P-30025 Relationship between plasma mycophenolic acid and its glucuronide concentrations, and adverse effects in renal transplant patients

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Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF) has been introduced into immunosuppressant protocols in kidney transplantation. Although MMF can produce several adverse effects in patients after kidney transplantation, it has not been clarified that the relationship between plasma MPA and its glucuronide (MPAG) concentrations, and adverse effects. Therefore, we investigated their plasma concentrations and hematological and gastrointestinal toxicity in the patients after kidney transplantation. Plasma MPA and MPAG concentrations were determined by HPLC. Pharmacokinetic parameters of MPA and MPAG were estimated from their plasma concentrations. The adverse effects were monitored after the transplant. Dose adjusted AUC of MPA and MPAG at 2 month following the kidney transplantation were about 1.5-fold higher than that at 1 month. Cytomegalovirus infection and diarrhea were caused with the increases of MPA and/or MPAG concentrations. These results suggested that several adverse effects in renal transplant patients with MMF might be related with the increases of plasma MPA and MPAG concentrations, and that monitoring of their plasma concentrations might be useful.

2P-021

腎移植患者におけるミコフェノール酸およびそのグルクロン酸抱合体の体内動態に及ぼす血液透析の影響

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【目的】代謝拮抗性免疫抑制薬のミコフェノール酸モフェチル（MMF）は生体内で活性体のミコフェノール酸（MPA）を生成する．さらにそのグルクロン酸抱合体（MPAG）は腸肝循環し，主に腎臓から排泄されることが知られている．最近，腎移植後早期においてMPAおよびMPAGの体内動態に大きな個体間差が存在することが報告されている．その要因のひとつに，移植腎に十分な利尿が得られるまで実施される血液透析の影響が報告されている．しかしながら，MPAおよびMPAGの体内動態に及ぼす血液透析の影響については詳細な検討がされていない．本研究では腎移植後に血液透析を必要とした症例を対象にMPAおよびMPAGの体内動態について評価した．

【方法】当施設において腎移植を施行した4例を対象として，移植後から定期的に採血を行った．血漿および尿中MPA，MPAG濃度はイオンペアHPLC法にて測定した．さらに得られた血漿中MPAおよびMPAG濃度から，血漿中薬物濃度下面積（AUC）ならびに経口クリアランスを算出した．また，尿中濃度および1日尿量からMPAおよびMPAGの尿中排泄クリアランス（CLrenal）を算出した．

【結果】MPAおよびMPAGのMMFの投与量を補正したAUCは，腎移植後早期において個体内の変動が認められた．さらに，血液透析の離脱後ではMPAおよびMPAGのAUC比（MPAG/MPA）は，離脱前に比べて低下する傾向が認められた．また，MPAおよびMPAGのCLrenalは血液透析の離脱後においてクレアチニンクリアランス（Ccr）の影響を受け，その比（CLrenal/Ccr）は血液透析の離脱前に比べ低下する傾向が認められた．

【考察】腎移植後早期のMPAおよびMPAGの体内動態に影響を及ぼす要因として，糸球体ろ過速度の変化や薬物相互作用が報告されている．本症例から腎移植後に実施される血液透析がその一因となる可能性が示唆された．さらにMPAとMPAGの体内動態に及ぼす血液透析の影響は両者で異なることが示された．腎移植後に血液透析を必要とする症例では，MPAの体内動態の変化に伴う過度の免疫抑制や移植腎の急性拒絶を回避するために血漿中濃度を指標としたMMFの投与量設計が必要であると考えられた．

Effects of Calcineurin Inhibitors on Pharmacokinetics of Mycophenolic Acid and Its Glucuronide Metabolite during the Maintenance Period Following Renal Transplantation

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Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF) has been introduced into renal transplant immunosuppressant protocols in combination with calcineurin inhibitors (CNIs) and steroids. This study compared the pharmacokinetic profiles of MPA and its major metabolite MPA glucuronide (MPAG) in combination with tacrolimus (TAC) or cyclosporine (CyA) during the maintenance period (>6 months) following renal transplantation. There was no difference between TAC and CyA-treated groups in MPA plasma concentration before drug administration (C_0). MPA C_0 in TAC and CyA-treated patients did not differ from that in patients who were not treated with a CNI. In patients treated with a CNI, MPAG C_0 was significantly greater in those treated with CyA compared with TAC. The MPAG/MPA ratio in CyA-treated patients was significantly greater than that in the TAC-treated group. We observed that C_0 of MPA was negatively correlated with that of TAC and CyA. Positive correlation between MPA C_0, MPAG C_0 and serum creatinine was stronger in patients treated with CyA compared with TAC. Our study suggests that CyA, but not TAC, inhibits enterohepatic circulation of MPAG as a secondary excretion pathway, and that renal function makes a major contribution to elimination of MPA and MPAG. We indicate that it may be necessary to estimate biliary excretion of MPAG to avoid the risk of intestinal injury in patients receiving combination therapy with TAC during the maintenance period.

Key words mycophenolic acid; calcineurin inhibitor; renal transplantation; maintenance period; therapeutic drug monitoring; individual variability

Mycophenolate mofetil (MMF) is a standard immunosuppressive agent used in combination with calcineurin inhibitors (CNIs) and steroids for the prophylaxis of acute rejection after organ transplantation.[1,2] Following oral administration, MMF undergoes rapid absorption and hydrolysis to mycophenolic acid (MPA). The active metabolite MPA is primarily metabolized by glucuronidation at the phenolic hydroxyl group to form the inactive metabolite 7-*O*-glucuronide conjugate (MPAG), which either undergoes enterohepatic cycling or urinary excretion as the major excretion product.[3] The contribution of this enterohepatic circulation to the overall pharmacokinetics of MPA is 37% in humans.[4]

As to elimination of MPA *via* enterohepatic circulation and glucuronidation, co-administrated CNIs may cause pharmacokinetic drug interactions with MPA and MPAG in triple immunosuppressive regimens including MMF.[5—9] Considering drug interactions, it has been reported that tacrolimus (TAC) can interfere with the activity of uridine diphosphate glucuronosyltransferase (UGT), which mediates conversion of MPA to MPAG,[10] and that cyclosporine (CyA) inhibits the biliary excretion of MPAG.[11—13] Steroids induce expression of hepatic glucuronosyltransferase and enhance the activity of UGT.[14] Co-administration of steroids decreases bioavailability and plasma concentration of MPA, and this causes lower levels of immunosuppression in renal transplant recipients.[15] However, these individual pharmacokinetic variability of MPA and MPAG have not been fully characterized using therapeutic doses of CNIs in renal transplant recipients during the maintenance period.

There is increasing evidence that therapeutic drug monitoring (TDM) of MPA might help to diminish adverse drug reactions of MMF and long term over-immunosuppression.[16,17] To obtain further information on pharmacokinetics of MPA and MPAG and their interactions with CNIs, we compared their pharmacokinetic profiles in combination with TAC or CyA and steroids in renal transplant recipients during the maintenance period.

MATERIALS AND METHODS

Demographic Characteristics of Patients and Study Schedule A total of 25 Japanese renal transplant recipients (>6 months post-transplantation, 16 men and 9 women, median age 43 years, range 14—60 years) from Hamamatsu University Hospital, Japan, were included in this study (Table 1). Twenty-two patients received a CNI-containing immunosuppressant regimen of TAC (Prograf®, $n=9$) or CyA (Neoral®, $n=13$), MMF (CellCept®) and prednisolone (PSL) (Predonine®, $n=21$). Three patients, who received a non-CNI-containing [CNI(−)] immunosuppressant regimen of MMF and PSL, showed intolerance to CNIs in initial immunosuppressive therapy. Until the present study, MMF had been orally administered without monitoring of plasma MPA and MPAG. Blood specimens were drawn into tubes containing EDTA at 12—14 h after oral administration of MMF or before the morning dosing (C_0) and 2 h after the morning dosing (C_2). All patients had been receiving the same immunosuppressive regimen including MMF for at least 1 month before testing. Each patient received information about the scientific aim of the study and gave their written informed con-

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Table 1. Demographic Characteristics of the Study Population

	Calcineurin inhibitor (−)	Tacrolimus	Cyclosporine
Number of patients (male/female)	3 (3/0)	9 (4/5)	13 (9/4)
Age (years)	40 (36—51)	40 (14—60)	45 (20—59)
Body weight (kg)	64.7 (63.9—66.4)	54.8 (45.0—72.3)	58.0 (39.4—74.0)
Donor type (living/cadaveric)	1/2	5/4	8/5
Serum creatinine (mg/dl)	1.73 (1.25—1.99)	1.30 (1.07—2.04)	1.40 (0.91—2.92)
Serum albumin (g/dl)	4.0 (3.9—4.2)	4.3 (3.8—4.4)	4.3 (3.9—4.6)
AST (IU/l)	14.0 (12.0—17.0)	14.0 (10.0—20.0)	15.0 (8.0—28.0)
ALT (IU/l)	12.0 (11.0—17.0)	11.0 (8.0—16.0)	10.0 (6.0—33.0)
Dose of MMF (mg/d)	1250 (1000—1750)	500 (250—2000)	1000 (250—1000)
Dose of CNI (mg/d)	—	3.0 (2.0—5.0)	150 (100—200)
Dose of PSL (mg/d)	5.0 (5.0—10)	5.0 (5.0—7.5)	5.0 (0—10)
Trough concentration of CNI (ng/ml)	—	4.6 (3.0—7.2)	41.9 (20.7—142)

Median (range).

sent. The study was approved by the Ethics Committee of Hamamatsu University Hospital.

