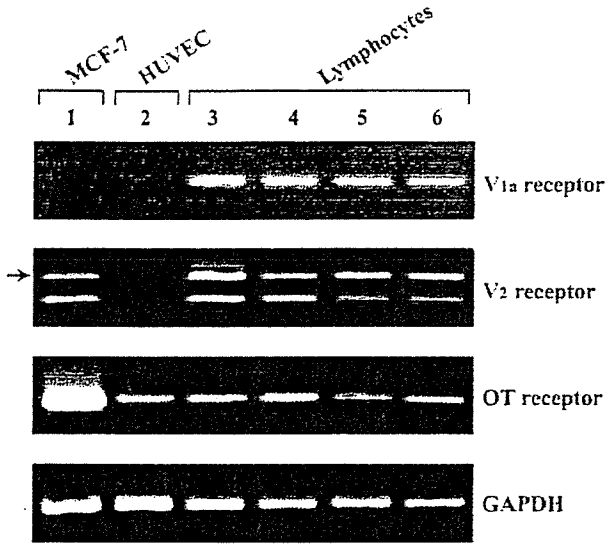

Fig. 1. Expression of arginine vasopressin (AVP) V_{1a} and V_2 and oxytocin (OT) receptors in human lymphocytes. MCF-7 cells (*lane 1*) and human umbilical vein endothelial cells (HUVEC, *lane 2*) were used as positive controls for expression of AVP/OT receptor mRNAs. *Lanes 3–6*, human lymphocytes 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).

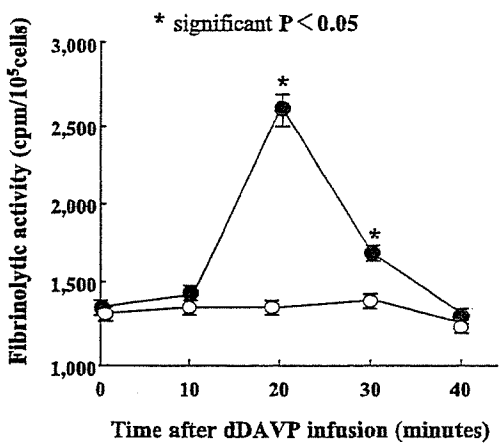

Fig. 2. Fibrinolytic activity in the medium of incubated peripheral blood lymphocytes after [deamino-Cys^1,D-Arg^8]-vasopressin (dDAVP) infusion (●) and without infusion (○). Values represent the average ± SD of 3 independent experiments.

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. 3*A*). However, AVP treatment did not result in the increased levels of fibrinolytic activity at any dose examined (Fig. 3*B*).

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. 4*C*).

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

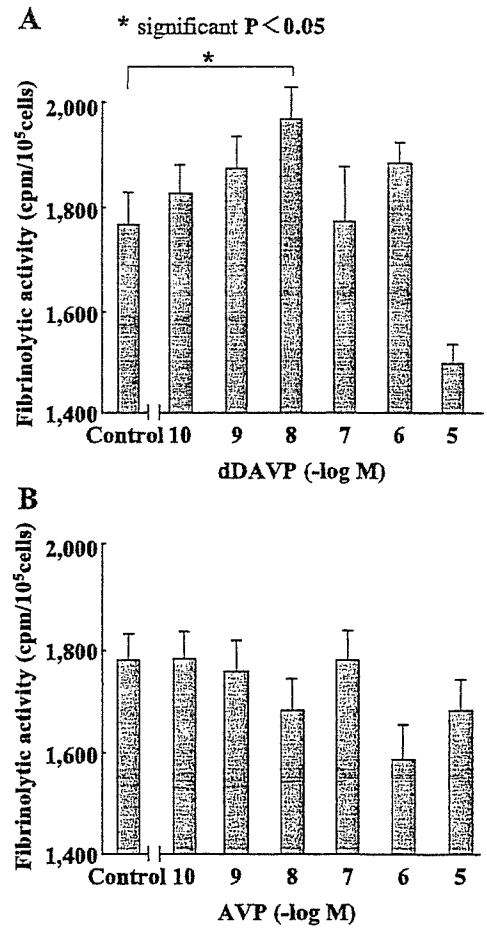

Fig. 3. Fibrinolytic activity in the medium after incubation of lymphocytes with dDAVP (*A*) and AVP (*B*). Values represent the average ± SD of 3 independent experiments.

