基本的には，PTDM の管理と治療は内分泌・糖尿病代謝の専門医の follow-up が必要と考える。移植患者は程度の差こそあれ，移植臓器障害の状態である。運動療法を含めた生活指導や薬物療法もそれを十分考慮に入れて患者指導・教育を行うべきと考える。

治療手段としては step-wise 療法を考慮すべきである。また，特に経口剤治療で注意すべき点は，腎機能障害を伴っている移植患者は，スルホニル尿素(SU)剤系による遷延性の低血糖発作やビグアナイド系(メトホルミン)などによる lactic acidosis に注意すべきと考える。高齢者，肝障害や腎障害を伴う移植患者はグリニド系製剤の投与を考慮すべきと考える。

3) その他の PTDM に伴う高血圧，高脂血症などの治療は大変重要であり，各々のガイドラインに従って治療すべきと考える。

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Induction of uPA release in human peripheral blood lymphocytes by [deamino-Cys1,d-Arg8]-vasopressin (dDAVP)

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[deamino-Cys1,d-Arg8]-vasopressin (dDAVP), known to be an arginine va-
sopressin (AVP) V_2 receptor agonist, is an agent that increases fibrinolytic
activity levels in plasma after its infusion into the human body. However,
mechanisms underlying an increase and exact localization of the extrarenal
dDAVP-responsive V_2 receptor remain unclarified. Two AVP receptors, V_{1a}
and V_2, and a related oxytocin (OT) receptor were found to be expressed in
human lymphocytes. Furthermore, we found an increase of fibrinolytic
activity in the medium of peripheral lymphocytes obtained from human
volunteers less than 20 min after dDAVP infusion. The increased activity was
also detected in the medium after incubating the lymphocytes in the presence
of dDAVP in vitro, being highest at 20 min after the incubation. In accord
with the increased fibrinolytic activity, the levels of urokinase-type plasmin-
ogen activator (uPA) in the medium were also increased. However, there was
no significant difference of plasminogen activator inhibitor-1 (PAI-1), pro-
uPA, and tissue-type plasminogen activator (tPA) concentrations in the
medium between dDAVP treatment and control. When lymphocytes were
preincubated with a V_2 receptor antagonist [Adamantaneacetyl1,O-Et-D-
Tyr2,Val4,Aminobutyryl6,Arg8,9]-vasopressin, the dDAVP-induced uPA in-
crease was diminished. In contrast, preincubation with a V_1 receptor antag-
onist, [β-Mercapto-β,β-cyclopentamethylenepropionyl7,O-Me-Tyr2,Arg8]-
vasopressin, prior to dDAVP treatment resulted in a greater increase of the
uPA concentration in the medium than with the dDAVP treatment alone.
Thus it was suggested that dDAVP may induce uPA release from human
lymphocytes via V_2 receptor-mediated reaction, and also via cross-talk
between V_1 and V_2 receptors.

arginine vasopressin; plasminogen activator; urokinase-type plasmin-
ogen activator; protease release

PROTEASE ACTIVITY IN HUMAN LYMPHOCYTES is an intriguing topic
because of its involvement in various senescence-associated
diseases, neural migration, or demyelination disorders (31–33),
although all of the proteases involved in these disorders have
not been well characterized. To elucidate the role of protease
activity, we recently established a method to search for agents
that increase the protease activity levels in lymphocytes freshly
prepared from human peripheral blood (34). In this new
method, fibrinolytic activity is estimated by incubating lym-
phocytes with ^{125}I-labeled fibrin as a substrate in the presence
of plasminogen. This cascade reaction amplifies the activity
levels and therefore is useful for detecting protease activation

events of stress response in the human body (34). In particular,
this reaction assay in vitro will reflect the protease activation
induced by drugs in vivo.

Arginine vasopressin (AVP) and oxytocin (OT) are cyclic
nonapeptides whose actions are mediated by stimulation of
specific G protein-coupled receptors classified into V_{1a} (vas-
cular), V_{1b} (pituitary), and V_2 (renal) receptors and OT recep-
tors (16, 18). All members of the family have been cloned, and
the affinity of cloned AVP and/or OT receptors for [deamino-
Cys1,D-Arg8]-vasopressin (dDAVP) and other ligands is well
described (19, 36). AVP directly elicits the contraction of
smooth muscle preparation via V_1 receptor activation. On the
other hand, V_2 receptors in renal tubular cells promote the
reabsorption of water (4). It has also been reported that, in
canine basilar artery, AVP causes an endothelium-dependent
relaxation via the V_{1a} receptor (9) and that, in experimental
animals (28) or humans (6), 4-valine-8-D-arginine vasopressin
or dDAVP causes a decrease in blood pressure that is not
mediated by prostaglandins (13, 16). In rat aortic strips,
dDAVP evokes endothelium-dependent vasorelaxation (40),
not via the authentic V_2 receptor but rather via the endothelial
V_1-like receptors, which may be functionally different from the
V_1 receptor in smooth muscle cells. OT is another posterior
pituitary hormone whose primary action is to stimulate uterus
contraction or milk ejection function via OT receptors (35). In
myometrium, binding of OT to high-affinity receptors stimu-
lates various biological responses, including inositol-triphos-
phate turnover and Ca^{2+} influx, similar to those induced by the
binding of AVP to the V_{1a} receptors. Interestingly, the uterus
contains not only OT receptors but also V_{1a} receptors of
approximately fivefold higher density in nonpregnant condi-
tions (2). OT has also been reported to enhance glomerular
filtration rate and to have a natriuretic effect (3). In addition,
OT has either diuretic or antidiuretic osmoregulatory effects
depending on the presence or absence of vasopressin, which
may be explained by the ability of OT to bind to the adenylate
cyclase-stimulating V_2 receptor in distal tubules and collecting
ducts (5, 15). Because some of the organs or cells express
different subtypes of AVP/OT receptors, the cross-talk be-
tween them may be involved in various unknown physiological
events.

dDAVP is known to be an agonist for one of the three types
of AVP receptors, namely V_2 (16, 18). It was reported that a
marked increase of fibrinolytic activity in plasma is observed
when dDAVP is infused in humans (17). This increase paral-

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leled that of immunoreactive tissue-type plasminogen activator (tPA), which is partly derived from vascular endothelial cells (12). It was also demonstrated that dDAVP induces the release of two coagulation factors, factor VIII and von Willebrand factor, from the vascular endothelium through the activation of a vasculoendothelial V_2-like receptor (11, 16, 26). However, the mechanism by which dDAVP causes the increase of fibrinolytic activity in plasma remains unclarified.

Human peripheral lymphocytes possess binding sites for dDAVP (38), although the expression of the receptor for binding dDAVP has not been determined. In addition, neither the role of V_2 receptor in lymphocytes nor the action of V_2 receptor agonist in peripheral blood lymphocytes has been documented until now.

In the present study, we determined the expression of mRNA species of AVP/OT receptors that can bind to dDAVP in human peripheral lymphocytes. Then, we examined whether peripheral lymphocytes obtained from human subjects with dDAVP infusion have the ability to increase plasminogen-dependent fibrinolytic protease activity and whether lymphocytes release the proteases when incubated with dDAVP in vitro.

MATERIALS AND METHODS

Agents. dDAVP and AVP were purchased from Ferring Pharmaceuticals (Copenhagen, Denmark). [β-Mercapto-β,β-cyclopentamethylenepropionyl[1],O-Me-Tyr^2,Arg^8]-vasopressin, [Adamantaneacetyl[1],O-Et-D-Tyr^2,Val^4,Aminobutyryl^6,Arg^{8,9}]-vasopressin, and fibrinogen were obtained from Sigma Chemical (St. Louis, MO). [^{125}I]Na (100 mCi/ml, 17 mCi/mg) was purchased from New England Nuclear (Boston, MA).

Infusion study. dDAVP was diluted with saline to allow for the intravenous delivery of 0.4 μg/kg in 100 ml over 10 min (6). Two milliliters of whole blood samples were withdrawn from cubital veins of 12 volunteers (6 males and 6 females from 40 to 60 yr old, mean age 47.3), with EDTA as an anticoagulant, every 10 min after dDAVP infusion. As a control, samples were taken from 12 age-matched volunteers (6 males and 6 females) without dDAVP infusion. Informed consent was obtained from all of the volunteers, and this study was approved by the Human Research Committee of Sakura National Hospital.

Preparation of lymphocyte samples. Lymphocytes were prepared from peripheral blood of volunteers principally according to the method described previously (32). Briefly, each (2-ml) blood sample was diluted with the same volume of phosphate-buffered saline [PBS; 10 mM sodium phosphate (pH 7.4) containing 135 mM NaCl] and put on 7 ml of Ficoll. The samples were centrifuged at 240 g for 20 min at room temperature. After centrifugation, the thin white layer of the lymphocyte fraction, termed "buffy coat," was collected and mixed with a fivefold volume of PBS. Then, it was centrifuged at 240 g for 10 min. The pellet was suspended in RPMI 1640 medium and incubated in a 60-mm dish for 20 min at 37°C for platelet attachment. Flow cytometric analysis (FACS) with anti-CD2 antibodies proved that the lymphocyte samples we used in this study contained ∼95% T cells (39). Contaminations by B cells and monocytes were negligible in this separation procedure by FACS that used anti-CD13, -CD14, -CD16, and -CD17 antibodies. The lymphocyte samples were diluted with RPMI 1640 medium to make solutions containing appropriate numbers of cells (10^4 to 10^6 cells/ml).

RNA isolation and RT-PCR. Total RNA was isolated from lymphocyte samples, MCF-7 human breast cancer cells, and human umbilical vein endothelial cells (HUVEC) by use of TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. MCF-7 cells and HUVEC were used as positive controls for expres-

sion of the three AVP receptors (V_{1a}, V_{1b}, and V_2) and the OT receptor, respectively (23, 37). After treatment with deoxyribonuclease I (Invitrogen) to eliminate possible DNA contamination, the first-strand cDNA synthesis was carried out by use of SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with 2 μg of total RNA and 0.5 μg of oligo(dT). Thereafter, a 1-μl aliquot of the first-strand cDNA was used together with 200 nM of each specific primer, PCR buffer (in mM: 10 Tris·HCl, pH 8.3, 50 KCl, and 1.5 $MgCl_2$), and 1 unit of recombinant *Taq* DNA polymerase (TaKaRa, Kyoto, Japan) to a total volume of 25 μl. The PCR primer sequences for AVP/OT receptors used in this study were exercised according to a previous report by Thibonnier et al. (37), and glyceraldehyde 3-phosphate dehydrogenase was used as a positive control for each RNA preparation. The amplification was performed in a TaKaRa thermal cycler (model TP-400) with the following steps: initial denaturation at 95°C for 5 min, followed by 35 cycles: 95°C for 30 s, 56°C for 1 min, 72°C for 1 min, and an additional extension at 72°C for 5 min. The PCR products were visualized after electrophoresis in a 2.0% agarose gel with ethidium bromide staining.