Materials MPA and *n*-butyl-*p*-hydroxy-benzoate (internal standard) were purchased from Sigma (St. Louis, MO, U.S.A.). MPAG was purchased from Analytical Services International (London, U.K.). HPLC-grade acetonitrile and tetra-*n*-butylammonium bromide were purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were reagent grade and commercially available.

Simultaneous Determination of MPA and MPAG After quantification of TAC by monoclonal microparticle enzyme immunoassay (Abbott Japan, Tokyo, Japan) and CyA by affinity column mediated immunoassay (Dade Behring, Newark, DE, U.S.A.), plasma was separated and stored at −40 °C. Simultaneous determination of plasma MPA and MPAG was performed by ion-pair HPLC/UV as previously described with a modification.[18] Plasma extracts with solid phase extraction cartridge (Sep-Pak C_{18}®; Waters, MA, U.S.A.) were injected onto an analytical column (TSKgel ODS-80Ts, 5 μm, 4.6 mm i.d.×150 mm, Tosoh, Tokyo, Japan). The mobile phase was a mixture of acetonitrile and 40 mM tetra-*n*-butylammonium bromide, in which the mixing ratio was 1 : 2 (v/v) for analysis and delivered at a flow rate of 1 ml/min. UV detection was set at 250 nm. The HPLC system consisted of a solvent delivery system (Model LC-10AT, Shimadzu, Kyoto, Japan), a UV–Vis detector (SPD-M10A VP, Shimadzu) and an auto-injector (Model SIL-10A XL, Shimadzu). This method was accurate and reproducible for MPA and MPAG, based on the results of small intra- and inter-assay coefficients of variation (<8.9%).

Data Analysis For the MPA, MPAG and MPAG/MPA ratio of C_0 and C_2, CNI-treated groups were compared using a nonparametric Mann–Whitney *U* test. Correlation between plasma concentration of MPA and MPAG and several factors, such as MMF dose, blood CNI C_0 and serum creatinine, was performed by regression analysis. Three CNI(−) cases were not included in these statistical tests. $p<0.05$ was considered statistically significant.

RESULTS

Effect of MMF on MPA and MPAG Plasma Concentration Figure 1 shows the dose-dependent effect of MMF on C_0 and C_2 of MPA and MPAG. MPA C_0 was significantly correlated with MMF dose in TAC- ($p=0.028$, $r=0.723$) and CyA-treated ($p=0.033$, $r=0.592$) patients. MPAG C_0 was also significantly correlated with MMF dose in TAC- ($p=0.012$, $r=0.785$) and CyA-treated ($p=0.049$, $r=0.555$) patients. The effect of MMF on C_2 of MPA and MPAG differed between TAC- [MPA ($p=0.001$, $r=0.911$), MPAG ($p=0.014$, $r=0.774$)] and CyA-treated [MPA ($p=0.092$, $r=0.634$), MPAG ($p=0.131$, $r=0.580$)] patients.

Effect of CNIs on MPA and MPAG Plasma Concentration Figure 2 shows the influence of CNIs on MPA C_0 and C_2 after administration of MMF. MPA C_0 did not significantly differ between the TAC- and CyA-treated groups. MPA C_0 in TAC- and CyA-treated patients did not differ from that of CNI(−) patients. CNIs had no influence on MPA C_2. Figure 3 shows the effect of CNIs on C_0 and C_2 for MPAG. MPAG C_0 was significantly increased in the CyA-treated compared with the TAC-treated group ($p=0.043$). The MPAG C_0 values of the three CNI(−) patients were higher than the median MPAG C_0 in patients treated with TAC. CNIs did not influence MPAG C_2 to the same extent as MPA C_2. Figure 4 shows the effect of CNIs on plasma MPAG/MPA ratio. MPAG/MPA ratio was increased significantly more in the CyA-treated group than in the TAC group at C_0 ($p=0.025$). The MPAG/MPA ratio in the CNI(−) patients did not differ compared with that in the CNI-treated groups. CNIs did not influence the MPAG/MPA ratio at C_2.

Correlation between Plasma Concentration of MPA or MPAG and CNI The correlation between C_0 of MPA or MPAG and C_0 of CNIs in renal transplant recipients is shown in figure 5. C_0 of MPA was negatively correlated with that of TAC ($p=0.343$, $r=-0.359$) and CyA ($p=0.102$, $r=-0.474$) at therapeutic doses, although not significantly. There was a weaker correlation between C_0 of MPAG and TAC ($p=0.740$, $r=-0.129$) and CyA ($p=0.228$, $r=-0.359$) compared with that of MPA.

Effect of PSL on Plasma Concentration of MPA and MPAG Figures 6 and 7 show the influence of PSL on plasma MPA and MPAG in renal transplant recipients. PSL did not affect plasma C_0 and C_2 of MPA and MPAG in a dose-dependent manner when given in combination with CNIs during the maintenance period (>6 months post-transplantation).

Correlation between Plasma Concentration of MPA and MPAG and Serum Creatinine Figure 8 shows the correlation between MPA C_0, MPAG C_0 and serum creatinine concentration. We observed a positive correlation between

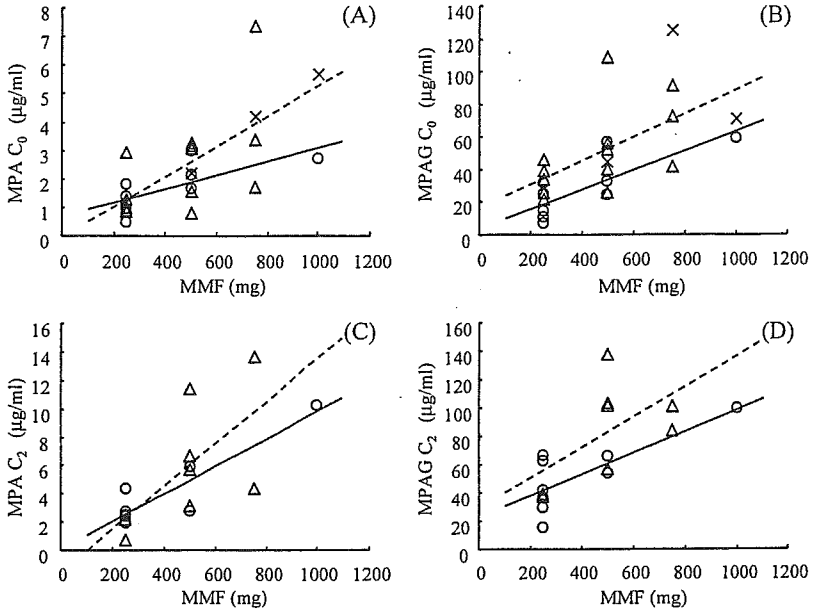

Fig. 1. Dose-Dependent Effect of MMF on C_0 of MPA (A) and MPAG (B), and C_2 of MPA (C) and MPAG (D) in Renal Transplant Recipients

The straight line indicates the regression of the TAC-treated group (O), and the dotted line indicates the regression of the CyA-treated group (△). × indicates the values of the CNI(−) group.

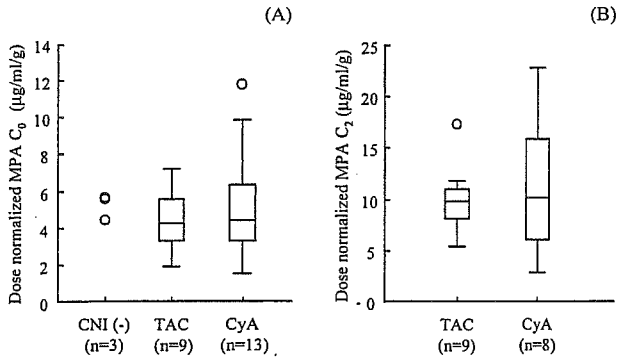

Fig. 2. Effects of TAC and CyA on C_0 (A) and C_2 (B) of MPA after Administrations of MMF in Renal Transplant Recipients

Each plasma concentration of MPA was normalized with the received dose of MMF. Box plots represent the median and 25th and 75th percentiles.