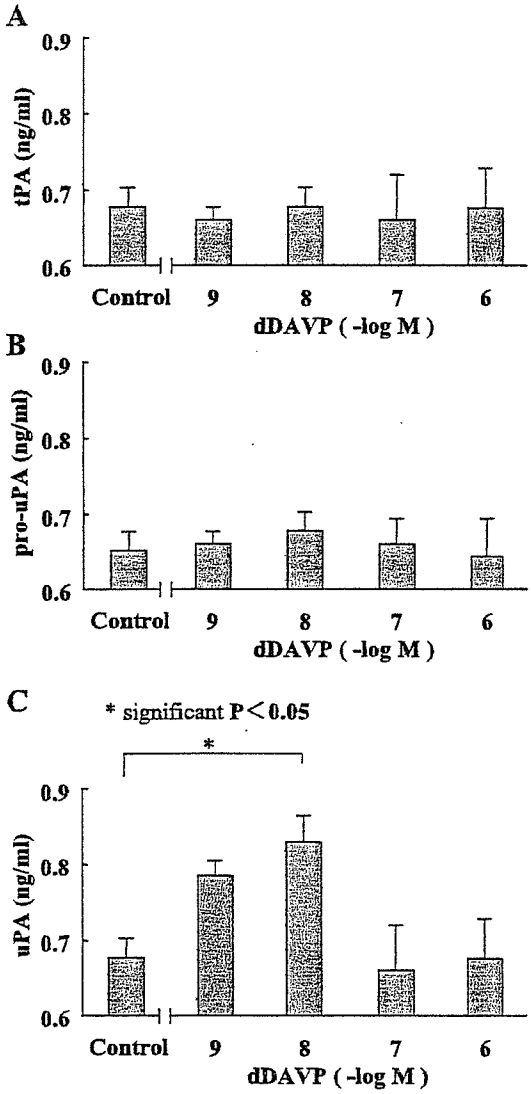

Fig. 4. Dose effect of dDAVP on tissue-type plasminogen activator (tPA, *A*), pro-urokinase-type plasminogen activator (pro-uPA, *B*), and uPA (*C*) concentrations in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.

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).

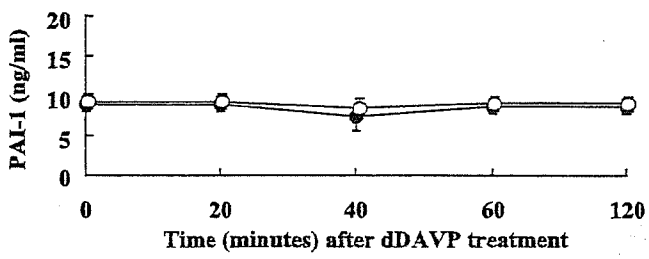

Fig. 5. Plasminogen activator inhibitor (PAI)-1 concentration in the medium after incubation of lymphocytes with (●) and without (○) 10^{-8} M dDAVP. Values represent the average ± SD of 3 independent experiments.

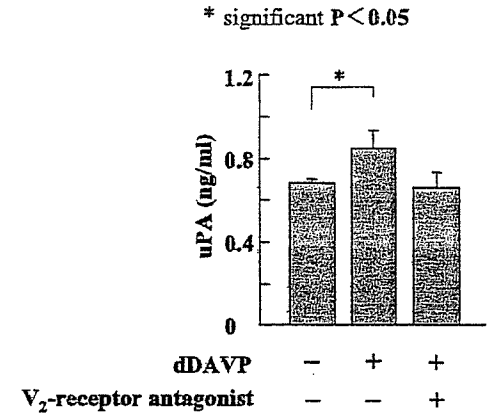

Fig. 6. Effect of the V_2 receptor antagonist on dDAVP-induced uPA increase in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.

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

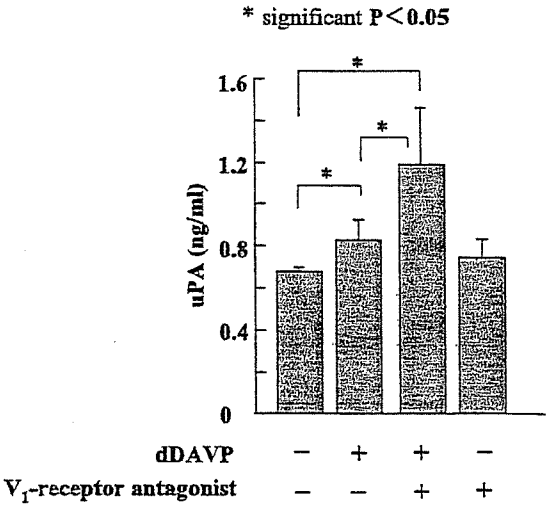

Fig. 7. Effect of the V_1 receptor antagonist on dDAVP-induced uPA increase in the medium after lymphocyte incubation. Values represent the average ± SD of 3 independent experiments.

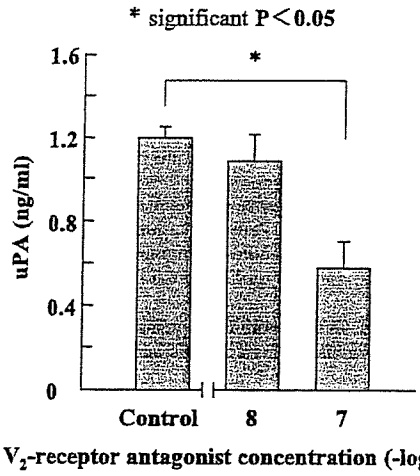

Fig. 8. Effect of the V_2 receptor antagonist on the V_1 antagonist and dDAVP combination-induced increase of uPA concentration in the medium after lymphocyte incubation. Lymphocyte samples were preincubated with the V_2 receptor antagonist (10^{-8} M and 10^{-7} M) for 20 min at 37°C. After preincubation 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. 3*A* and Fig. 4*C*). 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. 3*B*). 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.

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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GRANTS

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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.

Key words: oxidative stress, predialysis, chronic renal failure, redox state of albumin, creatinine clearance, mercapto group.

