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Butein (3,4,2′,4′-tetrahydroxychalcone) ameliorates experimental anti-glomerular basement membrane antibody-associated glomerulonephritis (3)

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Abstract

The antinephritic effects of butein (3,4,2′,4′-tetrahydroxychalcone) on original-type anti-glomerular basement membrane antibody-associated glomerulonephritis in rats were investigated. Butein was given to anti-glomerular basement membrane antibody-associated glomerulonephritic rats for 15 days after the induction of nephritis. Butein prevented proteinuria and histological alterations. The up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression and increase in leukocyte function-associated antigen-1 (LFA-1) positive cells in nephritic glomeruli significantly declined with butein treatment. In the further investigation to clarify the effects of butein on ICAM-1 expression, human umbilical vein endothelial cells were treated with butein in the presence of tumor necrosis factor-α (TNF-α) or phorbol 12-myristate 13-acetate (PMA). Butein prevented the up-regulation of ICAM-1 expression mediated by TNF-α or PMA on human umbilical vein endothelial cells in a dose-dependent manner. When human umbilical vein endothelial cells or neutrophils were treated with butein, the adhesion of neutrophils to human umbilical vein endothelial cells was suppressed. These data suggest that the antinephritic action of butein is due to inhibition of intraglomerular accumulation of leukocytes through the prevention of the up-regulation of ICAM-1 and the inhibition of a function of adhesion molecules on the surface of leukocytes.

Keywords: Butein; Anti-GBM nephritis; Intercellular adhesion molecules; Endothelial cells; Leukocyte, polymorphonuclear

1. Introduction

Butein (3,4,2′,4′-tetrahydroxychalcone), a chalcone derivative, is a flavonoid which is extracted from the flower *Butea frondosa*. Recent investigation has demonstrated that butein has an anti-proliferative action on human tumor cells (Yit and Das, 1994; Ramanathan et al., 1992) and an anti-oxidant effect (Sogawa et al., 1994). The structure of butein is analogous to that of genistein, a flavonoid which was isolated from the culture medium of *Pseudomonas* (Ogawara et al., 1986). It is reported that genistein suppresses the activity of $pp60^{v\text{-}src}$ and tyrosine kinase of the epidermal growth factor (EGF) receptor (Akiyama et al., 1989). Moreover, staurosporin, which inhibits the activities of src and protein kinase C (Saya et al., 1993; Tamaoki et al., 1986), suppresses the up-regulation of intercellular adhesion molecule-1 (ICAM-1) on TNF-α-stimulated human umbilical vein endothelial cells (Lane et al., 1990). These data suggest the possibility that butein might inhibit the up-regulation of ICAM-1 expression through the suppression of intracellular signal transduction of inflammatory cytokine.

However, our previous study (Hayashi et al., 1996) demonstrated that butein was effective against crescentic-type anti-GBM nephritis in rats and suppressed the accumulation of leukocytes in nephritic glomeruli. Adhesion molecules play an important role in the development of the inflammatory response and in the recruitment of leukocytes into tissues including nephritic glomeruli (Rothlein and Wegner, 1992; Brady, 1994; Isobe et al., 1992). Recent studies have demonstrated the importance of various adhesion molecules in the leukocytic accumulation in nephritic glomeruli and glomerular damage in human and experimental nephritis (Brady, 1994;Wuthrich et al., 1990; Hill et al., 1994). These studies have shown that ICAM-1, which is expressed on many cell types including endothelium and has as its ligand members of the $\beta 2$ integrin family, leukocyte function-associated antigen-1 (LFA-1)

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and macrophage activating complex-1, appears to function as the most important adhesion molecule in directing glomerular leukocytic infiltration. Furthermore, it is reported that monoclonal antibodies against ICAM-1, LFA-1, macrophage activating complex-1 and very late activating antigen-4, decrease urinary protein excretion and the accumulation of leukocytes in glomeruli in experimental anti-GBM nephritis (Mulligan et al., 1993; Kawasaki et al., 1993).

The aim of this study is to clarify the mechanism underlying the butein inhibition of leukocyte migration into nephritic glomeruli.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley strain rats, weighing approximately 170 g (Nihon SLC, Hamamatsu, Japan), were used in all experiments.