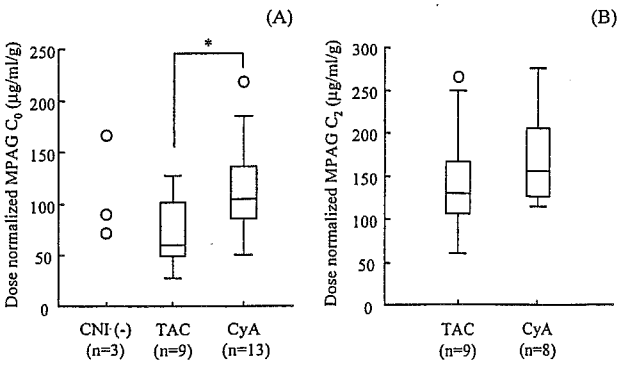

Fig. 3. Effects of TAC and CyA on C_0 (A) and C_2 (B) of MPAG after Administrations of MMF in Renal Transplant Recipients

Each plasma concentration of MPAG was normalized with the received dose of MMF. Box plots represent the median and 25th and 75th percentiles (* p=0.043).

plasma MPA C_0 and serum creatinine, and the correlation in CyA-treated patients (p=0.038, r=0.578) was significantly stronger than that in the TAC group (p=0.480, r=0.272). There was also a stronger positive correlation between

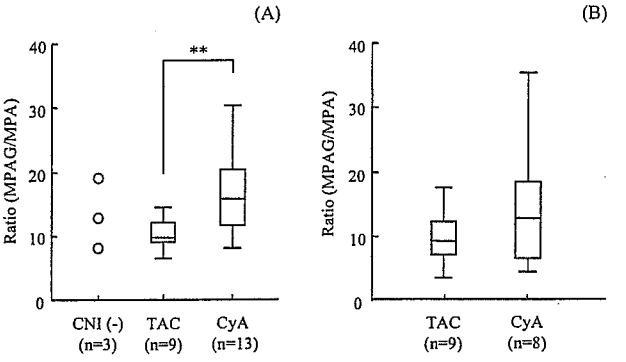

Fig. 4. Effects of TAC and CyA on the MPAG/MPA Ratio of C_0 (A) and C_2 (B) after Administrations of MMF in Renal Transplant Recipients

Each MPAG/MPA ratio was normalized with the molar concentration. Box plots represent the median and 25th and 75th percentiles (** p=0.025).

plasma MPAG C_0 and serum creatinine in CyA-treated (p=0.089, r=0.490) compared with TAC-treated patients (p=0.875, r=−0.061).

DISCUSSION

Triple immunosuppressive regimens of MMF, CNIs and steroids cause pharmacokinetic drug interactions with MPA and MPAG in renal transplant recipients.[5—8,15] However, TDM of MPA is not generally accepted during the treatment of renal transplant recipients, and the individual pharmacokinetic variability of MPA and MPAG have not been fully characterized for therapeutic doses of CNIs.

There was a significant relationship between MMF dose and MPA and MPAG C_0 in TAC- and CyA-treated patients. This suggests that it may be possible to use monitoring to adjust the targeted C_0 of MPA and MPAG during the maintenance period. C_0 in steady state reflects oral clearance and is a valuable index for avoiding acute renal transplant rejection and serious adverse drug reactions.[16] The dose-dependency

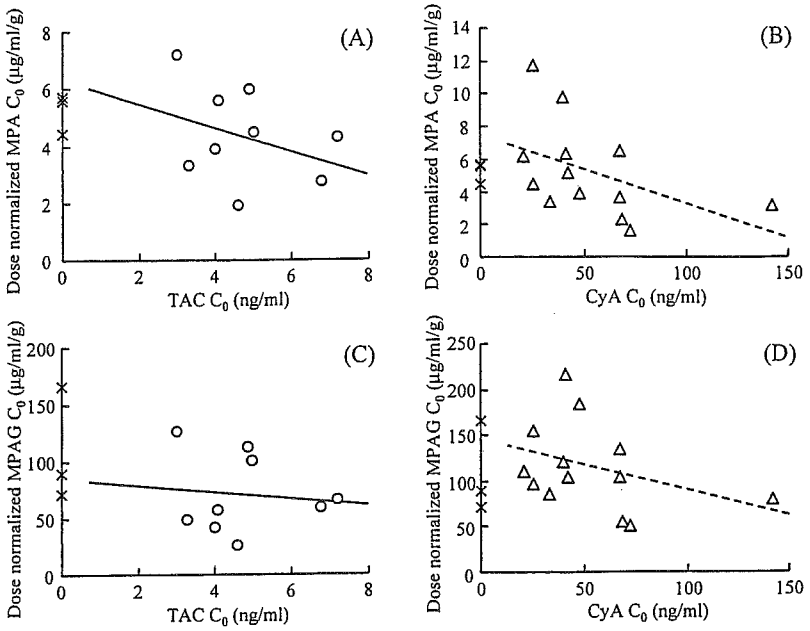

Fig. 5. Correlation between C_0 of MPA and TAC (A) or CyA (B), and between C_0 of MPAG and TAC (C) or CyA (D) in Renal Transplant Recipients

Each plasma concentration of MPA and MPAG was normalized with the received dose of MMF. The straight lines indicate the regression of the TAC-treated patients (○), and the dotted lines indicate the regression of the CyA-treated patients (△). × indicates the values of the CNI(−) patients.

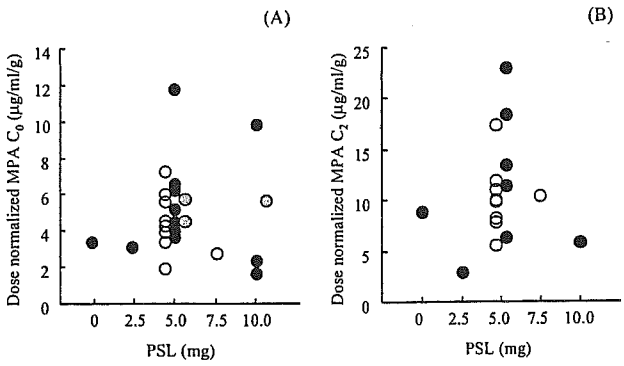

Fig. 6. Effect of Prednisolone on MPA C_0 (A) and C_2 (B) after Administrations of MMF in Renal Transplant Recipients

Each plasma concentration of MPA was normalized with the received dose of MMF. ○, TAC-treated group; ●, CyA-treated group; ⊙, CNI(−) group.

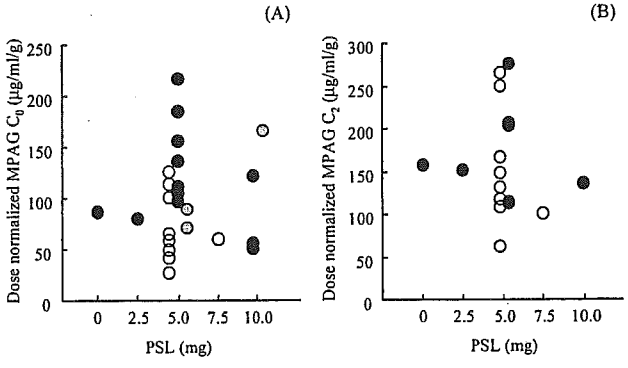

Fig. 7. Effect of Prednisolone on MPAG C_0 (A) and C_2 (B) after Administration of MMF in Renal Transplant Recipients

Each plasma concentration of MPAG was normalized with the received dose of MMF. ○, TAC-treated group; ●, CyA-treated group; ⊙, CNI(−) group.

of MMF for MPA and MPAG C_2 differed between the CNIs. Recently, it has been reported that MPA C_2 has a better correlation with area under the time–concentration curve (*AUC*) than C_0.[19,20] CNI C_2, especially that of CyA, is monitored as index as well as C_0 instead of *AUC* in some centers. *AUC* of MPA is related to drug exposure and is predictive of the likelihood of allograft rejection after renal transplantation.[21] From a clinical point of view, it would be better to estimate *AUC* using C_2 equation for all commonly used drugs (CNIs and MMF) in order to simplify TDM.

Most patients with diabetes mellitus have delayed gastric emptying of immunosuppressant drugs because of gastroparesis.[22,23] Although diabetic renal transplant patients exhibit increased time of maximum concentration for MPA, they do not have a significantly different dose-normalized *AUC* and clearance.[22] Delayed gastric emptying may have influenced MPA and MPAG C_2 in the two patients in the CyA-treated group with severe diabetes and in those with milder diabetes.