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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

国立病院機構千葉東病院臨床研究センター・内科
山田研一

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)，③B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(T_R)) 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

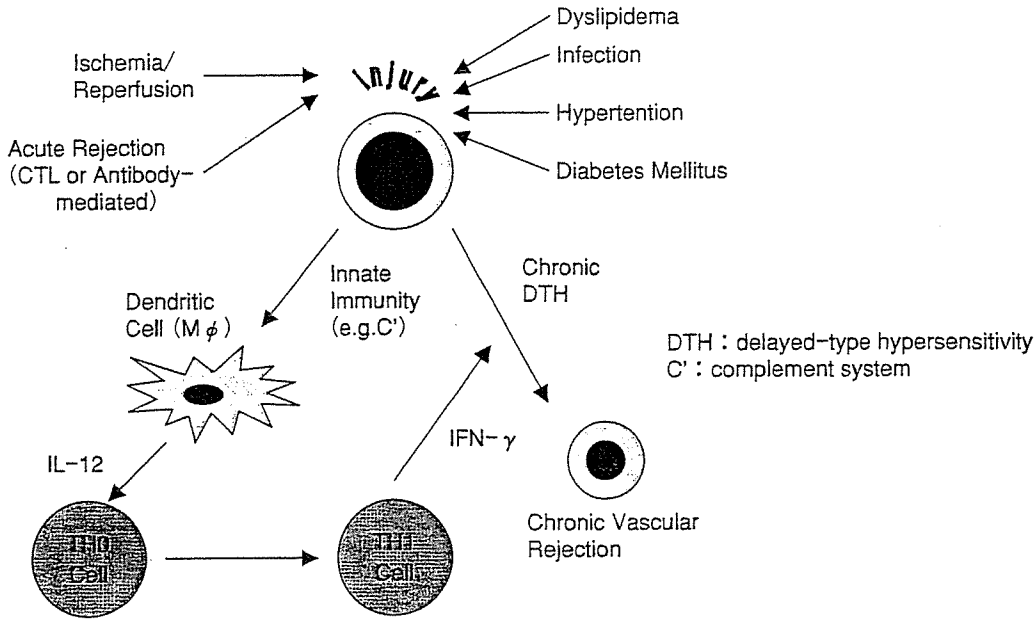

図 **Chronic vascular rejection** と免疫調節モデル

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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Nephrology
Dialysis
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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 multiligand 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 GTT 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

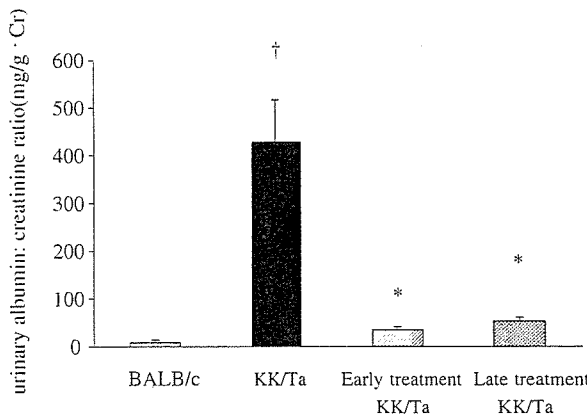

Fig. 1. Urinary albumin excretion at 28 weeks of age. Each bar represents means ± SEM from six mice in each group. †$P < 0.005$ *vs* BALB/c; *$P < 0.005$ *vs* KK/Ta without treatment.

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.

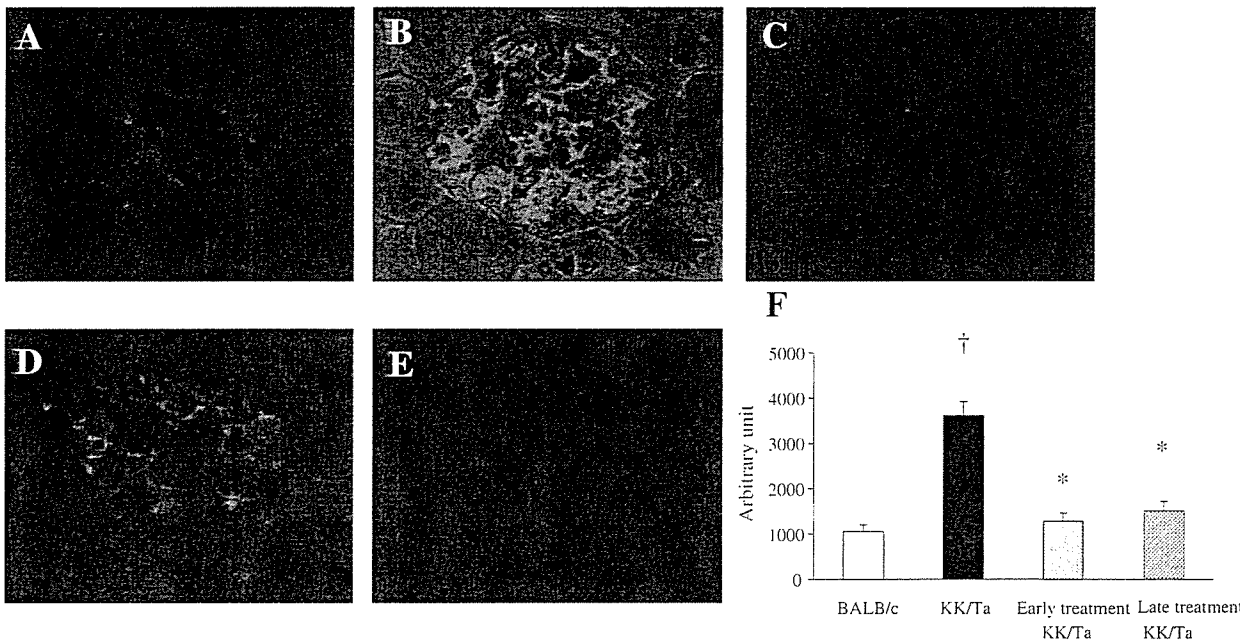

Fig. 2. Representative immunofluorescence staining for CML at 28 weeks of age. (**A**) BALB/c, (**B**) KK/Ta, (**C**) early treatment KK/Ta, (**D**) late treatment KK/Ta, (**E**) negative control. Original magnification: ×400. (**F**) Analysis of intensity in glomerular immunofluorescence. Each bar represents 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).