In vitro incubation of lymphocytes with or without drugs. Lymphocyte samples were incubated in RPMI 1640 medium with various concentrations of dDAVP or AVP for 20 min at 37°C in vitro. After the incubation, supernatants were obtained by centrifugation of samples for 5 min at 300 g and used for further analysis.

Preincubation of lymphocyte samples (10^6 cells/ml) with receptor antagonists was performed for 20 min at 37°C. After the preincubation, samples were further incubated with or without dDAVP (10^{-8} M) for another 20 min at 37°C and then centrifuged at 300 g for 5 min. The supernatant was used for the protease assay.

Assay of fibrinolytic activity. Fibrinogen was labeled with [^{125}I]Na by the chloramine-T method (7) and then used for preparing polystyrene tubes coated with ^{125}I-labeled fibrin (29). ^{125}I-labeled fibrinogen had a specific radioactivity of 1.0 mCi/mg protein.

The lymphocyte samples and RPMI 1640 medium without lymphocytes (as a control) were incubated in ^{125}I-fibrin-coated tubes at 37°C for 20 min in the presence of plasminogen. The released radioactivity (counts/min or cpm) of ^{125}I was counted as described previously (29). The radioactivity increased linearly during the incubation for 1 h.

Measurement of tPA, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 concentrations. The concentrations of tPA, pro-urokinase-type plasminogen activator (pro-uPA), and plasminogen activator inhibitor (PAI)-1 in the supernatant of the samples after in vitro incubation of lymphocytes were measured using the corresponding assay kits, Chromolize tPA Assay Kit, Chromolize uPA Assay Kit, and Imulyse PAI-1, respectively (all from Biopool International, Ventura, CA). The uPA assay was performed using AngioMax Human Urokinase (uPA) ELISA Kit (Angiopharm, O'Fallon, MO). The reaction was carried out at room temperature throughout the assay.

Statistical analysis. Values are presented as means ± SD. Statistical analysis was performed by Student's *t*-test with StatView software (SAS Institute, Cary, NC).

RESULTS

Expression of AVP/OT receptors in human lymphocytes. In the beginning of the study, the mRNA expression of specific AVP/OT receptors in human lymphocytes from peripheral blood was determined by RT-PCR analysis (Fig. 1). Expression of V_{1a}, V_2, and OT receptors was observed in all lymphocytes of four independent donors. On the other hand, we could not detect the expression of the V_{1b} receptor either in human lymphocytes or in MCF-7 cells (data not shown). Two amplified DNA bands were detected from lymphocytes as well as from MCF-7 cells when we analyzed the expression of V_2
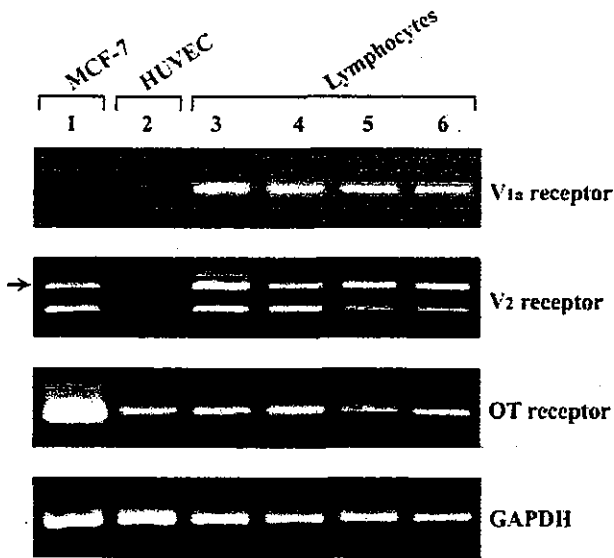
Fig. 1. Expression of arginine vasopressin (AVP) V_{1a} and V_2 and oxytocin (OT) receptors in human lymphocytes. MCF-7 cells (lane 1) and human umbilical vein endothelial cells (HUVEC, lane 2) were used as positive controls for expression of AVP/OT receptor mRNAs. Lanes 3-6, human lymphocytes from 4 independent donors. Arrow, amplified DNA corresponding to an alternative form of V_2 receptor mRNA. GAPDH, positive control for each RNA preparation.

receptor mRNA (Fig. 1). According to a previous report (23), MCF-7 cells express an alternative form of V_2 receptor containing the entire 106 bases of intron 2 in addition to a sequence for V_2 receptor mRNA as well as normal forms. Therefore, it was suggested that both normal and alternative forms of V_2 receptor mRNA are expressed in human lymphocytes.

Effect of dDAVP infusion on fibrinolytic activity. Each lymphocyte sample was prepared up to 40 min after infusion in humans with or without dDAVP. The levels of fibrinolytic activity in the medium of incubated lymphocytes were significantly increased and reached the peak at 20 min after the infusion, followed by a decrease to the basal level (Fig. 2).
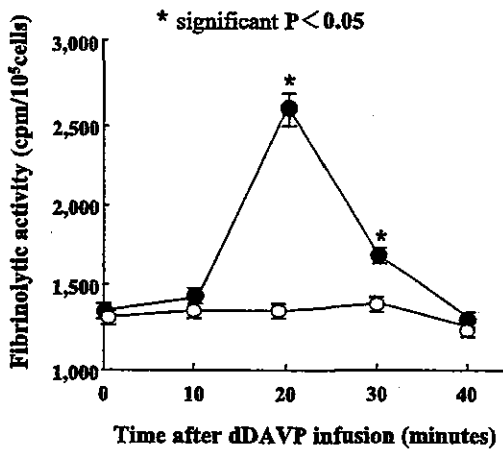
Effect of dDAVP or AVP treatment on fibrinolytic activity in vitro. To investigate the kinds of proteases released in the medium, lymphocyte samples were incubated with dDAVP or AVP. The levels of fibrinolytic activity in the medium were highest at 10^{-8} M of dDAVP, and the dose-response curve appeared to be bell-shaped (Fig. 3A). However, AVP treatment did not result in the increased levels of fibrinolytic activity at any dose examined (Fig. 3B).

Effect of dDAVP on tPA, pro-uPA, and uPA concentrations. We next measured the concentrations of tPA, pro-uPA, and uPA in the medium of lymphocyte samples incubated with dDAVP ($\leq 10^{-6}$ M). There were no significant differences in the tPA and pro-uPA concentrations between dDAVP treatment and control (Fig. 4, A and B). However, a significant increase in the levels of uPA was observed after incubating lymphocytes with dDAVP, showing a bell-shaped pattern with the highest level at 10^{-8} M dDAVP (Fig. 4C).

Effect of dDAVP on PAI-1 concentration. The uPA assay kit used in this study recognizes both PAI-1-free (active) and PAI-1-bound (inactive) uPA. To examine whether uPA was detected as an active form or as an inactive PAI-1-bound form, we measured levels of PAI-1 in the medium after incubating lymphocytes with dDAVP (10^{-8} M). No increase was observed in total levels of PAI-1 concentration up to 120 min

Fig. 2. Fibrinolytic activity in the medium of incubated peripheral blood lymphocytes after [deamino-Cys¹,D-Arg⁸]-vasopressin (dDAVP) infusion (●) and without infusion (○). Values represent the average ± SD of 3 independent experiments.
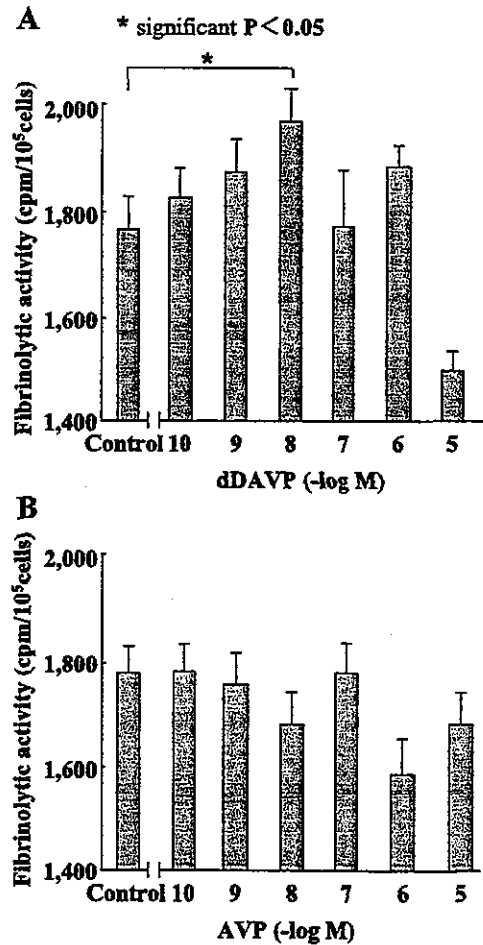

Fig. 3. Fibrinolytic activity in the medium after incubation of lymphocytes with dDAVP (A) and AVP (B). Values represent the average ± SD of 3 independent experiments.
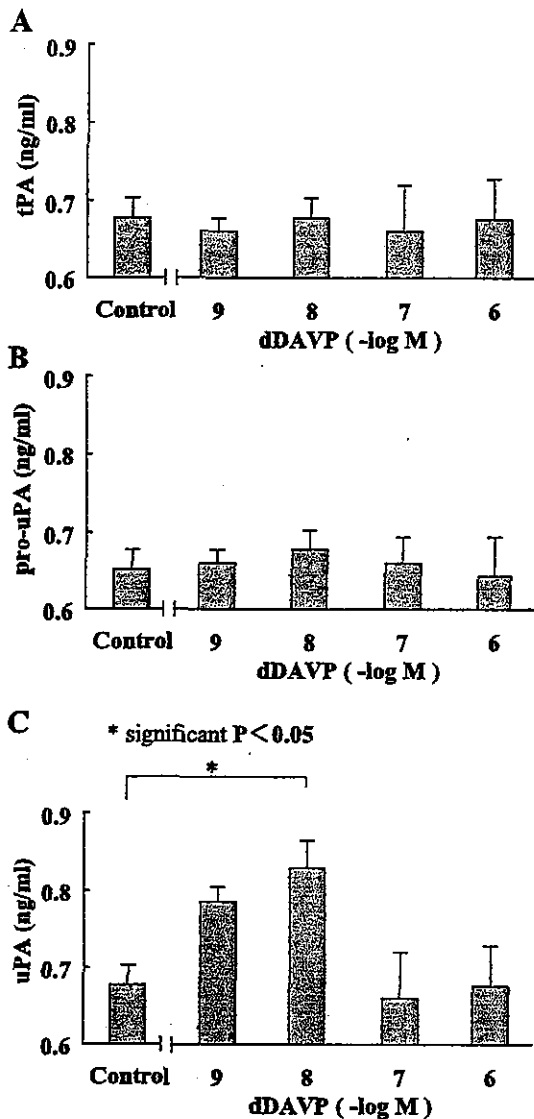
Fig. 4. Dose effect of dDAVP on tissue-type plasminogen activator (tPA, A), pro-urokinase-type plasminogen activator (pro-uPA, B), and uPA (C) concentrations in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.