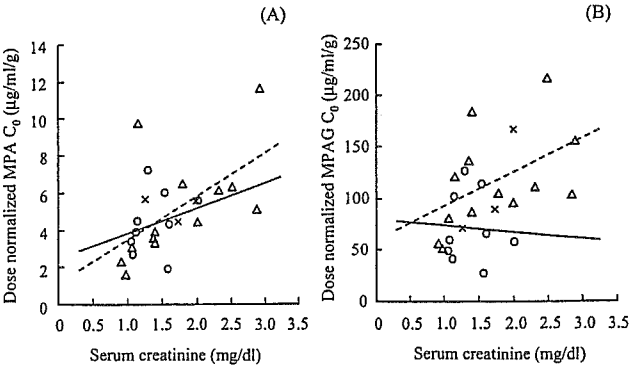

Fig. 8. Correlation between C_0 of MPA (A) and MPAG (B) and Serum Creatinine Concentration in Renal Transplant Recipients

Each plasma concentration of MPA and MPAG was normalized with the received dose of MMF. Straight lines indicate the regression of the TAC-treated patients (○), and the dotted lines indicate the regression of the CyA-treated patients (△). × indicates the values of the CNI(−) patients.

We did not find that combination of immunosuppressants and CNIs had any pharmacokinetic effect on MPA. Zucker *et al.* and Filler *et al.* reported significantly increased C_0 and *AUC* of MPA in recipients treated with TAC compared with CyA.[5,8] Smak Gregoor *et al.* reported that combining MMF with CyA reduced plasma MPA exposure compared with CNI(−) recipients.[9] Our study differed from some previous studies, in that the recipients were given a large range of MMF doses for several adverse drug reactions such as bone marrow suppression and cytomegalovirus infection. The secondary MPA peak caused by enterohepatic cycling had little influence on C_0 because blood sampling was carried out at 12—14 h after oral administration. We observed that MPA C_0 was negatively correlated to a small extent with therapeutic C_0 of CNIs in renal transplant recipients, and the correlation of MPA was stronger than that of MPAG in patients treated with CyA. Filler *et al.* reported that there was a significant negative correlation between dose-normalized *AUC* of MPA and CyA.[24] These data suggest that concentration of CNI, particularly CyA, influences clearance of MPA.

TAC and CyA differently influence the pharmacokinetics of MPAG and the MPAG/MPA ratio.[25,26] In addition, we showed that the MPAG/MPA ratio in CNI(−) patients was not higher than the median ratio in patients treated with TAC. It has been reported that CyA interferes with the enterohepatic circulation of MPAG, and that TAC may interfere with the activity of UGT.[10—13] Our results suggest that the higher MPAG/MPA ratio observed in the CyA-treated recipients was a consequence of inhibition of biliary excretion of MPAG by CyA. Kobayashi *et al.* reported that therapeutic doses of CyA, but not TAC, inhibited the biliary excretion of MPAG in rats, possibly *via* the multidrug resistance-associated protein 2 (Mrp2)/canalicular multispecific organic anion transporter.[12] We speculate that TAC-treated recipients may have frequent intestinal exposure and toxicity to MPA compared with CyA-treated recipients. Diarrhea occurs more frequently as an adverse effect of MMF in patients treated with TAC.[27—29] We suggest that estimating biliary excretion of MPAG is needed to avoid the risk of intestinal injury in combination therapy with TAC during the maintenance period.

Steroids have different effects on the bioavailability and plasma concentration of MPA between the early and maintenance post-transplant periods.[15] Renal transplant recipients were given high dose steroids to avoid allograft rejection in the early post-transplant period, and then maintained on a low dose or discontinued after tapering. We observed that co-administration of PSL (0—10 mg) did not influence the plasma concentration of MPA and MPAG. Our results indicate that concentrations of MPA and MPAG were influenced by the choice of CNI and its concentration rather than PSL dose during the maintenance period.

Some groups have reported that plasma concentration of MPA and MPAG changes with varying degrees of renal function in renal transplant recipients.[30—32] We observed that there was a positive correlation between MPA and MPAG C_0 and serum creatinine concentration. C_0 of MPA and MPAG was influenced by renal function in CyA-treated recipients but not in those treated with TAC. We speculate that CyA may inhibit enterohepatic circulation as a secondary excretion pathway, and, therefore, that renal function may contribute greatly to elimination of MPA and MPAG. In TAC-treated patients, many excretion pathways, such as enterohepatic circulation and other metabolic pathways,[33,34] may contribute to the concentration of MPA and MPAG. During the maintenance period, serum creatinine concentration may be predictive of the likelihood of avoiding over-immunosuppression with MPA.

TAC and CyA differently influenced the pharmacokinetics of MPAG, but not MPA during the maintenance period. In addition, we suggest that the concentration of CNIs, particularly CyA, may influence clearance of MPA. We indicate that CyA, but not TAC, may inhibit enterohepatic circulation of MPAG as a secondary excretion pathway, and that it may be necessary to estimate its biliary excretion to avoid the risk of intestinal injury in patients receiving combination therapy with TAC during the maintenance period. Our study provides some information on individual pharmacokinetic variability of MPA and MPAG in triple immunosuppressive regimens including MMF during the maintenance period.

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ピルジカイニドの血漿中濃度と臨床検査値および心電図変化

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【目的】クラスⅠ抗不整脈薬は血漿中薬物濃度の有効域が狭く副作用濃度と近接しているため、血漿中薬物濃度を測定しながら投与することが望ましい薬剤とされているが、現在のところ薬物治療モニタリング(TDM)は充分に行われていない。本研究ではピルジカイニド(Pil)投与中の患者における血漿中薬物濃度と臨床検査値および投与前後における心電図変化の関係を明らかにすることを目的とした。さらに、Pilの血漿中濃度が著しく高値であり副作用が認められた症例について報告する。

【方法】Pil服用中の患者21例を対象とし、血漿中薬物濃度をHPLC法を用いて測定した。また臨床検査値およびPil投与開始前後におけるQRS幅とQTc値の調査を行った。

【結果・考察】対象患者における血漿中Pil濃度は6.2-120.0μg/mLであり、そのうち4例(19%)が治療域よりも低値を、4例(19%)が高値を示した。また、投与量で補正した血漿中Pil濃度とクレアチニンクリアランスの間には相関を示す傾向が認められた(p=0.09)。さらに、Pil投与開始前後においてQRS幅とQTcには有意な変化は認められなかった。一方、血漿中Pil濃度が2.65μg/mLであった症例において、頭痛と口渇が認められ、QRS幅およびQTcが投与前に比べ延長していた。なお本症例においてPilの投与量減量に伴い血漿中薬物濃度は低下し、これらの症状は消失した。以上より、今回検討したPilの血漿中薬物濃度の範囲においては、Pilの投与により有意なQRS幅およびQTcの延長は認められないものの、血漿中薬物濃度が高値の症例で明らかな副作用が発現したことから、Pilの投与時にTDMを行うことが有用であると示唆された。

ORIGINAL ARTICLE

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NADPH oxidase inhibitor, apocynin, restores the impaired endothelial-dependent and -independent responses and scavenges superoxide anion in rats with type 2 diabetes complicated by NO dysfunction

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Objective: We investigated the effect of apocynin, an NADPH oxidase inhibitor, in the impairment of vascular responses in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (type 2 diabetic rat model) with or without (w/wo) *N*-nitro-L-arginine methyl ester treatment.

Methods: Male OLETF and littermate Long-Evans Tokushima Otsuka (LETO) (28 weeks old) rats were separated as follows: LETO w/wo apocynin (Gp C, Gp C-apo), OLETF w/wo apocynin (Gp DM, Gp DM-apo) and OLETF plus L-nitro arginine acetate ester w/wo apocynin (Gp DMLN, Gp DMLN-apo). Five days after, peritoneal macrophages were stimulated with thioglycolate. Two days after, they were evaluated.

Results: Plasma glucose and lipid levels remained unchanged. Acetylcholine-induced nitric oxide-dependent (NO-dependent) relaxation and nitroglycerin-induced NO-independent relaxation were improved in the Gp DMLN-apo, compared with that in Gp DMLN. Tone-related basal NO release and plasma NO_2^- and NO_3^- tended to be lower in Gp DM and Gp DMLN groups. The increased amount of superoxide anion released from macrophages in Gp DM and Gp DMLN was restored by apocynin. Intimal thickening was observed in aortae of Gp DM and Gp DMLN animals; however, there was little in aortae of Gp DM-apo and Gp DMLN$^-$ apo rats. Increased tumour necrosis factor-α (TNF-α) in the Gp DM and Gp DMLN was also restored by apocynin treatment.

Conclusion: Apocynin restores the impairment of endothelial and non-endothelial function in diabetic angiopathy in OLETF without changing plasma glucose and lipid levels. NO and O_2^- may play a role in this process by decreasing TNF-α levels.