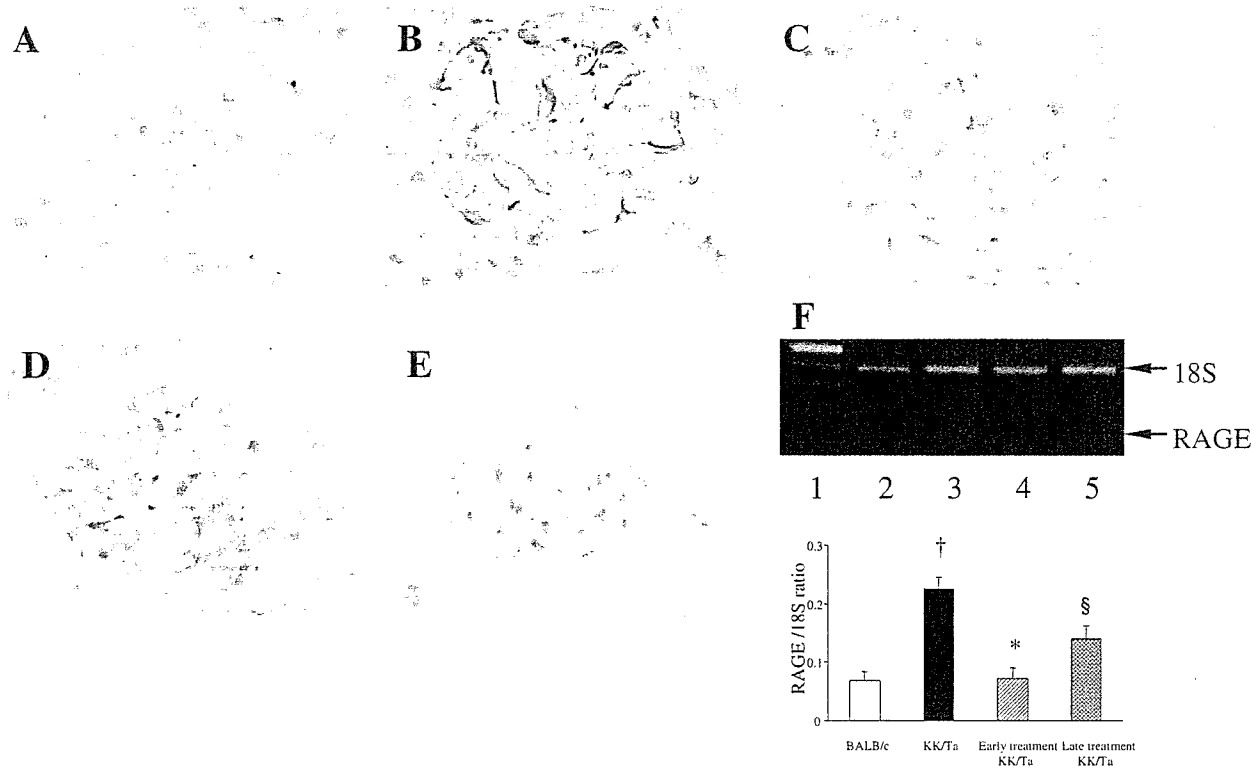

Fig. 3. Representative immunohistochemical staining and competitive RT–PCR for RAGE at 28 weeks of age. (**A**) BALB/c, (**B**) KK/Ta, (**C**) early treatment KK/Ta, (**D**) late treatment KK/Ta, (**E**) negative control. Original magnification: ×400. (**F**) The upper bands are 18S competitors (489 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.

