Author's response to reviews

Title: Effects of Mycophenolate Mofetil on Key Pattern of Coronary Restenosis: a Cascade of in vitro and ex vivo Models

Authors:

Rainer Voisard (rainer.voisard@medizin.uni-ulm.de)
Sandra Viola (sandra.viola@medizin.uni-ulm.de)
Verena Kaspar (verena.kaspar@medizin.uni-ulm.de)
Christian M Weber (christian.weber@medizin.uni-ulm.de)
Lutz von Muller (lutz.muller@medizin.uni-ulm.de)
Regine Baur (regine.baur@medizin.uni-ulm.de)
Iris Gastrock-Balitsch (iris.gastrock-balitsch@medizin.uni-ulm.de)
Vinzenz Hombach (vinzenz.hombach@medizin.uni-ulm.de)

Version: 2 Date: 14 March 2005

Dear BMC Editorial Team,

enclosed please find revision I of our manuscript entitled: Effects of Mycophenolate Mofetil on Key Pattern of Coronary Restenosis: a Cascade of in vitro and ex vivo Models.

The authors would like to thank the referees for fair and constructive criticism. The queries of the referees have been carefully addressed, changes in the manuscript have been clearly marked.

We hope that the manuscript can now be accepted for publication in BMC.

With best wishes

..

Priv.-Doz. Dr. Rainer Voisard
Effects of Mycophenolate Mofetil on Key Pattern of Coronary Restenosis: a Cascade of in vitro and ex vivo Models

Reviewer I:

Q1: Effects of MMF on ICAM-1 protein levels: Are there some informations about toxic effects of MMF which might partially explain the strong loss of ICAM-1 at MMF concentrations > 150 g/ml?

A1: Studies of cell vitality do certainly contribute to the quality of the study:

This sentence was added to the section methods, flow cytometry, page 7, paragraph 3:
The effects of MMF (50 g/mL - 300 g/mL) on vitality of HCAEC and HCMSMC were analyzed with propidium iodide (Sigma-Aldrich, Taufkirchen, D).

This sentence was added to the results, section effect of MMF on ICAM-1 protein levels: flow cytometry studies, page 10, paragraph 2:
In HCAEC no toxic effects were detected after adding of MMF in concentrations of 50 g/ml, 100 g/ml, and 150 g/ml, little toxic effects were found after adding of MMF in concentrations of 200 g/ml, 250 g/ml, and 300 g/ml. In HCMSMC no toxic effects were detected after adding of MMF in concentration of 50 g/ml - 300 g/ml.

This sentence was added to discussion, page 13, paragraph 2, line 8:
Due to the fact that little toxic effects were found after incubation of HCAEC with MMF in concentrations of 200 g/ml - 300 g/ml, the decrease of ICAM-1 protein levels in HCAEC may be partially explained by these toxic effects.

Q2: Although MMF significantly reduced ICAM-1 on endothelial cells, adhesion of monocytes to HUVEC (which is partially regulated by ICAM-1) was enhanced. Are there some explanations on this?

A2: That's a good point by the referee that cannot be easily explained. Inhibition of ICAM-1 expression after adding of MMF in a concentration of 50 g/ml was significant, but merely less than 20%. In the 3DLA model expression of ICAM-1 was slightly increased, however standard deviations were very high.

Q3: Were the adhesion experiments repeated using higher MMF concentrations (>50 g/ml)?

A3: Due to the cascade character of the in vitro and ex vivo models the effect of MMF in the 3DLA and organ culture model was merely studied with a concentration of 50 g/ml.

Q4: Although the authors speak in terms of significant and non significant, no informations are provided how the statistic evaluation has been carried out.

A4: The section statistical analysis was added in methods, last section: Statistical Analysis:
Data of northern blot and flow cytometry studies were presented as mean S.D. Statistical significance of differences between controls and drug-treated cells was determined by paired Students t-test. The Mann-Whitney rank-sum test was used to investigate the significance of differences in the 3DLA-model and the organ culture model. Statistical significance was accepted for P < 0.05.