Keywords: NADPH oxidase, nitric oxide, diabetes mellitus, superoxide

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Introduction

The atherogenic process is characterized by an early deficit in nitric oxide (NO) [1,2]. Chronic inhibition of NO, in addition to a high-cholesterol diet, induces severe atherosclerosis [3]. Coronary risk factors such as hyperlipidaemia and diabetes are known to impair NO function, and its impairment becomes severe as the number of risk factors suffered is increasing [3,4]. In diabetes, large-artery

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ischaemic disease is a major problem, especially in the elderly, and complications associated with other coronary risk factors are known to facilitate ischaemia as well as the growth of lesions [5]. The restoration of impaired endothelial function, represented by impaired endothelium-dependent relaxation (EDR) in the diabetic vessel, is important for the stabilization of atheroma. However, restoration of EDR in patients with diabetes has not been consistently observed [6,7]. Clinical and experimental evidence has not yet demonstrated the sufficient stabilization of diabetic vascular complications.

Superoxide production by vascular tissues and its interaction with NO plays an important role in diabetic and atherosclerotic vascular pathophysiology [8,9]. Abnormal vascular endothelial function and atherosclerosis are prominent features of diabetes mellitus, and evidence from experimental studies suggests that increased superoxide production accounts for a significant proportion of the NO deficit in diabetic vessels [10,11]. In addition to NO scavenging, superoxide may alter the activity and the regulation of endothelial NO synthase in endothelial cells, and superoxide may also have other potentially proatherogenic effects on smooth muscle cell proliferation, inflammatory cell recruitment and redox-sensitive gene expression [12].

Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases and NO synthases. NAD(P)H oxidases appear to be the principal source of superoxide production in several animal models of vascular disease, including diabetes [9]. Furthermore, NAD(P)H oxidase subunits and activity has been observed in human blood vessels, including atherosclerotic coronary arteries, suggesting that this oxidase system plays an important role in a number of vascular disease states [12]. Despite the importance of increased superoxide production in endothelial dysfunction and vascular disease in diabetes, the characteristics and mechanisms of vascular superoxide production in diabetes remain only partially defined [13–15]. Accordingly, we evaluated the sources and relative magnitude of superoxide production in arteries taken from type 2 diabetic rats with or without (w/wo) *N*-nitro-L-arginine methyl ester (L-NAME) treatment, which can severely damage endothelial function; these rats were thought to be a suitable because the model status are similar to that of diabetes complicated by other coronary risk factors. In particular, we sought to investigate the NAD(P)H oxidase system by using a NADPH oxidase inhibitor, apocynin, and we also examined the putative role of endothelial NO synthase (eNOS) dysfunction in contributing to vascular superoxide production. The underlying mechanism leading to the increase of superoxide produced by NADPH oxidase, eNOS, etc., is thought to involve TNF-α, which regulates NADPH oxidase and inducible NOS (iNOS) at the promotor level. Furthermore, we previously reported that TNF-α, which is known to be present in high levels in patients with atherosclerosis, destabilizes eNOS, and the destabilization was prevented by treatment with estradiol [16]. In addition, TNF-α levels are known to be increased in patients with poor blood glucose control and with insulin resistance, and thus TNF-α is thought to play a role in diabetic angiopathy [16]. This line of reasoning led us to measure the plasma levels of TNF-α in the rat models used in the present study.

Materials and Methods

Chemicals

Acetylcholine chloride (ACh), prostaglandin F2α (PGF2α), indomethacin and L-NAME (NO synthase inhibitor) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Nitroglycerin was obtained from Nihon Kayaku Co. Ltd. (Tokyo, Japan). Determination of TNF-α was carried out by enzyme-linked immunosorbent assay (ELISA) kit [16].

Animals

Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats (type II diabetic rats, 28 weeks old) and littermate Long-Evans Tokushima Otsuka (LETO) rats (28 weeks old) were obtained from the Otsuka Pharmaceutical Company (Tokushima City, Japan) [13], and they were divided into six groups as follows: Gp C, LETO was fed regular chow; Gp C-apo, LETO was fed regular chow with apocynin (NADPH oxidase inhibitor, 30 mg/kg/day); Gp DM, OLETF was fed regular chow; Gp DM-apo, OLETF was fed regular chow with apocynin; Gp DMLN, OLETF was fed regular chow with L-NAME (100 mg/kg/day) and Gp DMLN-apo was fed regular chow with apocynin plus L-NAME. The rats were housed with free access to water. Five days after treatment of each condition as described above, their peritoneal macrophages were stimulated with the peritoneal injection of thioglycolate [17]. Two days after the injection, they were exsanguinated and evaluated. All experiments were conducted in accordance with institutional guidelines for animal research.

Blood Sampling

The following measurements were also carried out before and after the treatment: body weight, fasting plasma glucose and insulin concentrations, serum total

cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol. The following factor, which might be involved in the mechanism, was also investigated: TNF-α. ELISA kits were used for the measurements (JIMRO, Takasaki, Japan).

Vascular Response

Seven days after treatment, the rats (each group n = 6) were sacrificed by exsanguination after being anaesthetized with pentobarbital (50 mg/kg IP). Vascular responses were investigated as described previously [18]. Briefly, aortae were cut into 2-mm wide transverse rings. Those were stretched to their optimal force, which was predetermined at the contraction to 122 mM KCl, and were mounted in chambers filled with Krebs' Henseleit solution at 37 °C. The response of aortic rings to ACh and normal triglyceride were determined under submaximal contraction by PGF2α (2.6×10^{-6} M). Tone-related basal NO release, contractile responses to L-monomethyl-arginine (L-NMA) was assessed under moderate tone (about 40% contraction by KCl) induced by PGF2α (0.8×10^{-6} M) [18]. In some studies, indomethacin (5×10^{-6} M) was added in to the chambers to rule out the contribution of prostanoids.

Measurement of Nitrite and Nitrate (NO_2^-/NO_3^-) and Detection of Aortic Superoxide Generation

Plasma NOx (sum of nitrite and nitrate, NO_2^-/NO_3^-) was measured using NO detector-HPLC system (ENO10; Eicom Co., Kyoto, Japan), which was used as previously reported [19].

Detection of Aortic Superoxide Anion (O_2^-) Generation from Aorta and Intracellular Redox State of Peritoneal Macrophages

Formation of O_2^- from the vessel was assayed by measuring the intensity of chemiluminescence probes in the presence of one of the Cypridina luciferin analogs, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo-1,2-a pyrazine-3-one (MCLA) [20]. In brief, the O_2^- generation signal from the 2 mm length of vessel was detected by a luminescence reader (BLR-201, Aloka Co., Tokyo, Japan). To ensure the specificity of MCLA to detect O_2^-, increasing concentrations of superoxide dismutase (1–50 U/ml) were added to the tissues. Intracellular redox state levels of peritoneal macrophages stimulated by thioglycolate for 2 days were measured with a fluorescent dye, CDCFH diacetate bis-AM ester, a non-polar compound that is converted into a non-fluorescent polar derivative (CDCFH) by cellular esterases after incorporation into cells. CDCFH is membrane-impermeable and rapidly oxidized to the highly fluorescent carboxydichlorfluorescein in the presence of intracellular hydrogen peroxide and peroxidases. The fluorescence intensity of each point was measured by flow cytometry and calculated with untreated control cells as a standard [21]. The excitation wavelength was 510–530 nm. Relative fluorescence intensities were measured.

Histological Evaluation of Atherosclerosis

Cross-sections of the aorta adjacent to segments for vascular responses were examined [22]. Briefly, the contours of the lumen and the internal elastic lamina (IEL) were traced. The mean surface involvement by atherosclerotic lesion (intimal thickening) per vessel (extent) was calculated after dividing the lesion circumference by the circumference of the IEL. The circumferences of the lesion and the healthy region were defined as the circumferences of the respective parts of the IEL. The area occupied by atherosclerotic lesions (total lesion burden: size/thickness) was defined as the percentage area bounded by the lumen and the IEL for luminal area.

Immunohistochemical Study

Tissues were sectioned to 5-μm slices [8]. Primary monoclonal antibodies (anti-macrophages, anti-smooth muscle cells, anti-endothelial NO synthase, anti-inducible NO synthase or anti-nitrotyrosine) were applied. Sections were incubated with biotinylated immunoglobulins and incubated with horseradish peroxidase-labelled avidin solution (Vecstatin Elite, Vector Laboratories, Burlingame, CA, USA). Negative controls included the substitution of primary antibody with irrelevant antibodies. There were no cross-reactions between anti-eNOS and anti-iNOS antibodies. Each field was scored for number of each antibody positive cells on the slides and analysed statistically as described by previous report [23]. Five samples were prepared from each rat.

Statistics

Data are presented as mean ± s.e.m. The student's *t*-test was used and Kruskal–Wallis one-way ANOVA was used for multiple comparisons. $p < 0.05$ is considered as significant. Statistical calculations were performed using STAT VIEW software (version 5.01-SAS Institute, Cary, NC, USA).