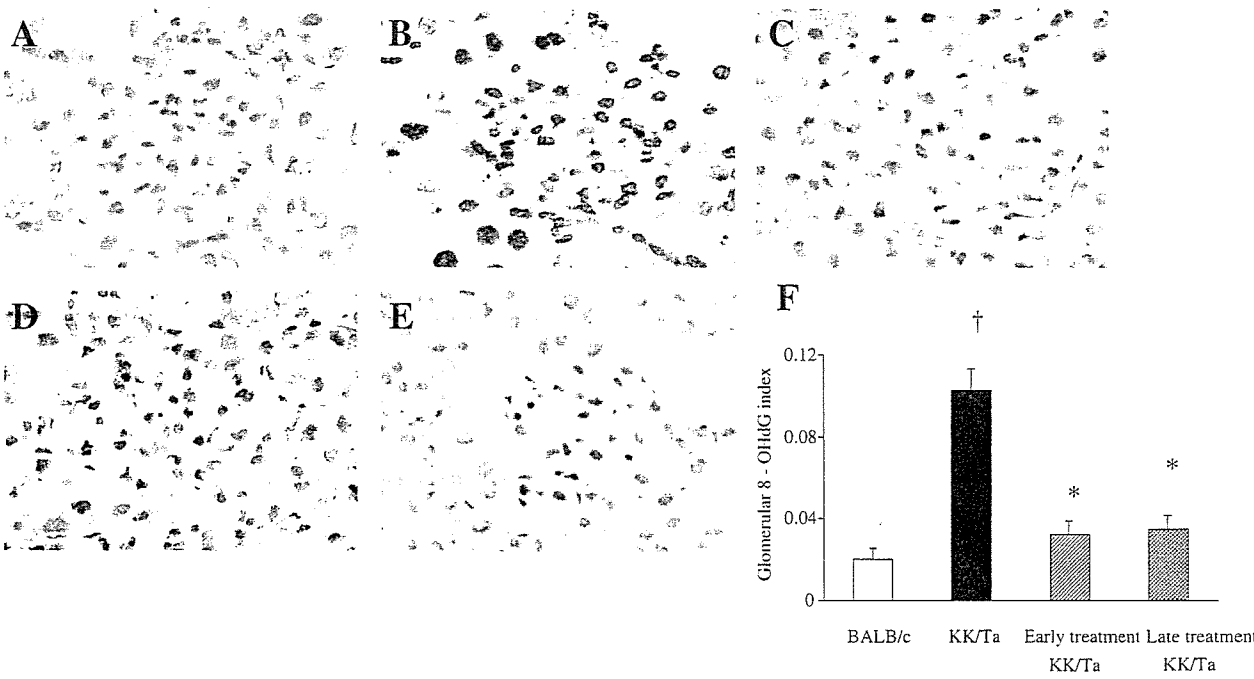

Fig. 4. Representative immunohistochemical staining for 8-OHdG at 28 weeks of age. (**A**) BALB/c, (**B**) KK/Ta, (**C**) early treatment KK/Ta, (**D**) late treatment KK/Ta, (**E**) negative control. Original magnification: ×400. (**F**) Glomerular 8-OHdG index was calculated as [positive area (μm^2)/glomerular 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.

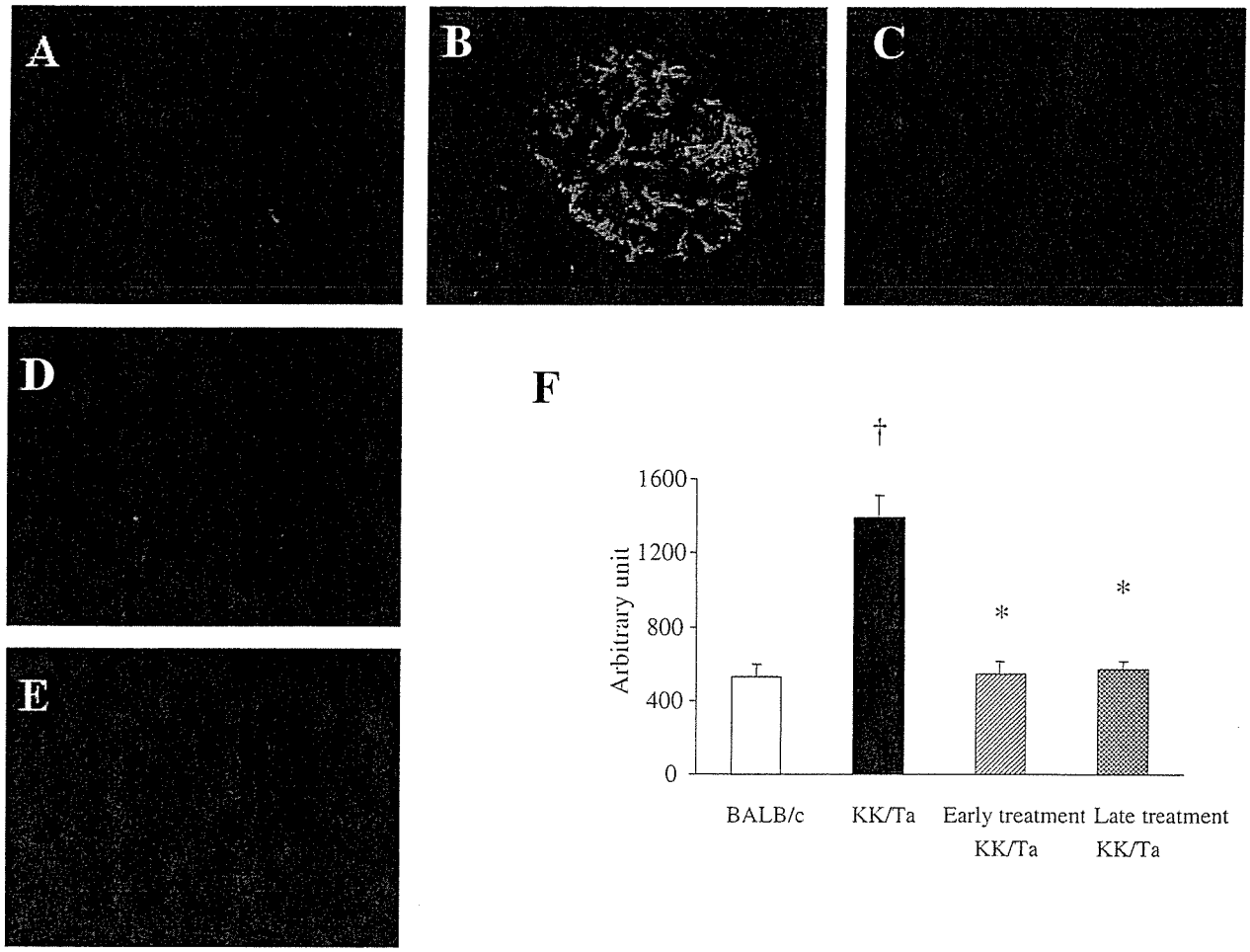

Fig. 5. Representative immunofluorescence staining for nitrotyrosine at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta. (D) late treatment KK/Ta. (E) negative control. Original magnification: ×400. (F) Analysis of intensity in glomerular immunofluorescence. Each bar represents means ± 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 *rac*1 or *rac*2. 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-