2.2. Drugs

In in vivo experiments, butein (3,4,2′,4′- tetrahydroxychalcone) (Dainippon Ink and Chemicals, Tokyo, Japan) and dexamethasone ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione, Sigma, St. Louis, MO, USA) were suspended in 1% gum arabic. Cyclosporin A (Sandoz, Tokyo, Japan) was dissolved in 5% ethanol in olive oil. In in vitro experiments, butein dissolved in dimethylsulfoxide at 10 mM was diluted in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) and cyclosporin A dissolved in ethanol with 20% Tween 80 at 10 mM was diluted in RPMI 1640. H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, Seikagaku, Tokyo, Japan) and genistein (5,7,4′-trihydroxyosoflavone, Wako, Osaka, Japan) were dissolved in RPMI 1640 at 100 μM. Staurosporin (Kyowa Medex, Tokyo, Japan) was dissolved in dimethyl sulfoxide at 100 μM. Recombinant human TNF-α and anti-cytokine neutralizing antibodies (rabbit anti-human TNF-α polyclonal antibody and rabbit anti-human interleukin-1β polyclonal antibody) were purchased from Genzyme (Cambridge, MA, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma.

2.3. Induction of original-type anti-glomerular basement membrane antibody-associated glomerulonephritis

Original-type anti-glomerular basement membrane antibody-associated glomerulonephritis in rats was induced by the injection of a nephritogenic dose (0.6 ml/animal, i.v.) of rabbit anti-rat glomerular basement membrane serum into their tail veins, as previously described (Hattori et al., 1994).

2.4. Evaluation of antinephritic effects of drugs

Each drug was administered to rats p.o. daily from the day after i.v. injection of anti-glomerular basement membrane serum to 15 days. Urine was collected on 1, 5, 11 and 15 days and the kidneys were taken on day 5 and 15 (Hayashi et al., 1994a). The antinephritic effect of test drugs was evaluated by comparing urinary protein excretion, histopathological parameters and ICAM-1 expression in the glomerulus with those of the nephritic control.

The urine sample was collected for determination of the urinary protein as previously reported (Hayashi et al., 1994a). The urinary protein content was determined by the method of Kingsbury et al. (1926) and expressed as mg/24 h urine.

For the light microscopic and immunohistochemical study, the kidneys were fixed in 10% formalin buffer, then embedded in paraffin and sectioned into 2–3 μm thick slices. For assessment of histopathological parameters, the sections were stained with hematoxylin and eosin. Hypercellularity and adhesion of Bowman's capsule to capillary walls (adhesion) in the glomeruli were observed under light microscopy with the previous method reported (Hayashi et al., 1994a). Hypercellularity suggests proliferation of intrinsic glomerular cells, because the accumulation of leukocytes is not observed in nephritic glomeruli at 15 days after the induction of nephritis (Hattori et al., 1994). For assessing hypercellularity, an equatorial cross-section was selected by random sampling methods and the number of nuclei was counted and expressed as the mean number per equatorial cross-section in 30 glomeruli/animal. Adhesion was graded as normal (0 point), mild (1 point), moderate (2 points) or severe (3 points), according to the extent of the alteration. The number of glomeruli corresponding to each score was represented as n_0, n_1, n_2 and n_3. The index of adhesion were calculated as follows: Index $= (1 \times n_1) + (2 \times n_2) + (3 \times n_3)/30$ glomeruli.

In the immunohistochemical study, the sections were sequentially incubated with monoclonal antibody to ICAM-1 or LFA-1 (CD11a) (Seikagaku, Tokyo, Japan), rabbit anti-mouse immunoglobulin G (IgG), and horseradish peroxidase-avidin biotin complex and the reaction was then developed with 3,3′-diaminobenzidine–tetrahydrochloride (Hayashi et al., 1994b). Each tissue section stained with the monoclonal antibody was analyzed with an image analyzer (TOYOBO Image analyzer V1, Toyobo, Tokyo, Japan) for the total area of ICAM-1 and the number of LFA-1 positive cells in glomeruli (30 glomeruli/section) and the results were expressed in mm^2 per glomerular cross-section (G.C.S.) or the number of cells per G.C.S. (Hayashi et al., 1994b).

Generally, ICAM-1 expression in glomeruli is scored as mild, moderate and severe. However, scoring ICAM-1 expression is not exactly quantitative because this method depends on a rough grading. The present method for quantitating ICAM-1 expression provides digital data and

is not obstructed by subjectivity. Therefore, we used this method for quantitating ICAM-1 expression in glomeruli in the present experiments. When we measured ICAM-1 expression in glomeruli, we avoided ICAM-1-positive cells, which were clearly regarded as leukocytes.