after the incubation (Fig. 5). We also made sure that the level of fibrinolytic activity in the medium after dDAVP treatment was decreased to the basal level by the addition of PAI-1 (data not shown).
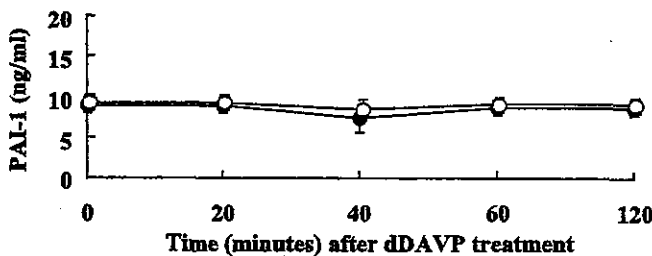

Fig. 5. Plasminogen activator inhibitor (PAI)-1 concentration in the medium after incubation of lymphocytes with (●) and without (○) 10^{-8} M dDAVP. Values represent the average ± SD of 3 independent experiments.
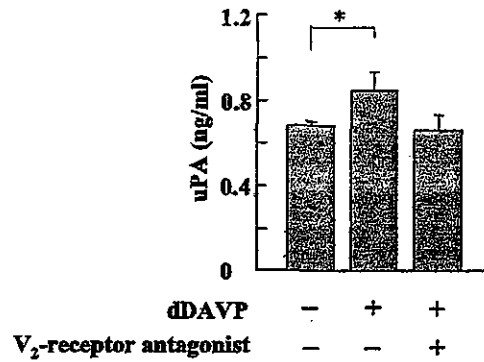

Fig. 6. Effect of the V_2 receptor antagonist on dDAVP-induced uPA increase in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.

Effect of receptor antagonists on dDAVP-induced uPA increase. We then examined whether the uPA increase in the medium was due to receptor-mediated response in lymphocytes. When lymphocytes were preincubated with the 10^{-8} M V_2 receptor antagonist [Adamantaneacetyl[1],O-Et-D-Tyr[2],Val[4], Aminobutyryl[6],Arg[8,9]]-vasopressin, uPA increase after incubating lymphocytes with dDAVP (10^{-8} M) was not detected as it had been without the antagonist (Fig. 6). We made sure that there was no change in uPA concentration after incubation only with the V_2 receptor antagonist. Preincubation with a V_{1a} receptor antagonist, [β-Mercapto-β,β-cyclopentamethylene-propionyl[1],O-Me-Tyr[2],Arg[8]]-vasopressin, resulted in more increased levels of uPA concentration than those by dDAVP treatment alone, although the antagonist by itself did not affect the increase (Fig. 7). However, the uPA increase by the combination of a V_{1a} receptor antagonist and dDAVP was undetectable by the 10^{-7} M V_2 receptor antagonist (Fig. 8).

DISCUSSION

In the present study, we observed increased levels of fibrinolytic activity in the medium after incubating human peripheral
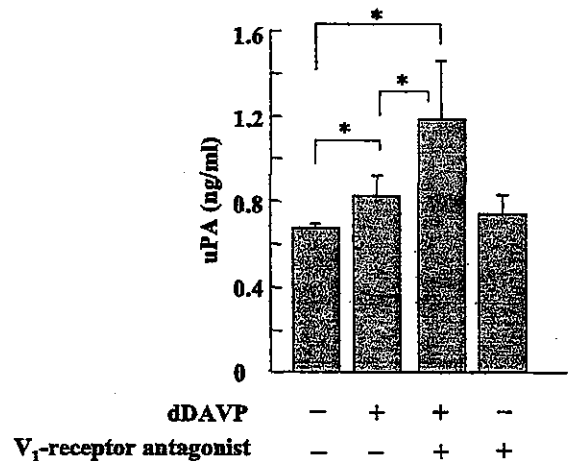

Fig. 7. Effect of the V_1 receptor antagonist on dDAVP-induced uPA increase in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.
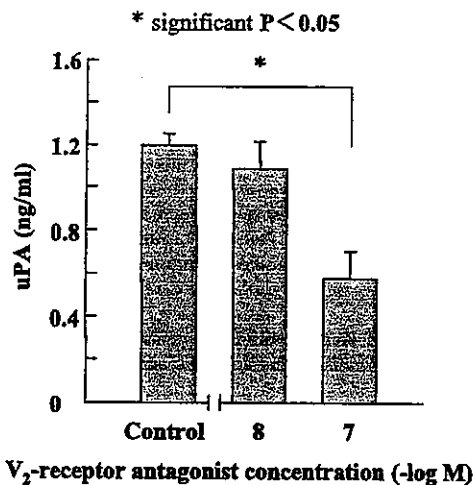
Fig. 8. Effect of the V_2 receptor antagonist on the V_1 antagonist and dDAVP combination-induced increase of uPA concentration in the medium after lymphocyte incubation. Lymphocyte samples were preincubated with the V_2 receptor antagonist (10^{-8} M and 10^{-7} M) for 20 min at 37°C. After preincubation with the V_2 receptor antagonist, samples were incubated with the V_1 receptor antagonist (10^{-8} M) for 20 min and then with dDAVP (10^{-8} M) for another 20 min at 37°C. Values represent the average ± SD of 3 independent experiments.

blood lymphocytes obtained from dDAVP-infused volunteers, possibly due to uPA released from lymphocytes. This uPA induction appeared to be involved in an AVP V_2 receptor-mediated reaction that is expressed in human lymphocytes (Fig. 1).

The levels of fibrinolytic protease activity in the medium were the highest when lymphocytes were incubated with 10^{-8} M dDAVP in vitro. The dDAVP-induced protease activity and increase in the uPA concentration showed similar dose-response curves, bell-shaped (Fig. 3A and Fig. 4C). No increase was observed in the levels of PAI-1 up to 120 min after the incubation (Fig. 5), and the level of fibrinolytic activity in the medium after dDAVP treatment was decreased to the basal level by the addition of PAI-1 (data not shown). In the absence of lymphocytes, the treatment of culture medium with dDAVP did not result in an increase of uPA concentration (data not shown). These findings suggest that the increased levels of the fibrinolytic protease activity correspond at least in part to an increase in uPA release in the medium after incubation of human peripheral blood lymphocytes with dDAVP.

The mechanism by which lymphocytes release uPA after dDAVP treatment remains unclear. We determined the population of receptors in our preparation by PCR and showed that lymphocytes express AVP V_{1a}, V_2, and OT receptors (Fig. 1). Human vascular endothelial cells have been shown to express OT receptors (37), and, in LLC-PK$_1$ renal epithelial cells, the uPA release by OT via V_2 receptor reaction has been reported (8). It could be explained by the sequential activation of various AVP/OT receptors. However, we propose that the V_2 receptor system makes a large contribution to this observed uPA release, because the preincubation of lymphocytes with the V_2 receptor antagonist completely inhibited the dDAVP-induced uPA increase in the medium (Figs. 6 and 8).

By contrast, the V_1 receptor antagonist enhanced the dDAVP-induced increase in uPA (Fig. 7). Some V_1 receptor antagonists demonstrate an agonist effect (21). The agonist

property of the V_1-receptor antagonist used in this study, [β-Mercapto-β,β-cyclopentamethylenepropionyl,O-Me-Tyr^2, Arg^8]-vasopressin, was reported to be involved in the activation of the phosphoinositide-signaling pathway (30). In our experiment, this V_1 receptor antagonist alone showed little effect on the uPA induction, although preincubation of lymphocytes with the antagonist enhanced uPA increase by dDAVP (Fig. 7). Interestingly, in Chinese hamster ovary cells transfected with the V_{1a} and the V_2 receptor cDNAs, the V_2 receptor-induced cAMP accumulation was potentiated by stimulation of the PLC pathway via the V_{1a} receptor (10). If this antagonist acts as an agonist to the V_{1a} receptor, the signal transduction system may lead to the stimulation of the V_2 receptor, resulting in the enhancement of the uPA induction. Another possibility is that the V_1 receptor in human peripheral blood lymphocytes has an inhibitory effect on the V_2 receptor function. In the presence of the V_1 receptor antagonist, this inhibition may have been cleared, so that the V_2 receptor fully functioned to induce the uPA increase by dDAVP.

The uPA increase under the combination of a V_1 receptor antagonist and dDAVP was undetectable after V_2 receptor antagonist preincubation at the highest dose (10^{-7} M) (Fig. 8). This result may suggest that the enhanced uPA induction is also a V_2 receptor-mediated reaction. Although the exact mechanisms of the enhancement and its inhibition remain unclear, the V_1 receptor may be involved in the V_2 receptor-mediated uPA induction.

In our study, AVP alone did not increase the levels of fibrinolytic activity at any dose examined (Fig. 3B). We also examined the effect of the V_1 receptor antagonist on uPA releasing activity by AVP, because we apprehended the possibility that the combination of the V_1 receptor antagonist and AVP might be able to increase the fibrinolytic activity. However, there was no difference in uPA concentration among lymphocytes treated only with AVP, those pretreated with the V_1 receptor antagonist, and control lymphocytes (data not shown). From this result, we might speculate that dDAVP has its own V_2-like receptor that is inhibited by the V_2 receptor antagonist and that pretreatment of the V_1 receptor antagonist helps dDAVP to bind its receptor more efficiently by occupying the neighboring V_1 receptors on lymphocytes. We showed two amplified DNA bands in analyzing the expression of V_2 receptor mRNA (Fig. 1). The expression of an alternative form of V_2 receptor mRNA in human peripheral lymphocytes might be the explanation for this receptor mechanism. RT-PCR analysis also showed that OT receptors are expressed in human lymphocytes (Fig. 1). Most of the V_{1a} receptor antagonists, including the one that we used in this study, have high affinity with OT receptors as well as with V_{1a} receptors. Although the affinity is higher for V_{1a} receptors, we could not completely exclude the possibility of cross-talk between V_2 and OT receptors. Very recently, a highly specific OT receptor antagonist, FE 200 440 (Ferring), was developed with an affinity for human cloned OT receptors that was ~300-fold that for V_{1a} receptors, whereas other OT receptor antagonists bind well to both receptors (22). When this newly developed antagonist is made available, we will be able to perform further experiments that should help to more fully explain the mechanism underlying uPA release through the AVP/OT receptor function in human lymphocytes.