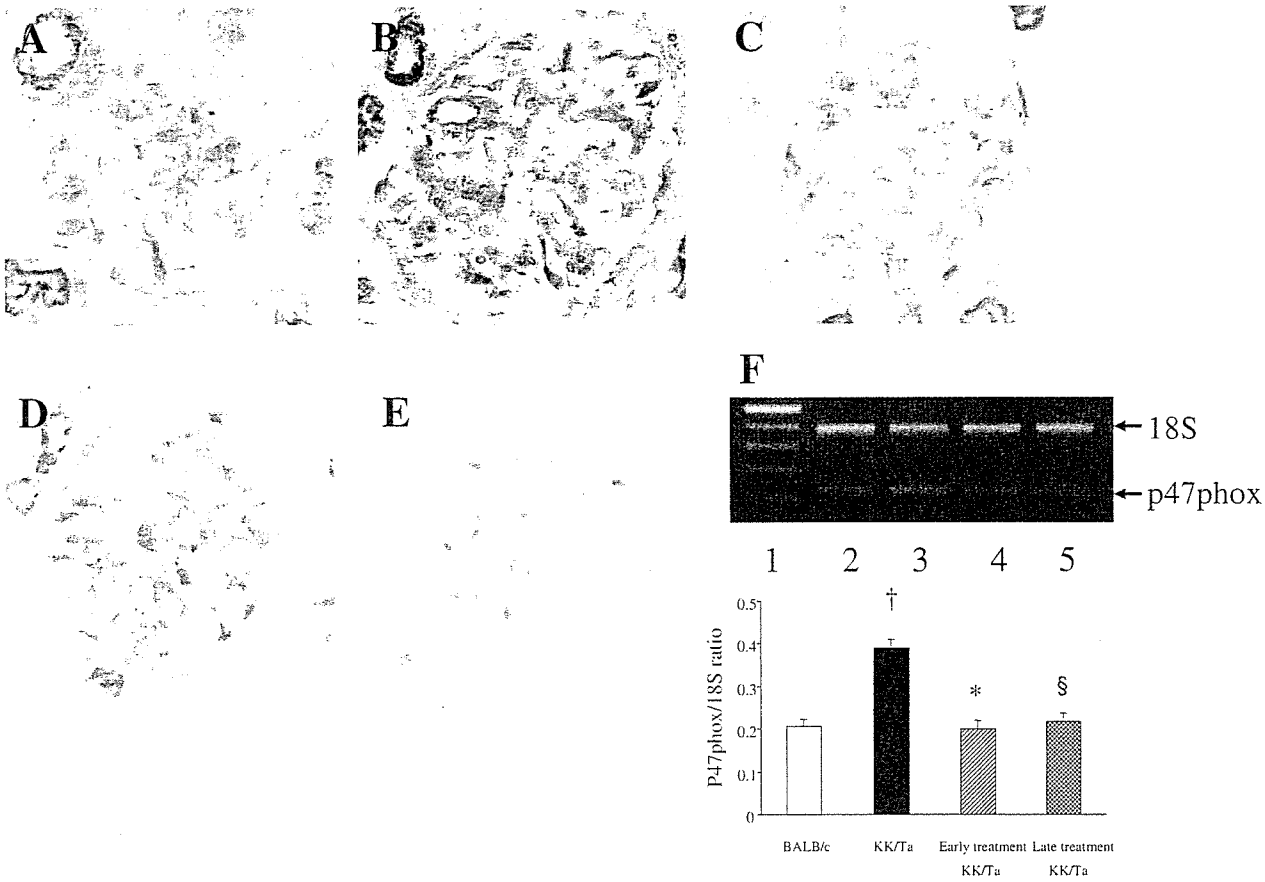

Fig. 6. Representative immunohistochemistry and competitive RT–PCR for NADPH oxidase p47phox subunit at 28 weeks of age. (**A**) BALB/c, (**B**) KK/Ta, (**C**) early treatment KK/Ta, (**D**) late treatment KK/Ta. (**E**) negative control. Original magnification: ×400. (**F**) The upper bands are 18S 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 superoxide 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 malondialdehyde-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