2.5. Cell culture

2.5.1. Human umbilical vein endothelial cells

Human umbilical vein endothelial cells were obtained from Curabou (Neyagawa, Japan, Japan). The cells were suspended in culture medium (MCDB131 with 2% fetal bovine serum, 10 μg heparin/ml, 10 μg endothelial cell growth supplement/ml, 10 μg EGF/ml, 1 μg hydrocortisone/ml, 50 μg gentamicin/ml and 0.25 μg amphotericin B/ml) (Curabou, Neyagawa, Japan) and grown in 75 cm^2 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA). Culture medium was changed twice weekly. Human umbilical vein endothelial cells were trypsinized when subconfluent, resuspended in culture medium and seeded into either new culture flasks or human-type-I collagen-coated plates (24- or 96-well) (Sumitomo Bakelite, Tokyo, Japan). Human umbilical vein endothelial cells were used from 3 to 6 passages.

2.5.2. Cell ELISA

Human umbilical vein endothelial cells (1×10^4 cells/well) were seeded into 96-well flat-bottomed human-type-I collagen-coated plates in 100 μl of M199 (Nissui Pharmaceutical) and allowed to reach subconfluency (approximately 1×10^5 cells/well). When human umbilical vein endothelial cells were subconfluent, 50 μl of the RPMI 1640, various agents (butein, genistein, cyclosporin A, H-7, staurosporin) or anti-cytokine neutralizing antibodies and 50 μl of human TNF-α (final concentration at 100 U/ml), phorbol 12-myristate 13-acetate (final concentration; 100 ng/ml) or medium was added to the appropriate wells to yield a final volume of 100 μl/well just after 100 μl of the medium from each well was removed. The cells were incubated for 4, 12, 20, 48 or 72 h at 37°C in 5% CO_2. The cell monolayers were washed twice with Hank's balanced salt solution (HBSS, Sanko Junyaku, Tokyo, Japan) and then fixed with 1% paraformaldehyde for 15 min at room temperature. After the fixed human umbilical vein endothelial cells were washed three times with HBSS, unbound sites were blocked by casein (block A™, Yukijirushi, Tokyo, Japan) and the cells were incubated at 37°C for 1 h. Anti-ICAM-1 monoclonal antibody (Genzyme), anti-vascular cell adhesion-1 (VCAM-1) molecule or anti-endothelial cell adhesion molecule-1 (ELAM-1) was added to each well and the plates were incubated at 37°C for 1 h. After the cells were washed with HBSS, a 1:2000 dilution of the secondary antibody (goat anti-mouse IgG (H + L) horseradish peroxidase conjugate, Bio-Rad, Richmond, CA, USA) in RPMI 1640 was added and was then incubated for 1 h at 37°C. The cells were washed with PBS and *o*-phenylenediamine (Sigma) development was determined by measuring the optical density at 490 nm with a Microplate reader (model 3550 Microplate reader; Bio-Rad).

2.6. Polymorphonuclear leukocyte preparation and labeling with ^{51}Cr

Polymorphonuclear leukocytes were collected from rats which were peritoneally injected with 1% (w/v) casein (Wako) for 15 h before leukocyte collection. The final pellet was suspended in M199 and the purity of polymorphonuclear leukocytes was about 95%, as determined by light microscopy. For studies of adhesion, polymorphonuclear leukocytes were radiolabeled with [^{51}Cr]sodium chromate (3.7 MBq/10^8 cells; Daiichi, Chiba, Japan) for 60 min at 37°C, were washed three times in M199 to remove extracellular ^{51}Cr and were resuspended in M199 containing 10% bovine serum albumin (Sigma).

2.7. Polymorphonuclear leukocyte adhesion to human umbilical vein endothelial cell monolayers

Human umbilical vein endothelial cells were grown to subconfluence in human umbilical vein endothelial cell medium on type I collagen coated 24-well tissue culture plates.