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To our knowledge, this is the first study to report that the levels of uPA increase in the medium after incubation of lymphocytes with dDAVP. uPA is an extracellular serine endoprotease with a multimodular structure; it has been critically involved in various biological activities, such as tissue remodeling and cell migration (14). The activities trigger a protease cascade, including digestion of the extracellular matrix and activation of latent growth factors, such as transforming growth factor-β and pro-hepatocyte growth factor (20, 27). Although the protease cascade is intimately associated with inflammation and tissue repair, little is known regarding the impact of lymphocytes on these processes. In this regard, it is interesting to note that human peripheral blood lymphocytes can produce uPA. Under normal physiological conditions, the level of plasma AVP concentration is much lower than that of the dDAVP we treated in our study. So, in normal conditions, the release of uPA from lymphocytes may not occur in humans. However, we emphasize the potential significance of lymphocytes releasing uPA under special conditions, such as inflammation, perhaps leading to the increasing sensitivities of the receptors on the activated T cells or the elevated concentration of vasopressin in tissues. Increased plasma concentrations and hypothalamic content and release of AVP were reported in inflammatory disease-prone Lewis rats (24, 25).

uPA released from human peripheral blood lymphocytes might be ubiquitous at the sites of inflammation or tissue repair; therefore, lymphocytes may promote tissue remodeling and angiogenesis. uPA, as an element of the fibrinolytic cascade, also takes part in regulating cell-mediated immunity in cardiac allograft acceptor mice. Histological analysis revealed that accepted cardiac allografts express uPA in mononuclear cells (1). In human renal allograft transplantation, there has been no study reporting the role of uPA on graft acceptance. If a difference in the activity of uPA release from lymphocytes exists between the renal transplant patients and normal subjects, it would be useful to know the pathophysiology of graft acceptance. Such kinds of studies are currently in progress, and the results will be reported elsewhere.

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CLINICAL NEPHROLOGY – EPIDEMIOLOGY – CLINICAL TRIALS

Oxidative stress is enhanced in correlation with renal dysfunction: Examination with the redox state of albumin

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Oxidative stress is enhanced in correlation with renal dysfunction: Examination with the redox state of albumin.

Background. Cardiovascular disease is known to be the most important complication among patients with renal failure, and oxidative stress has been proposed to play a major role as the source of such complications. Human serum albumin (HSA) is composed of human mercaptoalbumin (HMA) with cysteine residues having reducing powers, of reversibly oxidized human non-mercaptoalbumin-1 (HNA-1), and strongly oxidized human non-mercaptoalbumin-2 (HNA-2).

Methods. We used the "redox state of HSA" as a marker to investigate the current status of oxidative stress in predialysis patients with renal failure. The subjects were 55 nondialysis patients (31 males and 24 females) with chronic renal diseases, and having various degrees of renal function. The subjects' redox state of HSA was determined by a high-performance liquid chromatographic (HPLC) procedure, and the results presented in terms of the ratios between HNA-total(HNA-1 + HNA-2) and HNA-2.

Results. The values for each fraction of HNA-total (f(HNA-total)) and f(HNA-2) were increased with a decrease of renal functions, and a significant positive correlation with serum creatinine ($R = 0.529$, $P < 0.0001$ and $R = 0.618$, $P < 0.0001$) was detected. Multiple (forward stepwise) regression analysis using f(HNA-total) and f(HNA-2) as the criterion variables was performed, and creatinine was adopted as significant explanatory variable in both equations.

Conclusion. We found that even before dialysis, oxidative stress was enhanced in correlation with the level of renal dysfunction among patients with chronic renal failure. In the future, antioxidant strategies should become part of treatment for predialysis renal failure.

Cardiovascular disease (CVD) is the most important complication for patients with end-stage renal disease (ESRD). The mortality rate associated with CVD is extremely high, accounting for approximately 9% per year (approximately 45% of total death) among ESRD patients that undergo dialysis, and it is the primary cause of mortality. What characterizes the death of ESRD patients is the fact that mortality rate hardly seems to be reflected by age [1], unlike patients without renal failure.

While risks associated with CVD among ESRD patients who reach dialysis are widely acknowledged, those involving patients with renal failure before dialysis are in the process of being properly identified, although not well established yet. When compared with subjects with normal renal function, the frequency of CVD has been reported to be higher not only among dialysis patients but also among predialysis patients with renal failure [2], suggesting that factors promoting complications of the cardiovascular system may already exist in predialysis patients with renal failure.

Oxidative stress has recently been proposed to play a major role in the development of CVD among renal failure patients [3]. In the past, backgrounds similar to those of patients without renal failure, such as high blood pressure and atherosclerosis, have been postulated, but several reports have made it difficult to conceive these as major causes [2]. To date, increases in blood concentrations of reactants resulting from oxidative stress have mainly been reported among dialysis patients [4–7]. Further, other studies have reported that while administration of antioxidant substances, such as vitamin E and acetylcysteine, reduce cardiovascular system events among dialysis patients [8, 9], vitamin E did not exhibit apparent effects among patients without renal failure [10]. From these findings, that oxidative stress is, at least in part, involved in CVD among chronic renal failure patients, seems virtually unquestionable.

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総説　腎移植シリーズ

腎移植と移植臓器の動脈硬化

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はじめに

　近年の移植医療では，新規の免疫抑制剤の登場，移植医療技術の進歩や患者ケアの向上に伴い，急性拒絶反応は克服できるようになり，予後改善が目覚ましいものとなった。しかし反面，感染症発症の質的な変化と同時に，長期生着に伴う慢性移植腎障害が患者 QOL 向上を阻む課題としてクローズアップされてきている。国民生活様式の欧米化に伴い生活習慣病に関連した病態がさらに加味され，慢性期の移植腎障害を複雑にしている。欧米の主たる臓器移植である腎，心，肝移植での late graft loss の頻度は年 3～5 ％に達する[1~3]。本 review では大別して，臓器移植に伴う免疫学的機序，特に慢性拒絶反応に伴う動脈硬化と，非免疫学的機序の関与について概説する。理解を容易にするために，ヒト臓器移植，特に心移植慢性期における慢性拒絶による冠動脈硬化の病態と，加齢に伴う動脈硬化や動脈形成後再狭窄の病態との比較を提示する(表)(鈴木らによる報告[4])。

慢性拒絶反応と動脈硬化

　ここで言う「慢性拒絶反応」とは，純粋に host-anti-graft immune response による動脈や細動脈狭窄の原因となる慢性血管性拒絶反応(chronic vascular rejection)が主体となる。chronic vascular rejection の病像の実体とはどんなことだろうか。まず第一に，血管内腔の狭窄・制限が血管内膜肥厚に基因するとされる。血管内膜肥厚には血管平滑筋細胞の形質変換を伴う遊走と，増殖および細胞外基質の増生，一部白血球の遊走を認める。さらに近年の血管内視鏡による chronic vascular rejection の研究によると，adventitial scarring による動脈の拘縮(con-

表　動脈硬化の比較

	移植後冠動脈硬化	動脈形成後再狭窄	粥状冠動脈硬化
部位	びまん性，末梢性	部分的	部分的，中枢性
組織像	求心性内膜肥厚	求心性内膜肥厚	偏心性
石灰化	認められない。	認められない。	しばしば認める。
内弾性板	無変化	しばしば破裂	しばしば破裂
細胞浸潤	ときに認める。	ときに認める。	認められない。
進展速度	月単位	月単位	年単位
危険因子	不明	不明	高血圧症，糖尿病など
治療	再移植	PTCA，CABG	PTCA，CABG

（文献 4 より引用）

strictive　remodeling)も認められ，Tリンパ球やMφ(マクロファージ)も周囲に観察され，これらの細胞からの chemical mediator による局所傷害と細胞外基質増生が着目されている[5,6]。この constrictive remodeling は動脈硬化やバルーン再狭窄でも認められる。さらにこれらのことと同時に，血管は血管内皮依存性血管拡張障害 (vasoconstriction)を起こす。特に急性拒絶反応の既往はその傷害を強くする。この一連の反応がび漫性に求心性内膜肥厚を生じさせると同時に，細動脈から細動脈枝にまで末梢性に及ぶことが観察される(表)。

Chronic vascular rejection の免疫学的機序

　まず，この機序には三つの重要な役者の関与が認められる。① 抗原活性化された CD4＋cytokine 産生 "helper" T cells, ② 活性化 CD8＋ "cytolytic" T cells(CTL), ③ B リンパ球による抗体産生，である。活性化 CD4＋T 細胞は，CD8＋CTL の代謝の調節，IL-2 分泌作用を通じ直接的に，また CTL の分化調節のためにいわゆる dendritic　cell(Mφ)を活性化する。また，抗体産生 B　cell の分化と活性を調節，さらに抗原非依存性に Mφを活性化し，活性酸素種，NO, degradative engymes の分泌をもたらす。これら一連の反応は臓器組織移植の場合，DTH(delayed-type hypersensitivity)と考えられる。慢性のまた反復する活性化 CD4＋T 細胞は，DTH 誘発のサイトカイン，IFNγ, リンフォトキシンを，また eosinophil 活性化サイトカイン(IL-4, IL-5, IL-13), さらに免疫抑制性サイトカイン IL-10 や TGFβ の産生分泌を生じ，T リンパ球の subset の分化・産生，それぞれ TH1 cell, TH2 cell および TH3(または T regulatory(TR)) cell への分化・産生をきたす[7]。これらが相互にリンパ球活性化を調節し合っている。一方，CTL や抗体が慢性拒絶自体を直接的に誘発しているという証拠は現在のところまだないが，subclinical な進展性の acute smoldering rejection が慢性血管拒絶に関与している可能性は十分にある。

Chronic vascular rejection と非免疫学的機序の関与

　移植周術期の ischemia/reperfusion による傷害，hypoxia などは，同様の proinflammatory response を誘発する。また，免疫抑制剤に関連した高血圧(シクロスポリンによる交感神経系亢進)，さらに易感染性，特に CMV 感染と移植動脈硬化との関連性の報告がある[8]。免疫抑制剤に関連した dyslipidemia や糖尿病の合併も，血管障害を増長させる大きな要因になると思われる。さらに近年，生活習慣病に関連した metabolic syndrome に表現される因子が加味され，これらが移植患者の動脈硬化促進因子となり death with functioning graft などを誘発し，患者 QOL の低下につながっている可能性は否定できない。