Statistical significance was calculated de novo for the data of the effect of MMF on ICAM-1 mRNA levels. Expression of ICAM-1 was significantly (p = 0.01) increased after incubation of HCAEC with a concentration of MMF of 50 g/ml. The increase of expression of ICAM-1 in HCAEC after adding of MMF in concentrations of 100 g/ml, 150 g/ml, 200 g/ml, and 250 g/ml did not reach statistical significance. In HCMSMC the increase of ICAM-1 expression after adding of MMF did not reach statistical significance, no matter which concentration of MMF was added. These data were added in results, section effect of MMF on ICAM-1
mRNA levels: northern blot studies, page 9.

Q5: Methods, cell culture: HCMSMC should be written out in full the first time it appears in the manuscript.
A5: was done.

Q6: Where were HCMSMC derived from?
A6: Sorry, this information was missing. Both HCAEC and HCMSMC were purchased at Cambrex Bio Science (Vervier, B). The section cell culture in methods, page 6, was rewritten:

Cell Culture
HCAEC and HCMSMC were purchased at Cambrex Bio Science (Vervier, B). HCAEC were cultured in Endothelium Growth Medium (Cambrex) and identified by the typical cobble stone growth pattern and positive reaction against von Willebrand factor (Dakopatts). HCMSMC were grown in Smooth Muscle Cell Growth Medium (Cambrex). For identification of HCMSMC antibodies against smooth muscle a-actin (Renner, Darmstadt, D) were used. Human MC were isolated from the residual leukocytes of single donors using MACS cell-isolation kit (Milteny Biotec GmbH).

Q7: Methods, mycophenolate mofetil. How is MPL of 34 g/ml related to? Although a reference is given, I do not understand the link of MPL to MMF concentrations.
A7: The referee is perfectly right, it would be ideal to study exactly the effect of MMF in concentrations of 34 g/ml. The concentration of 50 g/ml is merely close to the MPL of 34 g/ml (SI/MPL-ratio: 1.47). This point is addressed in the chapter discussion, page 14, paragraph 2, line 2. The background is that MMF is available in a concentration of 50 mg/ml.

Q8: Results, effect of MMF on ICAM-1 protein levels: the term protein might be misleading as facs measures receptor surface expression.
A8: Protein levels was removed from the headline, page 9, section 3.

Reviewer II:

Q1: There is no description of the type of statistical analysis used, although p-values are reported throughout. In fig. 3A, the magnitude of error bars shown would suggest that there is no significant difference in adhesion with or without MMF at any of the time points, yet in the text on p.10 significance is claimed for times 1, 2, and 6 h. In fig 5A, the variance in BrdU labeling appears to indicate that none of the measurements in tissues derived from ballooned arteries are different from the non-ballooned control. With respect to experimental design, important controls are missing. For example, in fig. 1, 2, and 3 there are no results shown for treatment with MMF alone; this is especially important for the interpretation of the potentiating effects of MMF on TNF responses.
A1: The section statistical analysis was added in methods, last section: Statistical Analysis:
Data of northern blot and flow cytometry studies were presented as mean S.D. Statistical significance of differences between controls and drug-treated cells was determined by paired Students t-test. The Mann-Whitney rank-sum test was used to investigate the significance of differences in the 3DLA-model and the organ culture model. Statistical significance was accepted for P < 0.05.

Statistical significance of the data, as described in results, was controlled, the data are correct. Statistical significance was calculated de novo for the data of the effect of MMF on ICAM-1 mRNA levels. Expression of ICAM-1 was significantly (p = 0.01) increased after incubation of HCAEC with a concentration of MMF of 50 g/ml. The increase of expression of ICAM-1 in HCAEC after adding of MMF in concentrations of 100 g/ml, 150 g/ml, 200 g/ml, and 250 g/ml did not reach statistical significance. In HCMSMC the increase of ICAM-1 expression after adding of MMF did not reach statistical significance, no matter which concentration of MMF was added. These data were added in results, section effect of MMF on ICAM-1 mRNA levels: northern blot studies, page 9.