2.7.1. Effects of butein or genistein on adhesion molecules on the surface of human umbilical vein endothelial cells

Human umbilical vein endothelial cells were treated with butein or genistein and TNF-α (final concentration at

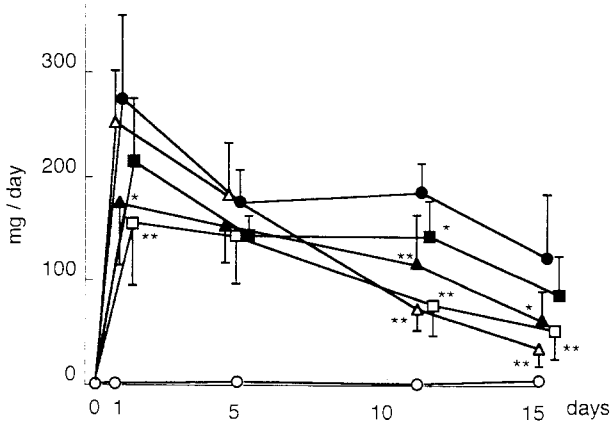

Fig. 1. Effect of butein on urinary protein excretion in original-type anti-glomerular basement membrane antibody-associated nephritis in rats. Test drugs were given p.o. daily during the period from the day of anti-glomerular basement membrane serum injection to 15 days. Each plot denotes the mean ± S.D. for 5 or 8 rats. Normal: ○; nephritic control: ●; nephritis + butein, 2 mg/kg: ■; nephritis + butein, 20 mg/kg: ▲; nephritis + butein, 200 mg/kg: □; nephritis + cyclosporin A, 20 mg/kg: △. * $P < 0.05$, ** $P < 0.01$, compared to nephritic control.

100 U/ml) or medium added simultaneously to each well. The plate was incubated for 12 h at 37°C in 5% CO_2. Polymorphonuclear leukocytes (0.4 ml of 1×10^7 cells/ml) were added to each well. The plates were incubated at 37°C and 5% CO_2 for 20 min. Cells were then washed with M199, 1 M NaOH was added to the wells and the radioactivity of the fluids was measured by a γ-counter.

2.7.2. Effect of butein on the function of adhesion molecules on the surface of human umbilical vein endothelial cells

Human umbilical vein endothelial cells were stimulated with TNF-α (100 U/ml) for 20 h. After being washed 3 times, the human umbilical vein endothelial cells were treated with butein for 1 h. After being washed, polymorphonuclear leukocytes (0.4 ml of 1×10^7 cells/ml) were added to each well. The plates were incubated at 37°C and 5% CO_2 for 20 min. The cells were washed with M199, 1 M NaOH was added to the wells and the radioactivity of the fluids was measured by a γ-counter.

2.7.3. Effect of butein on adhesion molecules on the surface of polymorphonuclear leukocytes

Polymorphonuclear leukocytes (0.4 ml of 1×10^7 cell/ml) were treated with or without butein for 1 h. After being washed, the polymorphonuclear leukocytes were added to human umbilical vein endothelial cells that had been stimulated with TNF-α (100 U/ml) for 12 h. The plates were incubated at 37°C and 5% CO_2 for 20 min. The cells were washed with M199, 1 M NaOH was added to the wells and the radioactivity of the fluids was measured by a γ-counter.

2.8. Statistical analysis

The data represent the means ± S.D. or S.E. and the results were statistically evaluated by analysis of variance. When the results were parametric, they were statistically evaluated by a Duncan multiple-range test. When the results were non-parametric, they were statistically evaluated by a Kruskal–Wallis test.