　Chronic vascular rejection についての想定される機序について図に示す。移植臓器の血管内皮細胞傷害が，免疫学的な攻撃(acute rejection など)による傷害を基礎に，ischemia/reperfusion による傷害，dyslipidemia, 高血圧，感染，IGT や糖尿病を含めた糖代謝異常による傷害が加味・装飾される。傷害された血管内皮細胞は，生理活性物質やサイトカインを活性化し，血管平滑筋細胞の形成変換，Mφも含めた分化・増殖を通じ動脈硬化形成に加担する。この場合，chronic vascular rejection は acute vascular rejection やその他の因子の慢性的，かつ smoldering な作用の結果と位置づけられるかもしれない。さらにその血管内皮細胞傷害は補体系を活性化し，Mφ(IL-12 分泌も含め)も活性化する。これらは TH1 cell システムの分化を誘発し，IFNγ, さらに TNFα, リンフォトキシンなどの分泌を促し，これらが viscious cycle となってさらに活性化する。このこと(TH1 predominant effect)は chronic DTH の病態であり，chronic vascular rejection(arteriosclerosis)が形成されると想定される。最近興味あることは，Mφのみならず活性化 T リンパ球自体による，HLA class II 依存性の血管平滑筋細胞
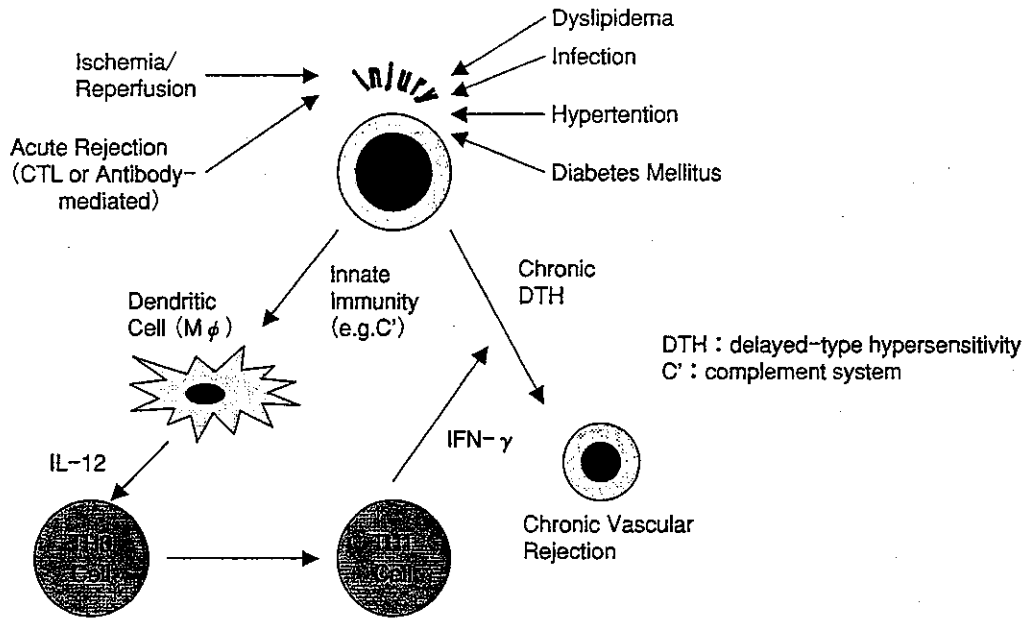
図 Chronic vascular rejection と免疫調節モデル

増殖の調節機序の存在[9]，また同じく活性酸素種の分泌，さらに蛋白分解酵素(セリンプロテアーゼであるウロキナーゼ)などの分泌も認められた[10]。chronic vascular rejection 形成には純粋な immunological response に non-immunological response の二重三重の反応機構が関与していることが明らかになり，今後の研究解明が期待される。

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Original Article

Candesartan reduced advanced glycation end-products accumulation and diminished nitro-oxidative stress in type 2 diabetic KK/Ta mice

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Abstract

Background. Angiotensin-II induces nitro-oxidative stress in patients with diabetic nephropathy. Peroxynitrite and reactive oxide species can accelerate formation of advanced glycation end-products (AGEs). We investigated the effects of candesartan, an angiotensin-II type 1 receptor blocker (ARB), on the formation of AGEs and nitro-oxidative stress in type 2 diabetic KK/Ta mouse kidneys.

Methods. KK/Ta mice were divided into three treatment groups: an early treatment group receiving 4 mg/kg/day candesartan from 6 to 28 weeks of age, a late treatment group receiving the same candesartan dose from 12 to 28 weeks of age and a group receiving the vehicle for candesartan. BALB/c mice treated with vehicle were used as controls. We evaluated at 28 weeks the renal expressions of carboxymethyllysine, the receptor for AGE (RAGE), the p47phox component of NADPH oxidase, endothelial nitric oxide synthase (eNOS), induced nitric oxide synthase (iNOS) and 8-OHdG and nitrotyrosine by immunohistochemistry and/or by competitive RT–PCR.

Results. Kidneys from KK/Ta mice showed increased formation of AGEs, nitro-oxidative stress and RAGE expression and these were attenuated by candesartan treatment. Protein and mRNA expressions of p47phox and iNOS were upregulated in KK/Ta kidneys, which also showed increased immunostaining intensities of 8-OHdG and nitrotyrosine. Treatment with candesartan attenuated all of these changes and prevented significant albuminuria. There were no significant differences in the expression of eNOS among the four groups.

Conclusions. These findings suggest that candesartan, an ARB, reduces AGE accumulation and subsequent albuminuria by down-regulating the NADPH oxidase p47phox component and iNOS expression and by attenuating RAGE expression in type 2 diabetic KK/Ta mouse kidneys.

Keywords: advanced glycation end-products; angiotensin-II-receptor antagonist; KK/Ta mouse; nitro-oxidative stress; NADPH oxidase p47phox; RAGE

Introduction

Increasing evidence indicates that advanced glycation end-products (AGEs) contribute to the pathogenesis of diabetic nephropathy. AGEs exert chemical, cellular and tissue effects through the formation of protein cross-links that alter the structure and function of extracellular matrix or that interact with specific receptors [1]. The best-characterized receptor for AGE, designated as RAGE (receptor for AGE), is a multi-ligand member of the immunoglobulin superfamily [1]. The binding of RAGE by AGEs activates several intracellular signalling pathways, including mitogen-activated protein kinase (MAPK), NF-κB and AP-1, and increases the production of cytokines, including TGF-β, VEGF and TNF-α [2]. Unless interrupted, this cascade of events leads to albuminuria and mesangial expansion and results in glomerular sclerosis. The AGE–RAGE pathway, therefore, represents a candidate molecular target for prevention and treatment of diabetic nephropathy.

Oxidative and nitrosative stresses are widely recognized as key factors in the development of diabetic nephropathy [3]. AGEs have long been associated with increased oxidative and nitrosative stresses in both *in vitro* [4] and *in vivo* [5] studies. Carboxymethyllysine (CML) is formed from sequential glycation and oxidation reactions (so-called glycoxidation).

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Alternatively, interactions between AGEs and RAGE induce cellular oxidative stress and upregulate renal inducible nitric oxide synthase (iNOS) expression [4,6]. Thus, the crosstalk between nitro-oxidative stress and the AGE–RAGE pathway establishes an amplification loop leading to further production of free radicals and AGEs.

Angiotensin-II-receptor blockers (ARB) and angiotensin-converting enzyme inhibitors (ACEI) have been reported to reduce the *in vitro* formation of AGEs [7]. A recent study demonstrated that the ARB olmesartan significantly reduced kidney pentosidine content in spontaneously hypertensive/ NIH-corpulent rats [8]. Although this study did not explore the mechanism of AGE-lowering by ARB, the drug may have caused decreased oxidative stress. An additional study recently showed that ACEI reduced the accumulation of AGEs and nitrotyrosine in experimental diabetic nephropathy [9].

The inbred KK/Ta mouse strain, established in Japan, is a diabetic strain that spontaneously exhibits type 2 diabetes associated with fasting hyperglyacemia, glucose intolerance, hyperinsulinaemia, mild obesity, dyslipidaemia and albuminuria [10]. Renal lesions in KK/Ta mice closely resemble those in human diabetic nephropathy [11]. Glomeruli from diabetic KK/Ta mice show diffuse-type and/or nodular-type hyperplasia of mesangial areas with mesangial cell proliferation. Immunohistological studies show an intense, specific fluorescence for albumin and γ-globulin along the glomerular capillary walls. As in human disease, albumin excretion in diabetic KK/Ta mice shows an increase at several weeks after the establishment of hyperglycaemia and this presumably represents intact leaking nephrons without overt reduction in the single nephron glomerular filtration rate [12]. Therefore, KK/Ta mice are considered to provide a suitable model for type 2 diabetes and the early phase of diabetic nephropathy in humans. Recently, we carried out a genome-wide linkage analysis of KK/Ta alleles and identified a susceptible KK/Ta locus (*UA-1*) responsible for the development of albuminuria on chromosome 2 [13,14]. In the present study, we focused on the interaction between the AGE–RAGE pathway and local nitro-oxidative stress in kidneys of the KK/Ta mouse in order to elucidate the *in vivo* AGE-lowering mechanism of ARB.

Subjects and methods

Animals and drug treatment

Malne diabetic KK/Ta and non-diabetic BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). The BALB/c mouse was found to be an appropriate control for the KK/Ta mouse in our previous study [13]. The mice were individually housed in plastic cages and were given free access to food (rodent pellet diet CE-2; 342.2 kcal/100 g containing 4.4% crude fat) and water or water treated with candesartan throughout the experimental periods. Fluid intake was measured every day. The dosages of candesartan were adjusted via the drinking water.

KK/Ta mice were divided into three groups as follows: (a) no treatment ($n = 6$), (b) early candesartan treatment at 4 mg/kg/day in drinking water ($n = 6$) given from 6 to 28 weeks and (c) late candesartan treatment at 4 mg/kg/day ($n = 6$) given from 12 to 28 weeks. Control BALB/c mice were followed concurrently. The dose of candesartan was selected on the basis of previous studies that showed a significant renoprotective effect [12]. KK/Ta and BALB/c mice were sacrificed at 28 weeks of age. Kidneys were removed and prepared for immunohistochemical examination and RNA extraction.

Clinical characteristics of animals

Body weight (BW), fasting glucose, systolic blood pressure (SBP), serum creatinine concentration and urinary albumin excretion of each mouse were serially monitored every 4 weeks. Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT). IPGTT was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the retro-orbital sinus were measured using Glutest E (Kyoto Daiichi Kagaku, Kyoto, Japan) at 0 (fasting blood glucose level) and 120 min after glucose injection. SBP was measured by a non-invasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan). Serum creatinine concentrations were enzymatically determined by an autoanalyser (Fuji Dry-Chem 5500; Fuji Film, Tokyo, Japan). The concentrations of urinary albumin were examined by Exocell's immunospecific enyzme-linked immunosorbent assay (Albuwell M kit; Exocell Inc., Philadelphia, PA, USA) and all samples were individually adjusted for creatinine excretion (Creatinine Companion; Exocell Inc., Philadelphia, PA, USA).