Results

Body Weight and Blood Sampling

The plasma biochemical profiles are shown in table 1. There were no significant differences among the six groups before or after treatment with regard to the body weight, serum total protein, fasting plasma glucose, total and HDL cholesterol and triglycerides. The plasma concentrations of TNF-α had significantly increased in the Gp DM and Gp DMLN groups, whereas no change was observed in the following groups: Gp C, C-apo, DM-apo and DMLN-apo (table 1). After treatment, the plasma concentration of NOx (sum of nitrite and nitrate) did not change significantly in Gp DM compared with Gp C, however, it was decreased significantly in Gp DMLN and Gp DMLN-apo.

Vascular Response

Basal NO release was decreased in Gp DM and Gp DMLN, but such a decrease was not observed in the following groups: Gp C w/wo apocynin, Gp DM-apo or Gp DMLN-apo (figure 1). Acetylcholine-induced NO-dependent relaxation was impaired in the Gp DM group and was particularly impaired in the Gp DMLN. However, this type of relaxation improved in the Gp DM-apo and Gp DMLN-apo, as compared to that in the corresponding groups without apocynin treatment (Gp DM and DMLN) (figure 1). Nitroglycerin-induced endothelium-independent relaxation was also slightly impaired in the Gp DM and DMLN groups, but no change was observed in the other groups.

Detection of Aortic Superoxide Anion Generation and Intracellular Radicals State of Peritoneal Macrophages

The chemiluminescence signals as superoxide anion production from aorta remarkably increased in Gp DM-LN and slightly increased in Gp DM as compared with LETO groups. O_2^- from aorta in Gp C was 0.35 ± 0.18 Kcpm/mg protein. Superoxide anion production decreased in Gp DM-apo when compared with Gp DM-LN (figure 2 upper). The amounts of intracellular redox released from peritoneal macrophages were increased in the OLETF groups, especially in the DMLN group, as compared with the LETO groups (figure 2 lower). However, these levels were restored in the DM-apo and DMLN-apo groups. In particular, DMLN-apo restored the amount of superoxide anion to the normal level.

Histological Evaluation of Atherosclerosis

Atherosclerotic changes were observed in the aortae from Gp DMLN rats (11.3 ± 2.1, $4.8 \pm 1.7\%$ in surface involvement, area occupied by lesion); however, there were few such changes observed in the aortae from the Gp DM (5.3 ± 1.4, $2.1 \pm 0.8\%$), Gp DM-apo (2.3 ± 1.1, $1.1 \pm 0.4\%$) and Gp DMLN-apo rats (4.7 ± 1.3, $1.4 \pm 0.5\%$).

Immunohistochemical Study

Monocytes/macrophages were attached to the endothelium and were distributed only to a small extent in the sub-intima of the Gp DM group rats; these cells were present in modest amounts in the sub-intima of the Gp DMLN animals, but not observed in the other groups (figure 3 upper). A few smooth muscle cells were observed in the sub-intima in the Gp-DM, Gp-DMLN, Gp DM-apo and Gp DMLN-apo groups, but not in the other two groups (data not shown). iNOS was slightly apparent in the sub-intima of the Gp DM and Gp DMLN, but absent in the other groups (figure 3 lower panel). Peroxynitrite, as visualized by staining with nitrotyrosine, was rarely present, but it was observed in small concentration in the sub-intima of the Gp DM and

Table 1 Biochemical and NO related profile

	B.W. (g)	T.P. (g/dl)	FBS (mg/dl)	T.Chol (mg/dl)	T.G. (mg/dl)	HDL-C (mg/dl)	NO_2^-/NO_3^- (μM)	TNF-α (pg/ml)
Gp C	633.0+24.3	8.1+1.2	83.0+21.3	75.2+9.8	31.8+4.2	30.4+7.2	36.4+3.5	13.1+3.6
Gp Capo	612.6+27.3	7.8+1.2	80.6+17.3	59.5+10.6	29.8+4.0	29.3+6.2	40.5+3.6	10.3+2.4
Gp DM	690.5+70.4	6.9+2.5	263.1*+35.0	87.8+14.1	34.9+4.1	29.5+7.7	33.2+4.1	25.5+4.5
Gp DM-apo	64.7+83.3	7.2+1.9	250.5+52.7	69.3+23.8	30.9+5.1	30.3+4.5	36.9+4.6	13.8*+2.5
Gp DMLN	625.0+10.7	6.9+1.7	252.0*+37.6	66.7+15.7	34.7+8.5	35.3+7.7	22.5*+4.5	32.8*+4.6
Gp DMLN-apo	612.5+101.4	6.9+1.8	251.1+47.2	74.9+19.1	35.3+4.2	38.1+7.1	25.8*+4.2	16.7#+2.6

B.W., body weight; FBS, fasting blood sugar; HDL-C, High-density lipoprotein cholesterol; T.Chol, Total Cholesterol; T.G., Triglyceride; T.P., Total Protein; TNF-α, tumour necrosis factor-α. *$p < 0.05$ vs. Gp C, #$p < 0.05$ vs. Gp DMLN.

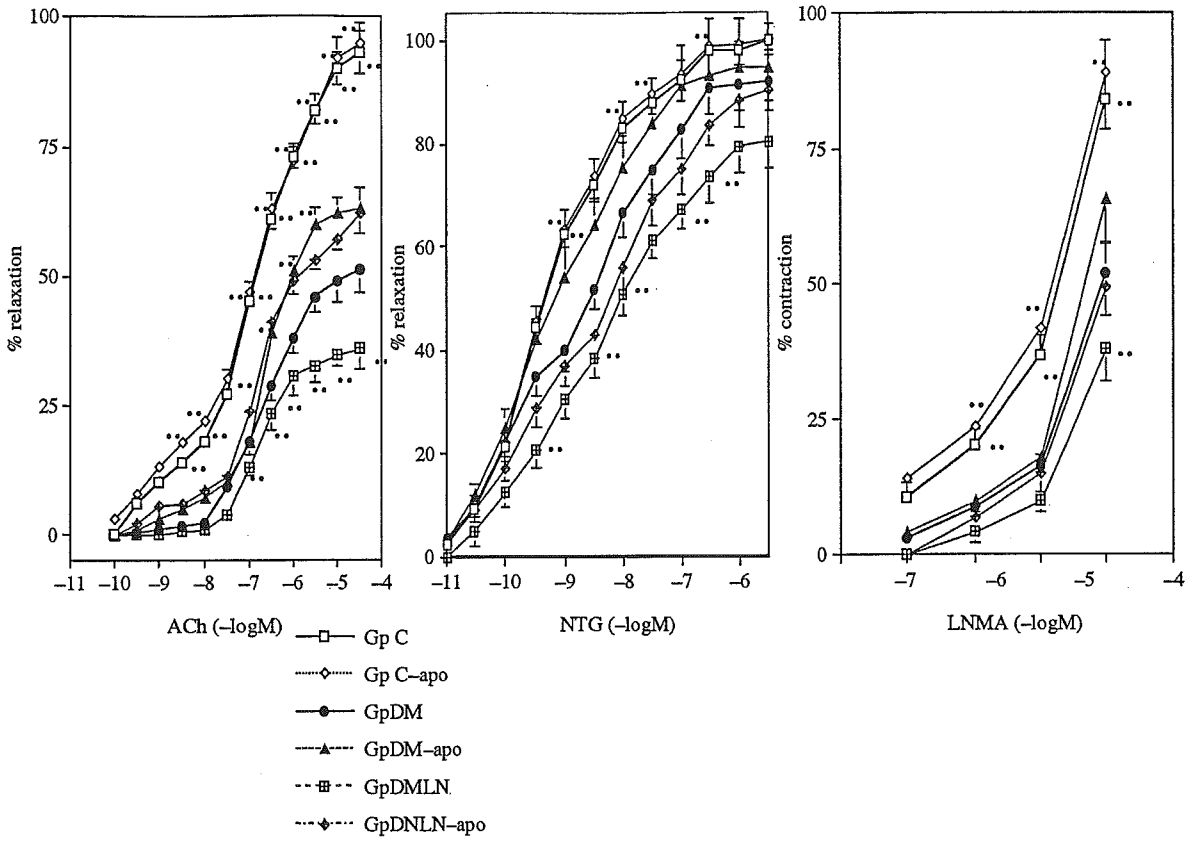

Fig. 1 Cumulative concentration response curves for each of the agonists in the abdominal aortae of six groups, which were precontracted by prostaglandin F2α: Gp C, Long-Evans Tokushima Otsuka (LETO) fed regular chow; Gp C-apo, LETO-fed regular chow with apocynin; Gp DM, Otsuka Long-Evans Tokushima Fatty (OLETF) fed regular chow; Gp DM-apo, OLETF-fed regular chow with apocynin; Gp DMLN, OLETF-fed regular chow with *N*-nitro-L-arginine methyl ester (L-NAME); Gp DMLN-apo fed regular chow with apocynin plus L-NAME. Left: Cumulative concentration-response curves for acetylcholine. Middle: Cumulative concentration-response curves for nitroglycerin. Right: Cumulative concentration-response curves for L-NMA; NOS inhibitor. % means the percentage vs. the magnitude of the contraction level by PGF2α. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Gp C.