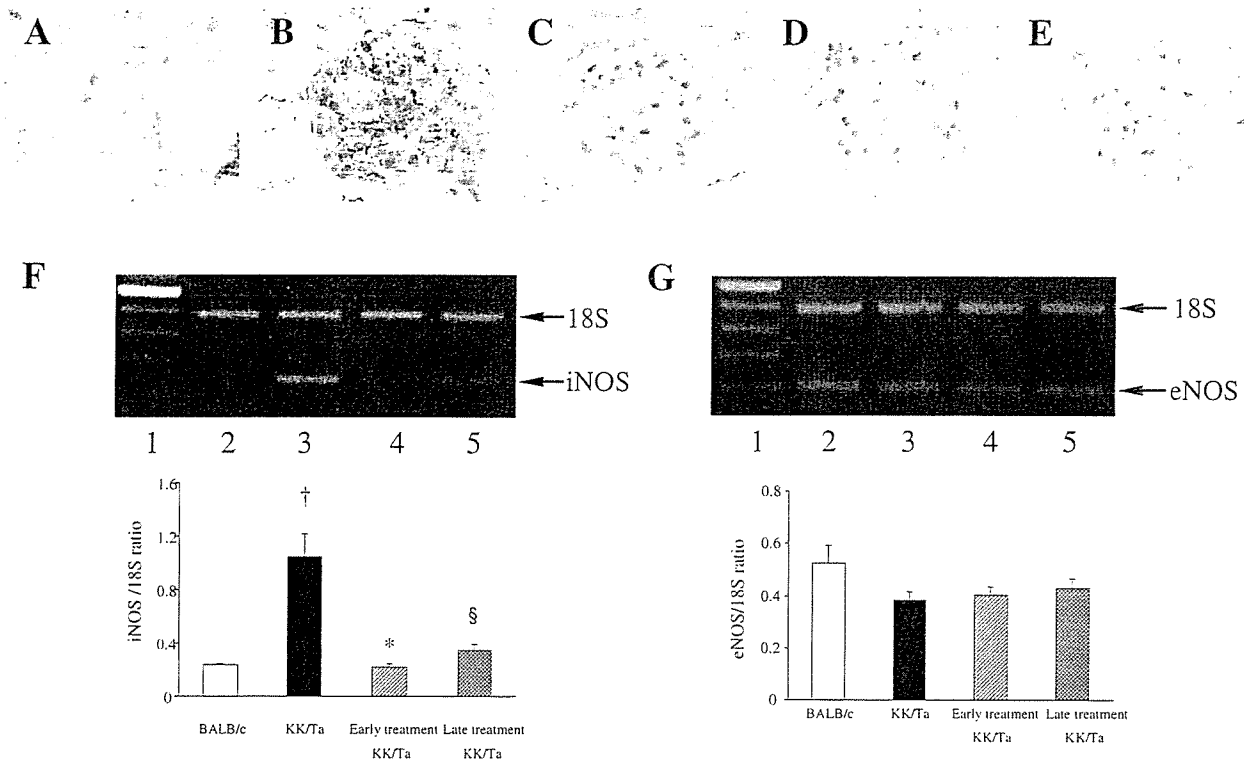

Fig. 7. Representative immunohistochemistry for iNOS and competitive RT–PCR for iNOS and eNOS at 28 weeks of age. (**A**) BALB/c, (**B**) KK/Ta, (**C**) early treatment KK/Ta, (**D**) late treatment KK/Ta, (**E**) negative control. Original magnification: ×400. (**F**) The upper bands are 18S competitors (489 bp) and the lower bands are iNOS (304bp). (**G**) The upper bands are 18S competitors (489 bp) and the lower bands are eNOS (209 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. $^{\dagger}P < 0.05$ *vs* BALB/c; $^{*}P < 0.005$ *vs* KK/Ta; $^{\S}P < 0.01$ *vs* KK/Ta.

mechanisms, such as $p21^{ras}$, erk 1/2 MAP kinases and NF-κB in an oxidant-sensitive manner [2]. Moreover, Sugimoto *et al.* [6] demonstrated that both CML and iNOS were detected in the glomerular mesangial areas in STZ-induced diabetic rats at 52 weeks of age and that they decreased after treatment with aminoguanidine, an inhibitor of AGE formation. They concluded that CML may enhance the expression of iNOS by stimulating TNF-α via RAGE [6]. These findings suggest that RAGE contributes to sustained generation of nitro-oxidative stress, which in turn accelerates the formation of AGE. Candesartan may down-regulate AGE-induced overexpression of RAGE by attenuating the activities of NF-κB and the JAK/STAT pathway since the RAGE gene promoter contains NF-κB and STAT binding sites that can be activated by angiotensin-II via the AT1 receptor [20].

In summary, the present findings demonstrated that nitro-oxidative stress and the production of AGEs are enhanced in the kidneys of type 2 diabetic KK/Ta mice. The AT1 receptor antagonist candesartan decreased nitro-oxidative stress by down-regulating NADPH oxidase p47phox and iNOS expression. Candesartan also modified the interaction between AGEs and RAGE by attenuating RAGE expression, which contributed to the reduction of AGE accumulation and subsequent albuminuria. Further studies are ongoing to elucidate the effects of ARB on other target molecules activated by the AGE–RAGE pathway.

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Conflict of interest statement. None declared.

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Original Article

Renoprotective Effect of Losartan in Comparison to Amlodipine in Patients with Chronic Kidney Disease and Hypertension—a Report of the Japanese Losartan Therapy Intended for the Global Renal Protection in Hypertensive Patients (JLIGHT) Study

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A 12-month, multicenter (57 clinical institutions), randomized, open-labeled trial was undertaken to compare the efficacy of the angiotensin II receptor antagonist losartan and the calcium channel blocker amlodipine in patients with proteinuric chronic kidney disease (CKD) and hypertension. A total of 117 patients (79, chronic glomerulonephritis; 14, diabetic nephropathy; 24, other CKD) were randomly allocated into two treatment groups. Losartan and amlodipine exerted the same efficacy for blood pressure (BP) control; however, losartan significantly reduced the 24-h urinary protein excretion at months 3, 6, and 12, with the reduction of 20.7%, 35.2%, 35.8%, whereas amlodipine did not change the amount of proteinuria over the 12-month study period. When patients were stratified into groups according to the level of BP control at 3 months, the reduction in urinary protein excretion by losartan was evident in the group for which a BP of $<140/90$ mmHg was achieved, as well as in the group for which the goal BP ($<130/85$ mmHg) for treatment of CKD was not achieved. When patients were stratified according to baseline urinary protein excretion, those with ≥ 2 g/day showed a reduction in proteinuria by losartan of 23.3%, 39.4%, and 47.9% at months 3, 6, and 12, and those with <2 g/day showed a reduction of 18.5% and 31.2% at months 3 and 6, respectively. No fatal adverse