Immunohistochemistry

Renal tissues were snap-frozen in optimum cutting temperature compound and cut into 3-μm-thick sections. Immunohistochemical studies were performed with the following commercially available antibodies: peroxidase-conjugated anti-CML monoclonal antibody (Transgenic, Kumamoto, Japan), anti-RAGE goat polyclonal antibody (Chemicon, Temecula, CA, USA), anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnologies, Lake Placid, NY, USA), anti-8-OHdG mouse monoclonal antibody (NOF Corp., Tokyo, Japan), anti-NADPH oxidase p47phox rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-iNOS and endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibodies (BD Transduction Laboratories, Lexington, KY, USA).

Frozen cryostat sections were fixed in acetone for 10 min and air dried. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide/methanol for 15 min. The sections were then blocked by blocking solution (2% fetal bovine serum, 0.2% fish gelatin and 10% normal serum in phosphate-buffered saline). After incubation with primary antibodies at 4°C overnight, the sections were incubated with anti-rabbit or anti-mouse Envision+ polymer reagents (DAKO, Carpinteria, CA, USA), or anti-goat polymer reagents (Histofine, Tokyo, Japan), at room temperature for 30 min. Bound antibody was visualized by light microscopy with diaminobenzidine. The omission of primary antibodies

served as negative controls for each antibody in this study. Quantification of immunostaining for 8-OHdG in glomeruli was calculated by a modification of the method described by Toyokuni *et al.* [15].

For immunofluorescent staining, the sections were incubated with anti-CML or anti-nitrotyrosine antibody overnight using the same methods as described above. For CML immunostaining, the sections were exposed to Alexa Fluor 488 tyramide (Molecular Probes, Eugene, OR, USA) for 30 min according to the · manufacturer's instructions. For nitrotyrosine immunostaining, the sections were exposed to Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR, USA) at room temperature for 2 h. The omission of primary antibodies served as negative controls for each antibody in this study. Immunofluorescent images were viewed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) equipped with a cooled (−25°C) charge-coupled device camera (PXL; Photometrics Ltd, Tucson, AZ, USA). Fluorescence intensities in 10 glomeruli from each mouse were analysed with IPLab Spectrum v. 3.0 software (Signal Analytics, Vienna, VA, USA).

Competitive RT–PCR

Kidneys were dissected and snap frozen in liquid nitrogen for total RNA extraction. RNA was extracted with Trizol (Total RNA Isolation Reagent; Life Technologies, Rockville, MD, USA) and the purity was checked by spectrophotometry and agarose gel electrophoresis. Competitive reverse transcription–polymerase chain reaction (RT–PCR) was carried out using the Quantum RNA kit according to manufacturer's instructions (Ambion Inc., Austin, TX, USA). Briefly, 1 μg total RNA was reverse transcribed with Superscript II RNase H–reverse transcriptase (Life Technologies, Rockville, MD, USA) using random hexamers as downstream primers. The obtained cDNA was further amplified by PCR. The sequences of the primers used for the amplifications were as follows:

RAGE: (sense) 5′-CAG GGT CAC AGA AAC CGG-3′
         (antisense) 5′-ATT CAG CTC TGC ACG TTC CT-3′

NADPH oxidase p47phox: (sense) 5′-TCC TGG TTA AGT GGC AGG AC-3′
         (antisense) 5′-CCA TGA GGC CGT TGA AGT AT-3′

eNOS: (sense) 5′-GAC CCT CAC CGC TAC AAC AT-3′
         (antisense) 5′-CTG GCC TTC TGC TCA TTT TC-3′

iNOS: (sense) 5′-GAG GGA AGG AGG TCA AGT CC-3′
         (antisense) 5′-AAG GTA GGA TGG GTG GTG TT CC-3′

The PCR parameters were 30 s denaturation at 95°C, 30 or 90 s annealing at 52–60°C and 60 or 90 s extension at 72°C for 33–35 cycles. A mixed ratio of 18S ribosomal RNA primers and competimers (usually primer:competimer ratios from 1:9 to 2:8, depending on the genes) was used to amplify rRNA as an internal control under the same conditions as the genes of interest. The PCR products were resolved on 2% agarose gel, stained with ethidium bromide and analysed by the VersaDoc™ Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by analysis of variance using Stat View 4.0. Differences with P-values of <0.05 were considered statistically significant.

Results

Candesartan reduced elevations in urinary albumin excretion

In untreated KK/Ta mice, urinary albumen excretion was markedly higher than in BALB/c mice at 28 weeks of age. Candesartan significantly reduced albuminuria in KK/Ta mice. There were no significant differences in urinary albumin excretion between the two candesartan groups (Figure 1).

Mean BW and fasting as well as 120 min blood glucose levels during IPGTT in KK/Ta mice were significantly higher than in age-matched BALB/c mice. Candesartan treatment did not affect BW or blood glucose levels. SBP in both the early and late treatment groups was markedly decreased ($P < 0.05$). Mean levels of serum creatinine were not significantly different among the four groups at 28 weeks of age (Table 1).

Candesartan reduced CML accumulation and RAGE expression

Immunofluorescent staining revealed that 28-week-old KK/Ta mice had enhanced accumulation of CML, especially in the glomerular mesangium. Candesartan treatment markedly reduced this accumulation, but there were no differences between the early and late treatment groups (Figure 2).

Co-localization experiments using anti-synaptopodin immunoglobulin G revealed that RAGE expression was increased in the glomerular podocytes of KK/Ta mice compared with BALB/c mice (data not shown). RAGE expression was not found in other glomerular
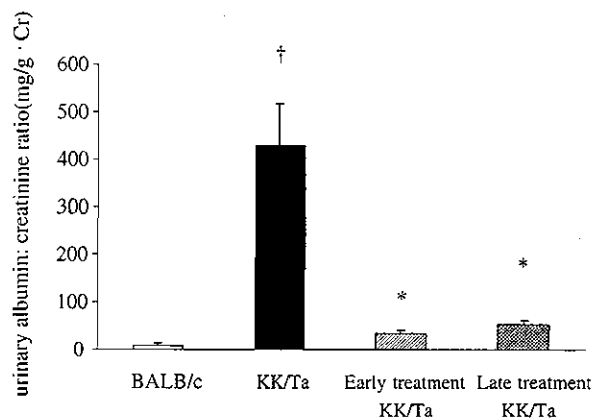

Fig. 1. Urinary albumin excretion at 28 weeks of age. Each bar represents means ± SEM from six mice in each group. $^{†}P < 0.005$ vs BALB/c; $^{*}P < 0.005$ vs KK/Ta without treatment.

Table 1. Biochemical and metabolic parameters at 28 weeks of age

	BALB/c (n = 6)	KK/Ta (n = 6)	Early treated KK/Ta (n = 6)	Late treated KK/Ta (n = 6)
Body weight (g)	31.0 ± 0.4	43.2 ± 1.1[a]	44.6 ± 0.9[a]	43.6 ± 0.8[a]
Fasting blood glucose levels (mg/dl)	77.7 ± 2.6	92.0 ± 9.2[b]	107.7 ± 3.3[b]	104.5 ± 6.4[b]
Blood glucose levels at 120 min (mg/dl)	124.7 ± 11.1	390.5 ± 61.1[c]	417.8 ± 30.2[c]	353.6 ± 43.2[c]
Mean blood pressure (mmHg)	91.6 ± 2.3	90.8 ± 3.1	83.1 ± 1.8[d]	84.7 ± 1.8[d]
Serum creatinine (mg/dl)	0.48 ± 0.06	0.69 ± 0.11	0.57 ± 0.09	0.55 ± 0.16

Data are expressed as means ± SEM.
Glucose levels were measured at 0 and 120 min after intraperitoneal glucose injection.
[a]$P < 0.0001$; [b]$P < 0.05$; [c]$P < 0.001$ vs BALB/c; [d]$P < 0.05$ vs KK/Ta and BALB/c.
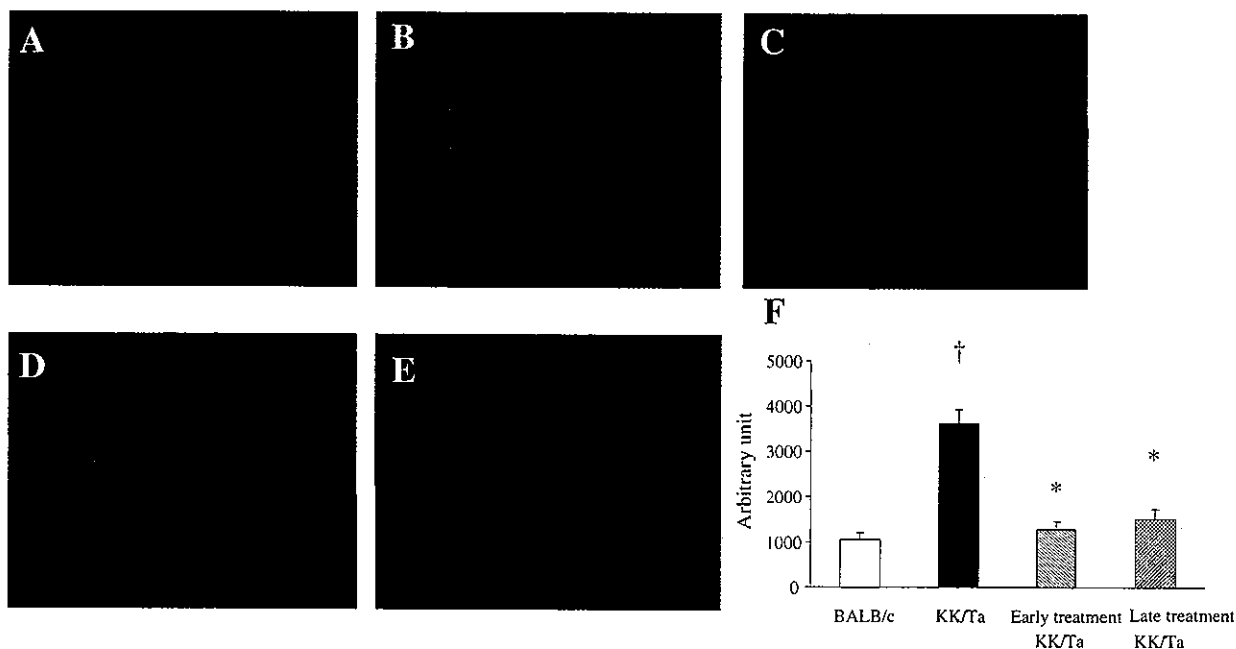

Fig. 2. Representative immunofluorescence staining for CML at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) Analysis of intensity in glomerular immunofluorescence. Each bar represents means ± SEM from six mice in each group and 10 glomeruli for each mouse. [†]$P < 0.005$ vs BALB/c; *$P < 0.005$ vs KK/Ta.

intrinsic cells. In mice treated with candesartan, only podocytes with glomeruli showed weak RAGE expression (Figure 3A–E).