DM-LN groups, but was not observed in the sub-intima of the other four groups. The staining area was almost same as that observed by iNOS antibody (data not shown).

Discussion

Abnormal vascular endothelial function and intimal thickening (atheromatous change) are common among the patients with diabetes mellitus, and increased superoxide production appears to be account for a significant proportion of the observed NO deficit status associated with diabetes [8–11]. Although potential sources of vascular superoxide production include NADPH-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases and endothelial and inducible NO synthases, NADPH oxidase appears to be the principal source in several animal models of diabetes [9,12,13]. For this reason, we investigated the effect of apocynin, an NADPH oxidase inhibitor, in a diabetes model w/wo severe endothelial dysfunction. In the present study, there was only a slight release of superoxide anion from macrophages in the LETO groups w/wo apocynin; this release was increased in OLETF rats, and especially in the L-NAME-treated OLETF rats. However, apocynin treatment (Gp DM + apo and GP DMLN + apo) restored the levels of superoxide anion to the control level observed in the LETO groups. Furthermore, morphological changes in response to diabetes plus NO dysfunction were not evident in the apocynin treatment groups.

Our findings suggest two important and potentially related mechanisms that underlie these functional deficits. First, increased superoxide production in atherosclerosis and other pro-atherosclerotic states likely reduces NO bioactivity by a direct scavenging mechanism. The

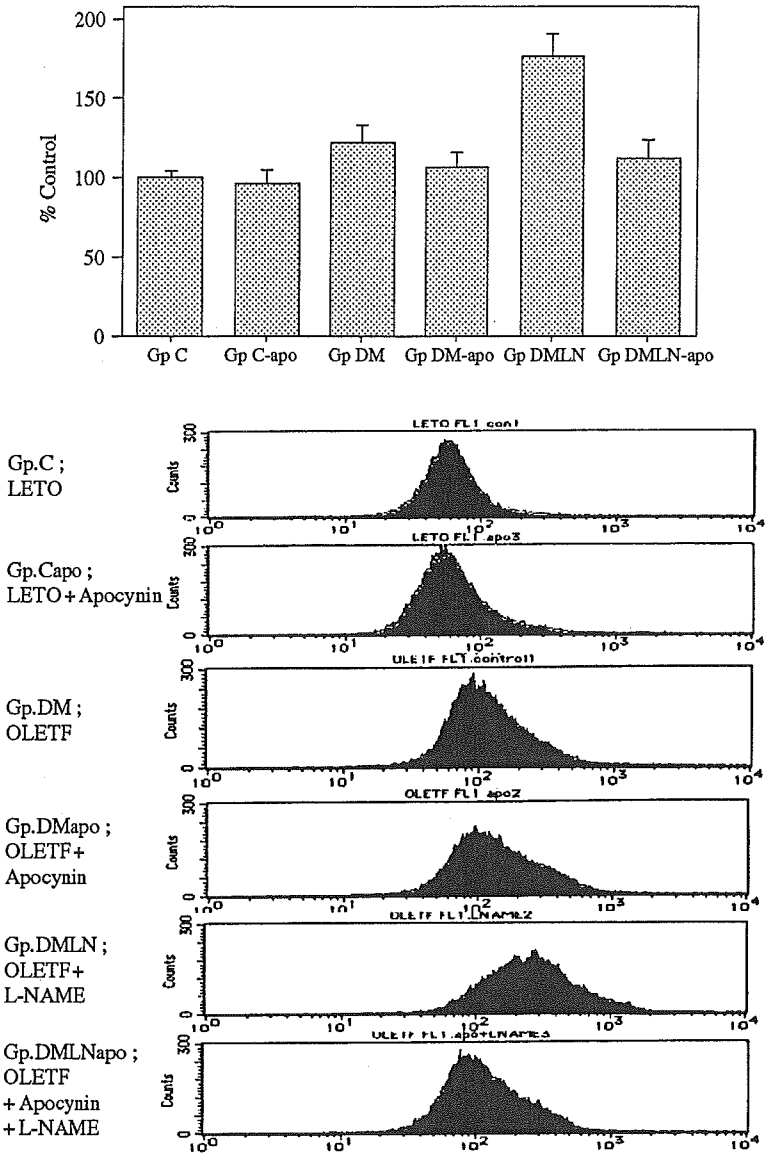

Fig. 2 Upper: Formation of O_2^- from vessel was assayed by MCLA methods. Gp C, Long-Evans Tokushima Otsuka (LETO) fed regular chow; Gp C-apo, LETO fed regular chow with apocynin; Gp DM, Otsuka Long-Evans Tokushima Fatty (OLETF) fed regular chow; Gp DM-apo, OLETF fed regular chow with apocynin; Gp DMLN, OLETF fed regular chow with *N*-nitro-L-arginine methyl ester (L-NAME); Gp DMLN-apo fed regular chow with apocynin plus L-NAME, $^*p < 0.05$ vs. of O_2^- from Gp C vessels. Lower: Superoxide anion release from peritoneal macrophages, which was evaluated by FACS Scan.

expression of NADPH oxidases have been shown in vascular cells and macrophages in atherosclerotic coronary arteries [11,12]. The effect of NADPH oxidase in this diabetes model was clearly demonstrated by the effectiveness of apocynin treatment, which basically reversed the impairment of endothelial function in the relevant groups in this study and decreased superoxide anion release from diabetic vessels and also decreased it from macrophages. The other mechanism underlying such functional deficits would be the uncoupling of NOS. Although eNOS in the vascular endothelium and iNOS in macrophages produce NO, they may be sources of superoxide production under certain conditions, due to the enzymatic uncoupling of L-arginine oxidation and oxygen reduction by the oxygenase and reductase domains of eNOS, respectively [24]. Recent studies have suggested that the reduced availability of the cofactor tetrahydrobiopterin (BH4) may result in eNOS or iNOS uncoupling, in turn responsible for the imbalance between NO production and superoxide production in diabetic vascular lesions [25]. It has been observed in diabetic vessels that hyperglycaemia increases NOS-dependent superoxide production in human endothelial cells and that mediates eNOS dysfunction in endothelial cells [26,27]. In studies of experimental diabetes in rats