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events were experienced in either drug group. We conclude that losartan reduced proteinuria in patients with CKD and hypertension. This positive effect may contribute to the renal protective benefit of losartan, and is beyond the magnitude of BP control. (*Hypertens Res* 2004; 27: 21–30)

Key Words: losartan, angiotensin, proteinuria, hypertension, renoprotection

Introduction

On the basis of understanding the role of angiotensin II in circulation and renal functions, the relevance of intervention of the renin-angiotensin system (RAS) for therapy of hypertension and kidney diseases has so far been extensively discussed (*1*, *2*). High blood pressure (BP) strongly affects the structure and functions of nephrons, and inversely, impaired renal function elevates the systemic BP level in patients with kidney diseases. Angiotensin converting enzyme (ACE) inhibitors are now one of the most frequently used drugs for hypertension, and a number of evidences are available with regard to the effect of ACE inhibition to ameliorate kidney diseases, especially proteinuria as a symptom (*3*). Indeed, in many clinical studies dealing with kidney diseases, proteinuria has been adopted as a surrogate endpoint, because proteinuria is not merely a marker of permselectivity of the glomerular membrane, but is toxic to the kidney *per se*, and plays a key role in the progression of kidney diseases, eventually leading to end-stage renal disease (ESRD) (*4–7*).

With reference to the effect of ACE inhibitors, the use of angiotensin II receptor antagonists for the treatment of kidney diseases has also been discussed. The RENAAL study, an international multicenter clinical trial of the angiotensin II receptor antagonist losartan, was published in 2001 (*8*). This trial studied the effect of losartan in patients with type 2 diabetic nephropathy. The results clearly demonstrated that losartan retarded the elevation of serum creatinine and decreased the rate of onset of ESRD. On the other hand, the effects of intervention of the actions of angiotensin II in patients with non-diabetic chronic kidney disease (CKD) and hypertension has been still a subject of debate with regard to relation to BP lowering effect. Any pharmacotherapy to lower BP may be effective for protection of renal functions; however, whether blockade of angiotensin II receptors confers renal protection in excess of that due to BP control has not been clearly answered. There is thus need of accumulation of evidences of comparative study with other classes of antihypertensive drugs in patients with CKD and hypertension. For this reason, we have performed a 12-month study comparing the effects of the angiotensin II receptor antagonist losartan and the calcium channel blocker amlodipine. A portion of the results were previously disclosed as an interim report at 3 months (*9*) with the full analysis set (FAS) (*10*). We here report our final results based on the final selection of patients by the Coordinating Committee. Our findings show that, although losartan and amlodipine exerted the same degree of BP control, only losartan induced a significant reduction in urinary protein excretion over the 12-month observation period.

Methods

This study was a 12-month, multicenter, randomized, open-labeled, clinical trial designed to compare the effect of the angiotensin II receptor antagonist losartan and the calcium channel blocker amlodipine to reduce proteinuria in patients with CKD and hypertension. Fifty-seven affiliated clinics in Japan contributed to this study. The overall design of the study has been described previously in an interim report presented at 3 months (*9*). Males and female outpatients, aged 20–74 years, who had CKD and hypertension and who met the following criteria during the 8-week pretreatment screening period were eligible for the study:

1) CKD: serum creatinine (Scr) levels of $1.5 \leq Scr < 3.0$ mg/dl in males of body weight (BW) ≥ 60 kg, and of $1.3 \leq Scr < 3.0$ mg/dl in females, or males of BW < 60 kg.

2) Hypertension: systolic BP (SBP) ≥ 140 mmHg or diastolic BP (DBP) ≥ 90 mmHg as measured in a sitting position at least two separate times at their visits to clinics.

3) Proteinuria: urinary protein excretion of ≥ 0.5 g/day.

The overview of study design is shown in Fig. 1. The randomization method was modified by dynamic balancing for Scr, the 24-h urinary protein excretion that was measured at the time of registration, and presence or absence of diabetic nephropathy, so that patients were allocated to the two groups avoiding significant difference of baseline characteristics in average. Patients of the two groups received either losartan 25 mg as a starting dose to up to 100 mg once daily, or amlodipine 2.5 mg as a starting dose to up to 5 mg once daily, respectively. However, in cases in which a patient's compliance was judged by investigator(s) to be sufficiently good for the administration of a higher dose, either 50 mg of losartan or 5 mg of amlodipine was adopted as a starting dose.

The target BP was $< 130/85$ mmHg, and patients were not allowed combination therapy with other antihypertensive agents during the first 3 months. However, after 3 months, if a BP of $< 130/85$ mmHg was not achieved, antihypertensive combination therapy with α-blockers, β-blockers, α/β-blockers, diuretics (excepting potassium-sparing diuretics), and other calcium channel blockers were considered as appropriate. Guidance was given to patients to maintain their usual diet, especially for those under dietary restrictions. The study protocol was reviewed and approved by the Institutional Review Boards of all clinics contributing to the study. Written informed consent was obtained from all enrolled pa-