Competitive RT–PCR demonstrated significant overexpression of RAGE in KK/Ta mouse kidneys. Candesartan markedly suppressed this overexpression to produce levels similar to those in BALB/c control mice (Figure 3F).

Candesartan treatment attenuated nitro-oxidative stress

To explore effects of candesartan on nitro-oxidative stress, expressions of nitrotyrosine and 8-OHdG were evaluated by immunohistochemistry. 8-OHdG, a marker of oxidative DNA damage, was overexpressed in the nuclear region of glomerular and tubular cells in KK/Ta mice (Figure 4). An accumulation of nitrotyrosine, an index of the nitrosylation of protein

by peroxynitrite and/or superoxide, was found in the glomeruli and particularly within the mesangium of KK/Ta mice (Figure 5). Candesartan treatment significantly decreased the accumulation of both 8-OHdG and nitrotyrosine (Figures 4 and 5).

Candesartan treatment attenuated the enhanced expression of NADPH oxidase p47phox and iNOS, but not eNOS

The p47phox cytosolic component of NADPH oxidase was expressed in podocytes, mesangial cells and in the basolateral membrane of tubules in BALB/c mice. Its expression at these sites was elevated in KK/Ta mice compared with BALB/c mice and these elevations were attenuated by candesartan treatment. Candesartan also caused a consistent attenuation of p47phox mRNA expression (Figure 6).
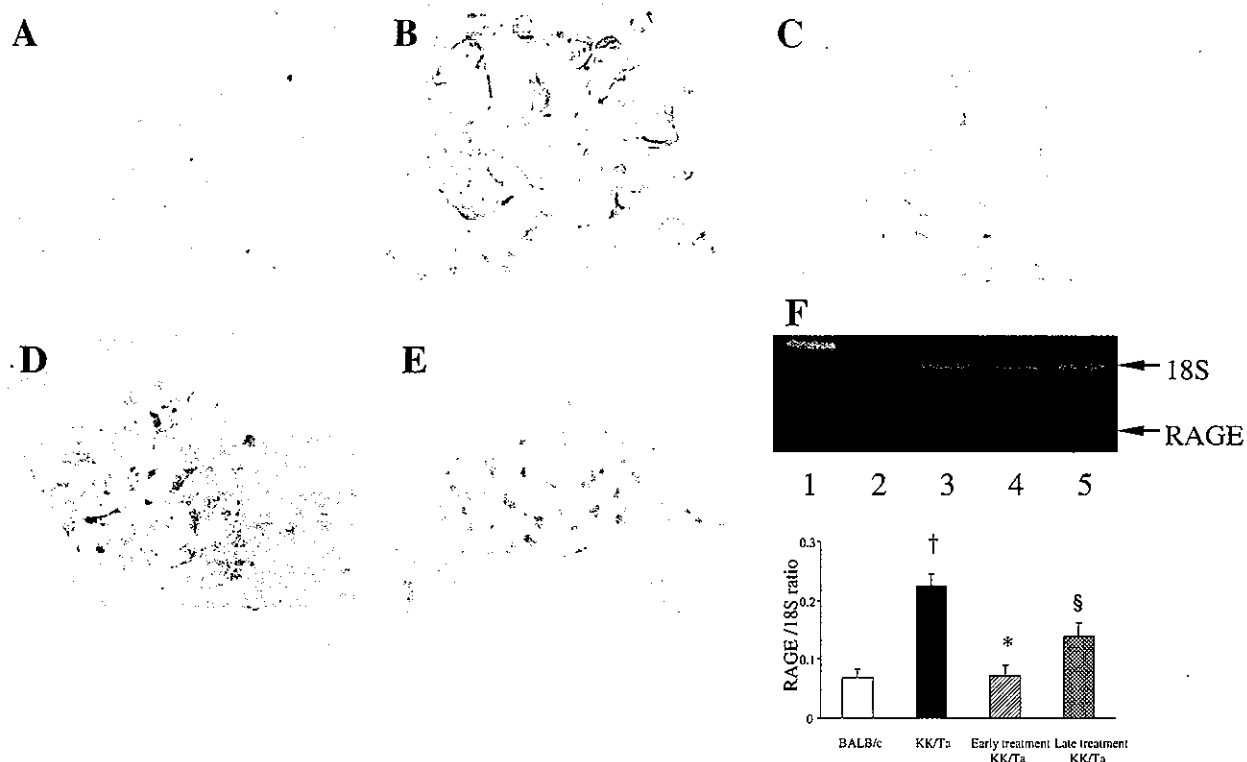
Fig. 3. Representative immunohistochemical staining and competitive RT–PCR for RAGE at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) The upper bands are 18S competitors (489 bp) and the lower bands are RAGE (246 bp). Lane 1, 100 bp ladder marker; lane 2, BALB/c; lane 3, KK/Ta; lane 4, early treatment KK/Ta; lane 5, late treatment KK/Ta. Each bar represents means ± SEM from six mice in each group. $^{\dagger}P < 0.005$ *vs* BALB/c; $*P < 0.005$ *vs* KK/Ta; $^{\S}P < 0.05$ *vs* KK/Ta.
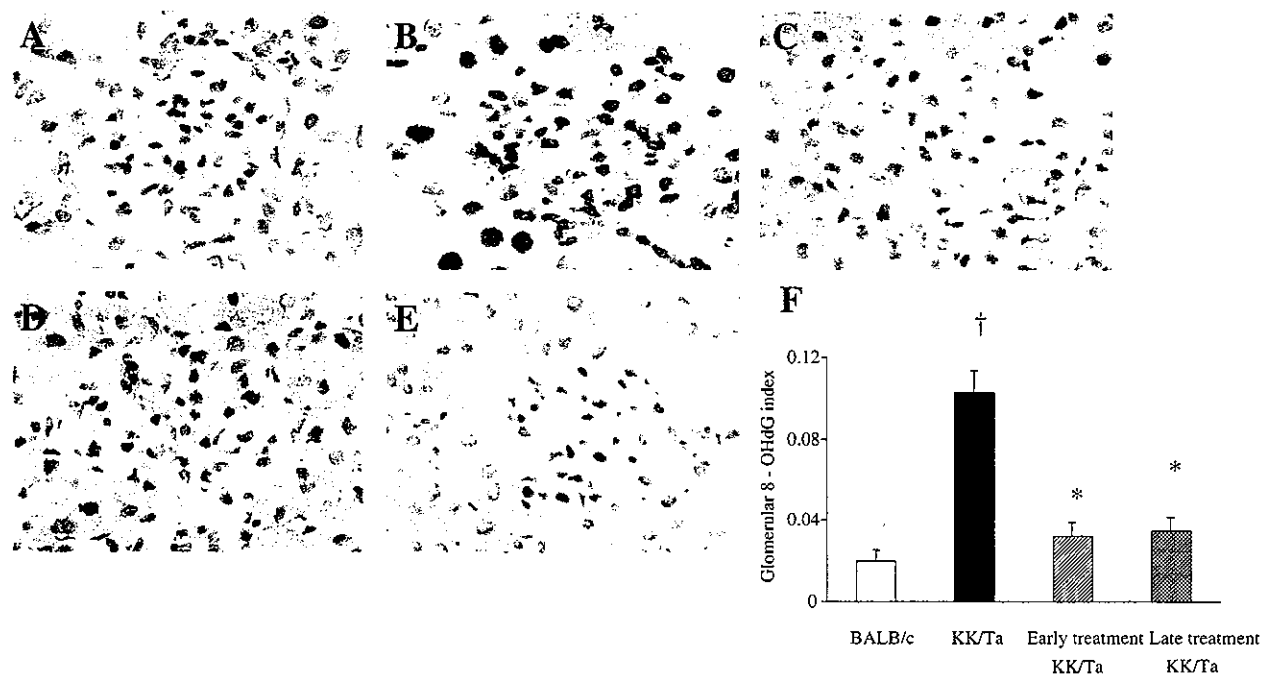