Standard deviations in the organ culture model are highly increased both in the ballooning/MMF and the control group. This was probably caused by the preparation procedures in the laboratory. This point is addressed in discussion, page 14, paragraph 1, line 9 and 10.
Q2: Page 7: How were cells recovered from adherent culture preliminary to flow cytometry? Was trypsin used? Also, since results of flow cytometry are given in terms of mean fluorescence as opposed to positive cells, what advantage does this analysis have over immunoblotting?

A2: The following sentences were added in methods, section flow cytometry, page 7, paragraph 1, line 1 and in paragraph 2, line 1:
For flow cytometry analysis of the expression of ICAM-1 in HCAEC and HCMSMC, cells were trypsinized and seeded into 6-well dishes (5x10⁴ cells).
After MMF/TNF-a treatment, cells were washed twice with phosphate-buffered saline (pH 7.2) and trypsinized.
Effects of MMF on expression of ICAM-1 are presented both as effects on RNA- and protein-level.

Q3: Page 9: Although the methods section stated that northern blots were controlled by measuring GAPDH expression, there is no evidence in the results that this normalization was performed.

A3: The following sentence was added in results, section effect of MMF on ICAM-1 mRNA levels: northern blot studies, page 9, paragraph 3:
Both in HCAEC and HCMSMC, expression of GAPDH after adding of MMF in concentrations of 50, 100, 150, 200, and 250 g/ml was identical with untreated controls.

Q4: A strength of the manuscript is the authors effort to use concentrations of inhibitor (in this case MMF) in vitro that are relevant to those associated with effects in vivo. Thus 50 g/ml MMF was often used, but a complete rationale for the choice of this concentration was not given. Where does hydrolysis of MMF to MFA occur, in the plasma or intracellularly? The introduction (p12) seems to indicate that plasma levels of MFA have been measured to gauge the absorption of MMF; is this correct? Can the authors be confident that conversion of MFA has taken place in all of their experimental systems, such as the absence of an effect can be distinguished from the absence of the inhibitor? Could the efficiency of this conversion vary according to cell type?

A4: The concentration of 50 g/ml is merely close to the MPL of 34 g/ml (SI/MPL-ratio: 1.47). This point is addressed in the chapter discussion, page 14, paragraph 2, line 2. The background is that MMF is available in a concentration of 50 mg/ml and was further diluted in 1:10 steps.

The second part of the question is very helpful to discuss the data of the in vitro and the ex vivo models of the cascade. In discussion, page 14, paragraph 1, line 8, the following sentences were added:
Due to the fact that the ester linkage of MMF is rapidly hydrolysed in the plasma to MPA (10), it can be excluded that an inactive form of MMF caused the missing inhibitory effect. However high standard deviations and the absence of perfusion in the model may have contributed to the negative effect. In the presented coronary ex vivo model of restenosis (9,19) the solved drug gets into contact with the adventitial side, the endothelial side, and both frontal sides of the artery segment. Therefore our group has reported earlier that the model mimics a simultaneous intra/extravascular drug administration (19). However due to the absence of perfusion the contact between the endothelial side of the artery segments and the culture medium is limited. Limited nutrition of this area may be critical because ballooning injury and reactive cell proliferation&neointimal hyperplasia are expected to occure predominantly in this region of the vessel wall.

Ref. 19 was added in references, page 19:

Q5: There are numerous errors of grammar and syntax in the text. Also, the figure legends and figure labeling need to be made more informative.

A5: Errors of grammar and syntax in the text were corrected, figure legends and figure labeling was made more informative.