during bursting pressure measurements occurred at the site of the leak.

There was a significant difference in bursting pressure between the MMF and control groups on day 2 ($P = 0{\cdot}044$) and day 4 ($P = 0{\cdot}011$) (*Fig. 1*). The bursting pressure in the MMF/IGF-I and control groups was similar, implying that IGF-I may improve the negative effect of MMF.

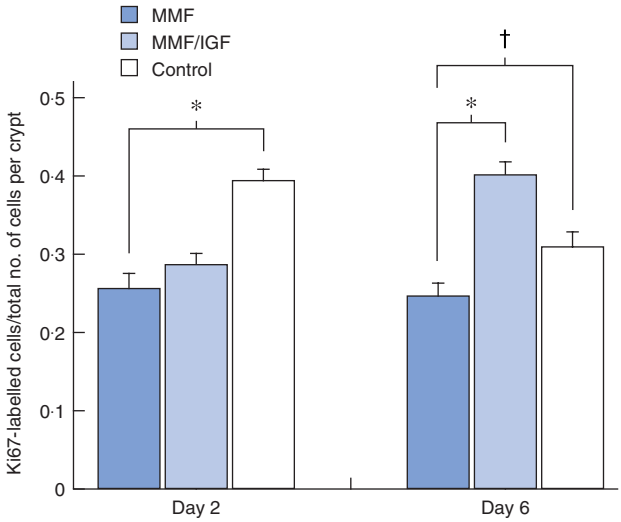

Fig. 5 Ratio of Ki67-labelled cells/total number of cells per crypt in mycophenolate mofetil (MMF), MMF/insulin-like growth factor (IGF) I and control groups. Values are mean(s.e.m.). *$P < 0{\cdot}001$, †$P = 0{\cdot}016$ (two-tailed Mann–Whitney *U* test)

However, the improvement was not statistically significant ($P = 0{\cdot}072$ on day 2; $P = 0{\cdot}195$ on day 4). As shown previously[6], there were no significant differences between groups on day 6.

Microscopic pathological assessment

The colonic crypt depth was significantly lower in the MMF group than in the control group on days 2 and 4 (both $P < 0{\cdot}001$) (*Fig. 2*). Furthermore, colonic mucosa in MMF/IGF-I rats was thicker than that in the MMF group on day 4 ($P < 0{\cdot}001$), indicating a tendency for IGF-I to reverse the negative effect of MMF on mucosal

proliferation. However, no significant differences were found on days 2 and 6.

Mucosal proliferation

BrdU staining indicated that there was a significantly higher number of proliferating cells in control than MMF rats on days 2 and 6 (both $P < 0{\cdot}001$) (*Figs 3* and *4*). Furthermore, colonic crypts in animals that had concurrent treatment with MMF and IGF-I contained significantly more proliferating cells than those treated with MMF alone on days 2 and 6 (both $P < 0{\cdot}001$). There were also significantly more BrdU-labelled cells in control than in MMF/IGF-I rats ($P = 0{\cdot}004$ on day 2; $P = 0{\cdot}022$ on day 6).

Similarly, staining with Ki67 revealed a greater number of proliferating cells in control than MMF rats (*Fig. 5*). There were also more Ki67-labelled cells in MMF/IGF-I compared with MMF rats, but the difference was significant only on day 6 ($P < 0{\cdot}001$).

Discussion

In recent years, MMF together with tacrolimus has been the most commonly used discharge regimen for solid-organ transplant recipients[19]. The appearance of colonic ulcers[20] and perforations[21] during MMF treatment suggests that it influences tissue maintenance or repair[22], and there is experimental evidence that it impairs anastomotic healing[6]. In the present study, measurement of bursting pressure, colonic crypt depth and proliferating cells has further demonstrated a negative effect of MMF on mucosal healing and anastomotic stability.

IGF-I has pleiotropic effects[23] including enhanced intestinal proliferation and healing[14,24,25]. Exogenous administration may reverse steroid-, aspirin- and methotrexate-induced alteration of mucosal repair[26,27]. In the present study, bursting pressures in rats treated with a combination of MMF and IGF-I were similar to those of control animals. Administration of IGF-I improved mechanical stability in animals treated with MMF, but a significant increase was not demonstrated, possibly because the sample size was too small (type 2 error). The early leaks may be attributed to a technical problem rather than to the influence of the agents used. However, the leak rate was similar in the three groups and did not affect the statistical results. In addition, there was no uniform correlation between leaks and low bursting pressures.

Enhanced mucosal thickness as determined by colonic crypt depth correlated well with the increase in proliferating (BrdU- and Ki67-stained) cells in the early postoperative period. The more pronounced thickening of the mucosa in animals treated with a combination of MMF and IGF compared with those receiving MMF alone suggests that impairment induced by MMF is neutralized by IGF-I.

The exact mechanisms by which IGF-I counteracts the effects of MMF are unknown. The current concept of mucosal growth and repair encompasses a wide range of growth factors and matrix components, signalling molecules, factors regulating cell–cell or cell–matrix adhesion or detachment, and molecules that regulate cytoskeletal functions[28]. Because both IGF-I[11] and MMF[29] exert their effects at least partially by either inducing or diminishing DNA and RNA synthesis respectively it may be postulated that they interact at this level.

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