Fig. 4. Representative immunohistochemical staining for 8-OHdG at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) Glomerular 8-OHdG index was calculated as [positive area (μm^2)/glomerular total area (μm^2)]. Each bar represents means ± SEM from six mice in each group and 10 glomeruli from each mouse. $^{\dagger}P < 0.005$ *vs* BALB/c; $*P < 0.005$ *vs* KK/Ta.
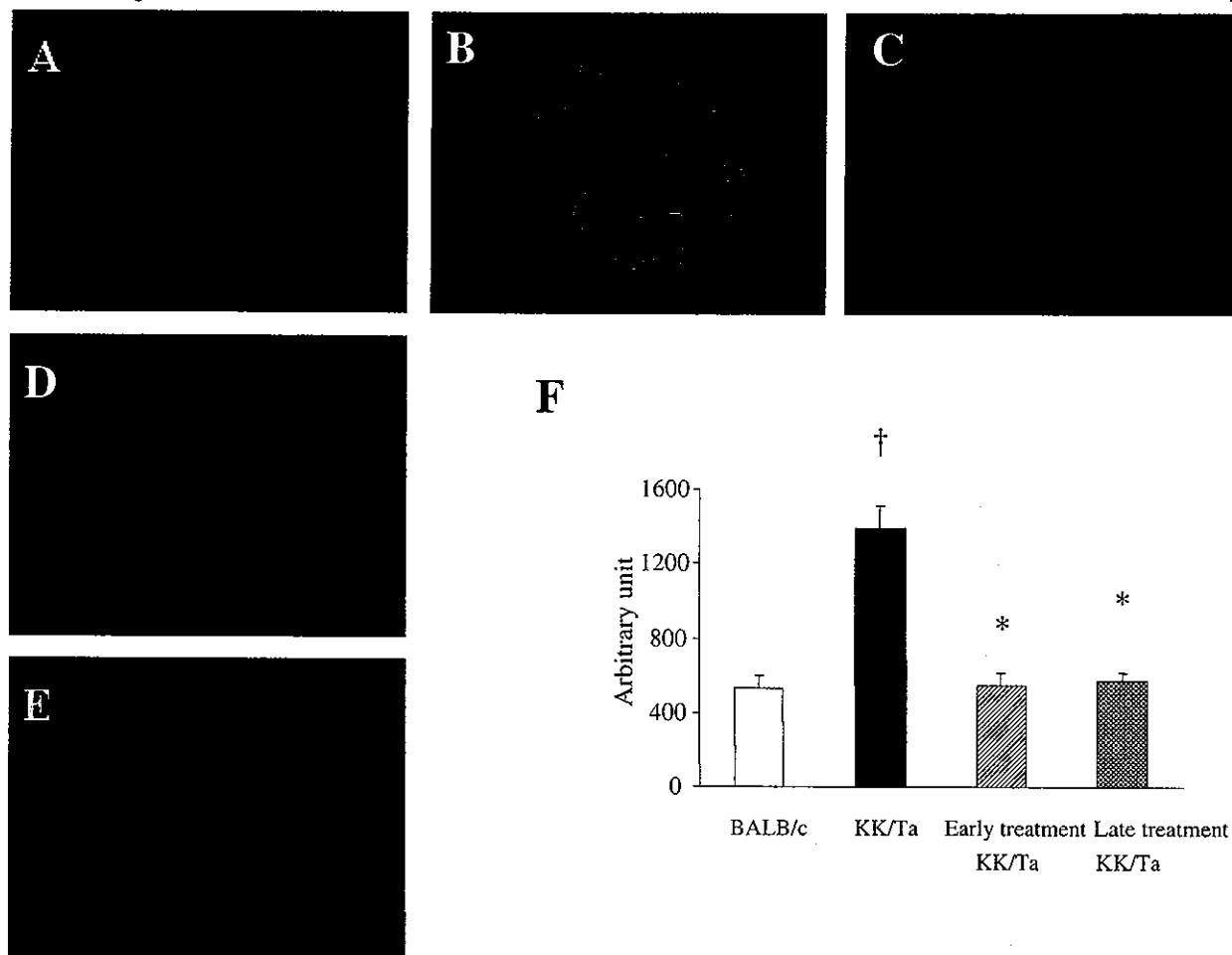
Fig. 5. Representative immunofluorescence staining for nitrotyrosine at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) Analysis of intensity in glomerular immunofluorescence. Each bar represents means ± SEM for six mice in each group and 10 glomeruli from each mouse. $^{\dagger}P < 0.001$ vs BALB/c; $^{*}P < 0.005$ vs KK/Ta.

Both the expressions of iNOS mRNA and protein were induced in KK/Ta mouse kidneys at 28 weeks of age. Administration of candesartan reduced the overexpression of iNOS at transcription and translation levels (Figure 7F). However, there were no significant differences in eNOS mRNA (Figure 7G) or protein (data not shown) expression among the four groups.

Discussion

In the present study, the three KK/Ta mouse groups showed mild glomerular mesangial expansion at 12 weeks of age. By 28 weeks, the untreated KK/Ta group exhibited moderate glomerular mesangial expansion with glomerular hypertrophy. However, these changes were markedly reduced in the candesartan groups. Renal tissues from the control BALB/c mice showed normal histological findings [12]. CML accumulation and RAGE expression were increased in KK/Ta mouse kidneys and these were attenuated by candesartan treatment. This attenuation occurred in the context of

reductions in both glomerular and tubular 8-OHdG and nitrotyrosine, which are markers of oxidative and nitrosative stress. Although these effects of candesartan tended to be stronger in the early treatment group, the differences did not attain statistical significance.

NADPH oxidase and nitric oxide synthase (NOS) are the major sources of superoxide production in the kidney. Angiotensin-II has been reported to upregulate the in vitro synthesis of NADPH oxidase and NOS in various cell types, including glomerular endothelial cells, mesangial cells, podocytes and tubular epithelial cells. NADPH oxidase consists of a membrane-associated cytochrome b_{558} composed of one p22phox subunit and one gp91phox subunit and at least four cytosolic subunits that include p47phox, p67phox, p40phox and the small GTPase rac1 or rac2. Superoxide is generated after phosphorylation of the cytosolic p47phox subunit, which plays a crucial role in agonist (angiotensin-II, PMA and TNF-α)-induced NADPH oxidase activation [16]. All three NOS isoforms are found in the kidney. eNOS is typically expressed in endothelial cells along the renal vascular tree. iNOS is induced by various cytokines in mesan-
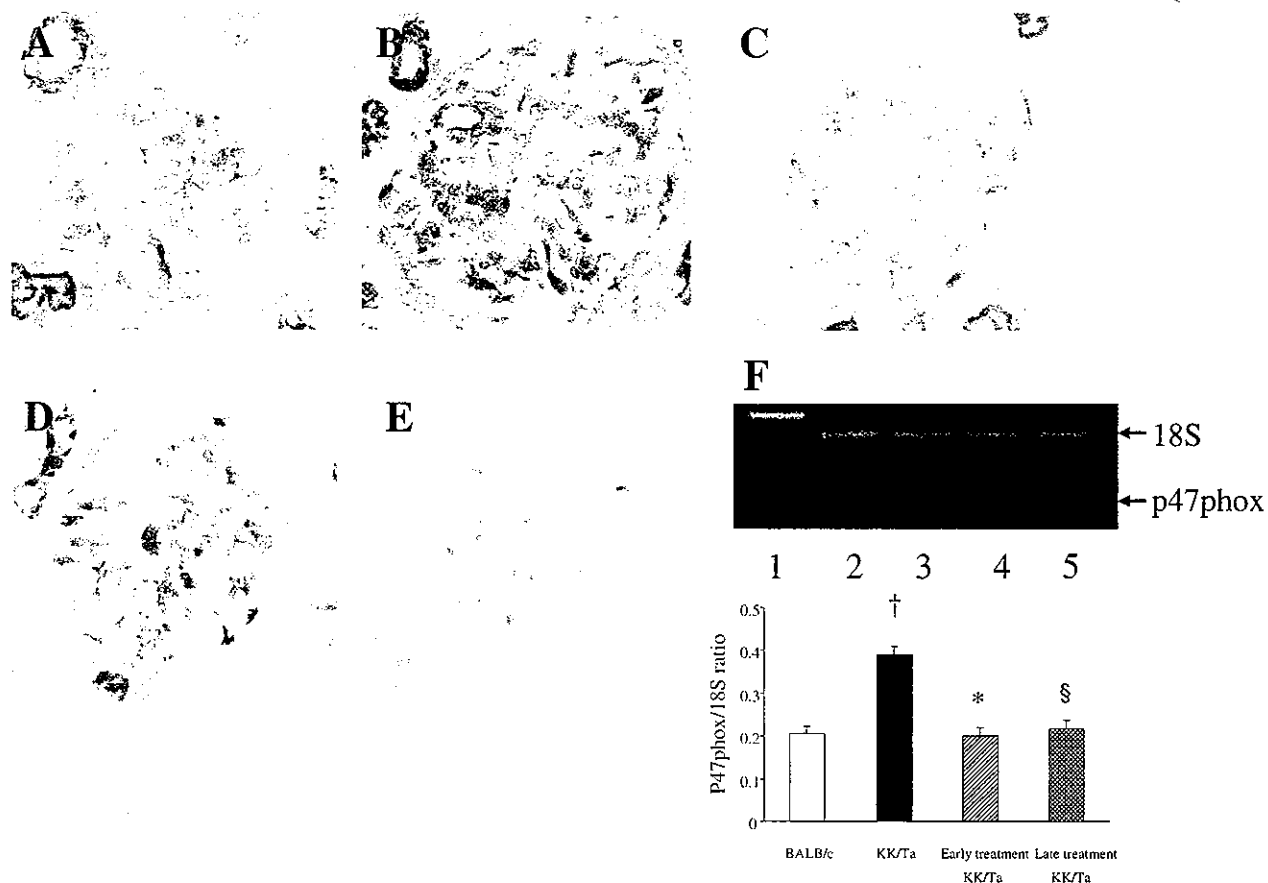
Fig. 6. Representative immunohistochemistry and competitive RT–PCR for NADPH oxidase p47phox subunit at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) The upper bands are 18S competitors (489 bp) and the lower bands are p47phox (225 bp). Lane 1, 100 bp ladder marker; lane 2, BALB/c; lane 3, KK/Ta; lane 4, early treatment KK/Ta; lane 5, late treatment KK/Ta. Each bar represents means±SEM from six mice in each group. $^{\dagger}P<0.001$ vs BALB/c; $*P<0.0005$ vs KK/Ta; $^{\S}P<0.001$ vs KK/Ta.

gial and tubular cells. High levels of neuronal NOS (nNOS) are located in the macula densa [17]. Increased expressions of renal p47phox and eNOS have been reported after 2 weeks of streptozotocin (STZ)-induced diabetes and these were attenuated by either quinapril or candesartan [3]. In the present study, the expressions of p47phox and iNOS were increased in kidneys of type 2 diabetic KK/Ta mice, which is consistent with findings in rats having long-term diabetes [6]. However, eNOS expression was not detected in KK/Ta mouse kidneys at 28 weeks of age. Since prior increases in eNOS expression have been associated with glomerular hyperfiltration at the early stage of experimental diabetic nephropathy [3], the expression pattern of NOS may relate to differences in the type of diabetes, existence of hyperinsulinaemia, long duration of hyperglycaemia or hyperglycaemia-induced endothelial damage [18]. iNOS mediates the synthesis of large (nM) amounts of nitric oxide (NO) following stimulation by inflammatory cytokines or disturbances in the cellular milieu. NO has a high affinity for the super-oxide anion and their interaction forms peroxynitrite (ONOO). Therefore, blockade of AT1 receptors by candesartan may decrease the generation of superoxide

and ONOO by down-regulating p47phox and iNOS expression in diabetic KK/Ta mouse kidneys.

Oxidation influences AGE formation at different stages, such as before and after the formation of Amadori products [6]. Recent observations demonstrated that ONOO can induce CML formation through oxidative cleavage of Amadori products and also through generation of reactive α-oxoaldehydes from glucose [19]. Horie et al. [5] found that CML and pentosidine accumulate in expanded glomerular mesangial matrices and nodular lesions in diabetic nephropathy and that they co-localize with malondial-dehyde-lysine, a lipoxidation product. They suggested that local oxidative stress contributes to the in situ generation of AGE in diabetic nephropathy [5]. In our present study, the effect of AGE inhibition might have depended, at least in part, on the prevention of local oxidative stress, even though candesartan treatment did not influence hyperglycaemia.

CML is a major AGE in diabetic nephropathy and its accumulation upregulates RAGE expression on podocytes. The interaction between AGEs and RAGE activates NADPH oxidase species in podocytes and mesangial cells, thereby triggering signal transduction