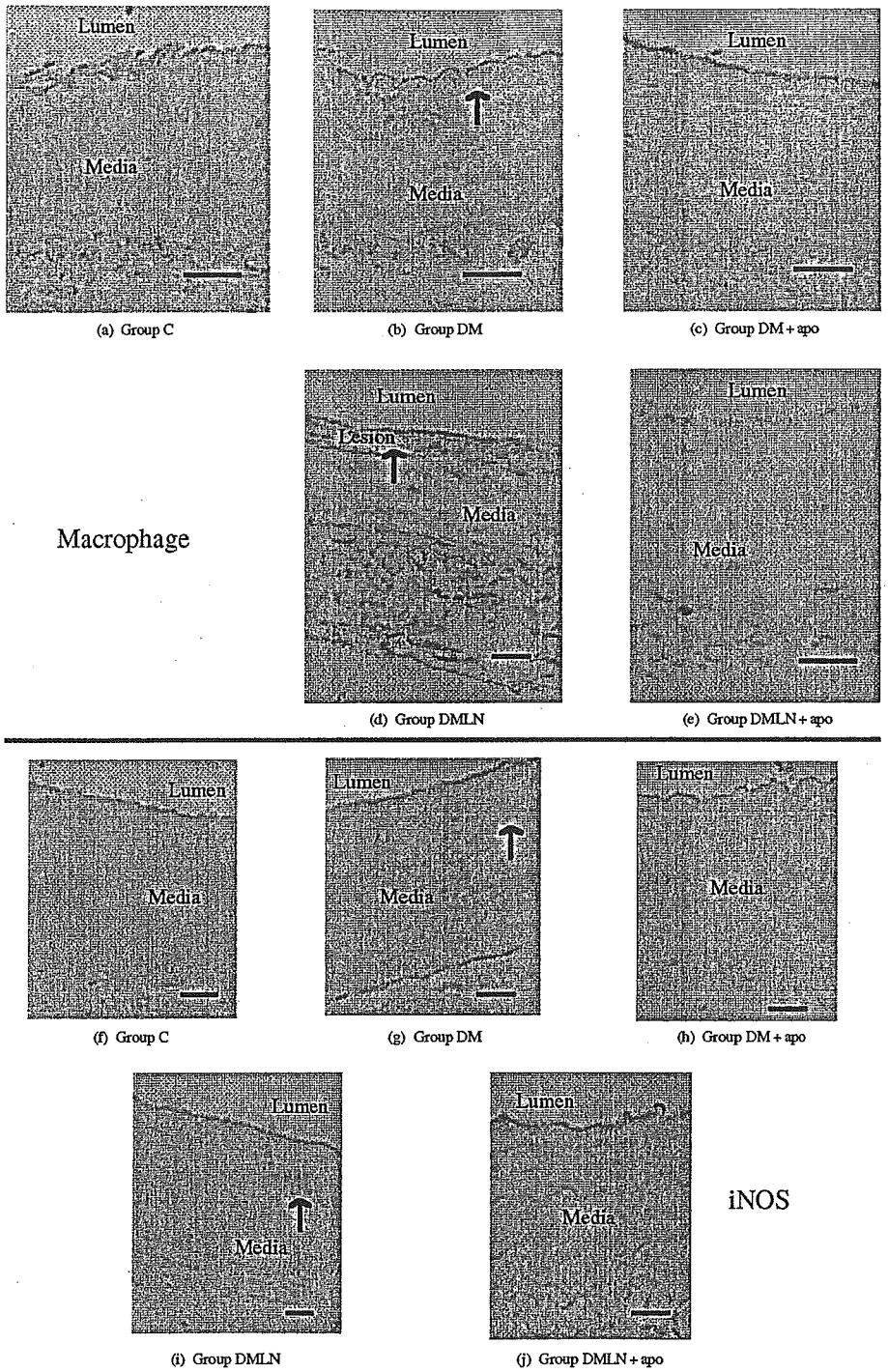

Fig. 3 The representative immunohistochemical staining of abdominal aortas. Upper: A section stained with a monoclonal antibody against macrophages (arrow) from (a) Gp C, Long-Evans Tokushima Otsuka (LETO); (b) Gp DM, Otsuka Long-Evans Tokushima Fatty (OLETF); (c) Gp DM-apo, OLETF with apocynin; (d) Gp DMLN, OLETF with *N*-nitro-L-arginine methyl ester (L-NAME); and (e) Gp DMLN-apo with apocynin plus L-NAME (original magnification ×150). Bar is 50 μM. Lower: A section stained with a monoclonal antibody against the marker of induced NO synthase (arrow) from (f) Gp C; (g) Gp DM; (h) Gp DM-apo; (i) Gp DMLN and (j) Gp DMLN-apo (original magnification ×100). Bar is 50 μM. Lumen: luminal area, Media: media, Lesion: atheromatous lesion.

and in atherosclerotic apolipoprotein E-knockout mice, in which both increased NAD(P)H oxidase activity and NOS dysfunction have been found to contribute to both increased total vascular superoxide production and reduced NO bioactivity [28]. Therefore, the up-regulation of vascular superoxide production by NAD(P)H oxidases may in turn lead to eNOS or iNOS uncoupling via the oxidation of BH4, which reduces NO production and further increases endothelial superoxide production.

In the present study, we studied the effect of L-NAME treatment on OLETF rats w/wo apocynin in order to investigate severe endothelial dysfunction in a diabetic animal model complicated by other coronary risk factors. Chronic NO inhibition by L-NAME treatment facilitates high-cholesterol-diet-induced atherosclerosis, and L-NAME treatment induces intimal thickening in rat models [3,29]. Landmark clinical trials such as the United Kingdom prospective diabetes study and 4S have demonstrated that strict control of complicated coronary risk factors such as hypertension and hypercholesterolemia are as important as the control of plasma glucose levels in the prevention of ischaemic cardiovascular events. One of the common mechanisms underlying these treatments is improving or restoring endothelial function such as NO bioavailability [30,31]. In our present study, it is interesting that apocynin successfully improved endothelial dysfunction and inhibited superoxide release from activated macrophages.

Apocynin (4′-hydroxy-3′-methoxy-acetophenone or acetovanilone), a non-toxic compound isolated from the medicinal plant *Picrohiza kuroa*, is a potent inhibitor of NADPH oxidase in stimulated human neutrophils (IC50: 10 mM) [32]. An additional interesting aspect of apocynin is its very low toxicity (LD50: 9 g/kg oral administration in mice) [33]. Apocynin inhibits neutrophil NADPH oxidase activity, thereby preventing the production of oxygen radicals [34–37]. Apocynin was reported to inhibit NADPH oxidase activity by the disassociation of p47 phox, which was the membranous component of NADPH oxidase from Gp 91 [36]. Our preliminary experiments using cultured macrophages and endothelial cells showed the same results (data not shown). This is the first report to investigate the usefulness of this NADPH oxidase inhibitor as a drug in a model of diabetic angiopathy. Our results suggest that the mechanism leading to this effect involves a decrease in the plasma TNF-α concentration. In the present study, blood glucose levels did not change in any of the groups. In this context, it should be noted that TNF-α has been shown to up-regulate NADPH oxidase in endothelial cells and diabetic vessels, and TNF-α was shown to inhibit the activation of eNOS, which in turn indirectly enhances eNOS uncoupling [38,39]. In liver, free radicals from NADPH oxidase in hepatic Kupffer cells play a predominant role in the pathogenesis of early alcohol-induced hepatitis by activating NF-kappa B, which activates the production of cytotoxic TNF-α [40]. Like this, superoxide anion from NADPH oxidase from endothelial cells and macrophages can activate NK-kappa B and then produce TNF-α.

In conclusion, we found that macrophages from diabetic rats produce high levels of superoxide. The level of superoxide was especially increased in diabetic rats with severe endothelial dysfunction resulting in NOS-inhibitor treatment. Apocynin, an NADPH oxidase inhibitor, reversed the endothelial dysfunction and prevented the atherosclerotic, morphological changes in diabetic rats regardless of whether or not they were subjected to NOS inhibition.

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The treadmill exercise-tolerance test is useful for the prediction and prevention of ischemic coronary events in elderly diabetics

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Abstract

Background: Approximately 80% of cases of ischemic heart disease (IHD) occur in patients with nonstenotic coronary arteries, and few studies have systematically assessed exercise testing (TMT) as a predictor of risk in the elderly. **Methods:** TMT was carried out using a protocol for the independent and active elderly (n=176). After 4.1±0.5 years follow-up, logistic regression analysis was performed for each coronary risk factor such as diabetes mellitus (DM) and hypercholesterolemia (HC). According to the results, patients were divided into Gp HC, hypercholesterolemic patients; Gp DM, diabetics; Gp HC+DM, hypercholesterolemic diabetics; and Gp C, nonhyperlipidemic and nondiabetics. Sensitivity and specificity of TMT for IHD (significant stenosis or acute coronary syndrome) were analyzed. **Results:** Odds ratios for each risk factors are as follows: DM, 4.167; HC, 4.485; and DM+HC, 8.652. Notably, TMT was 17.59. Age was a significant risk, but hypertension was not. Positive ischemic signs in TMT were observed in 52.7%, 28.6%, 33.3%, and 16.3% in the Gp HC+DM, HC, DM, and C groups, respectively. Only three participants complained of chest pain during the TMT. Significant stenosis was observed in 75.0%, 71.4%, 69.2%, and 60.0% of coronary angiography (CAG)-receiving patients of Gp HC, DM, HC+DM, and C. During the observation term, acute coronary syndromes occurred in 4.7%, 3.3%, 5.5%, and 0% of patients in the Gp HC, DM, HC+DM, and C groups, respectively. The sensitivity of TMT for IHD was higher than 66.7% and specificity was higher than 94.1% in each group. **Conclusion:** An exercise tolerance test in the elderly, especially for diabetics and hypercholesterolemic patients, is useful for the diagnosis of IHD.

1. Introduction

Recent mega-trials have revealed that strict control of complicated coronary risk factors such as hyperlipidemia is important for the prevention of diabetic vascular lesions (Jonsson, Cook, & Pedersen, 1999). Exercise stress testing is an accepted means of estimating and diagnosing cardiovascular disease, as well as of predicting cardiovascular and all-cause mortality (Gianrossi, Detrano, Mulvihill, et al., 1989). However, approximately 80% of cases of ischemic heart disease (IHD) occur in patients with nonstenotic coronary arteries, and these cases cannot be predicted by an exercise-tolerance test (Bezerra, Higuchi, Libby, Ramires, et al., 2001). Furthermore, few studies have systematically assessed exercise testing as a predictor of risk in the elderly. Diabetic coronary lesions are known to have long segmental narrowing, and the incidence of IHD seems to be especially increased in patients who have had diabetes for more than 10 years (Al-Attar, Mahussain, & Sadanandan, 2002; Stein, Weintraub, Gebhart, et al., 1995). We have speculated that an exercise-tolerance test would be useful for the evaluation and prevention of IHD in elderly diabetics, if it could be carried out in a safe manner. We therefore modified the protocol of the exercise burden for the treadmill exercise-tolerance test (TMT) to make it more suitable for elderly patients.

The present study focused on the relationship between the frequency of cardiovascular ischemia, the exercise-tolerance test, and coronary risk factors in the elderly.

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