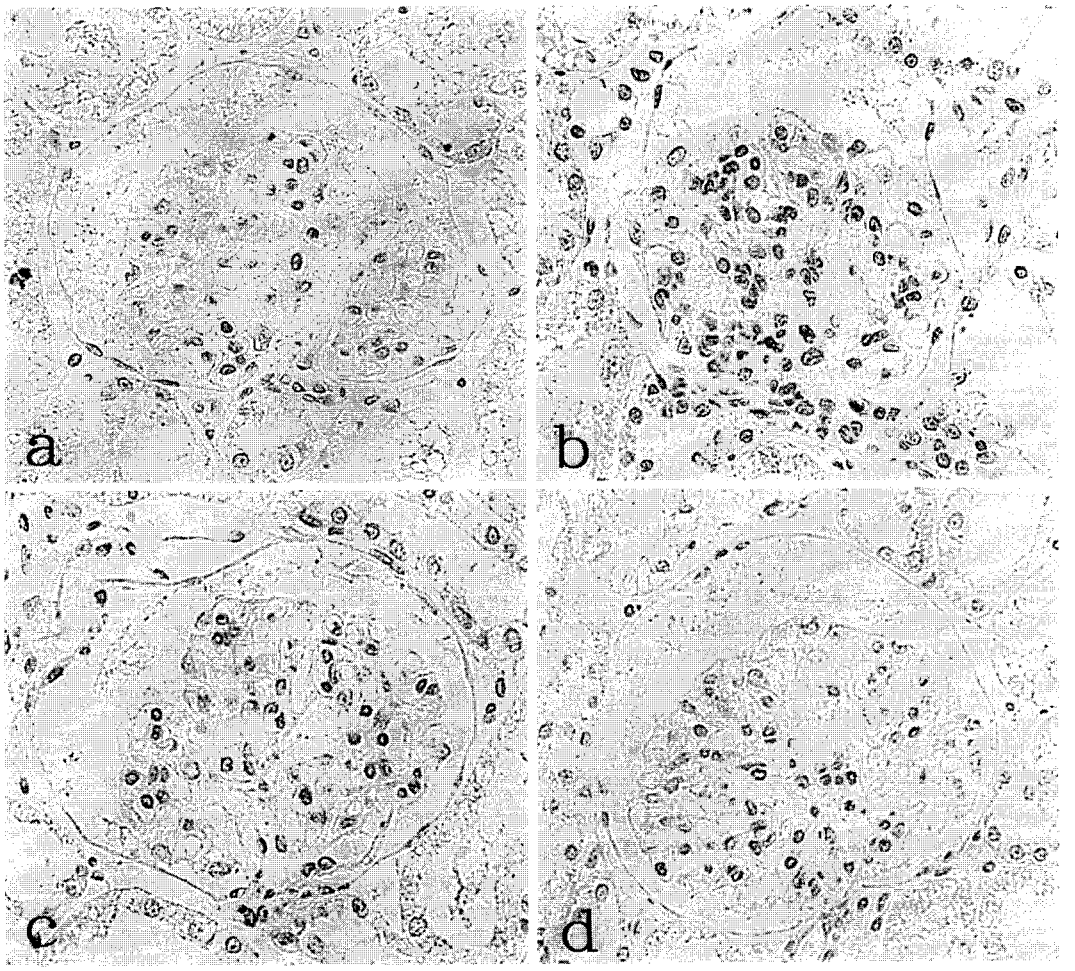

Fig. 2. Photographs of glomeruli from rats of the normal group (a); nephritic control group (b); group given butein (200 mg/kg) (c); group given cyclosporin A (20 mg/kg) (d). Test drugs were given p.o. daily during the period from the day of anti-glomerular basement membrane serum injection to 15 days (hematoxylin and eosin stain, ×400). Note that hypercellularity and adhesion to the capillary wall of Bowman's capsule are markedly less in the group treated with butein or cyclosporin A than in the nephritic control group.

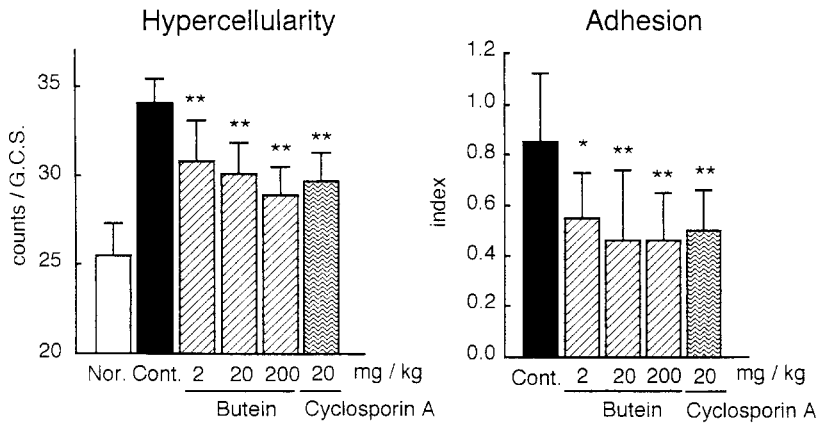

Fig. 3. Effect of butein on histopathological changes in the glomeruli in original-type anti-glomerular basement membrane antibody-associated glomerulonephritis in rats. Test drugs were given p.o. daily during the period from the day of anti-glomerular basement membrane serum injection to 15 days. The value for the adhesion of the normal group is almost 0. Nor., normal; Cont., nephritic control; G.C.S., glomerular cross-section. * $P < 0.05$, ** $P < 0.01$, compared to nephritic control.

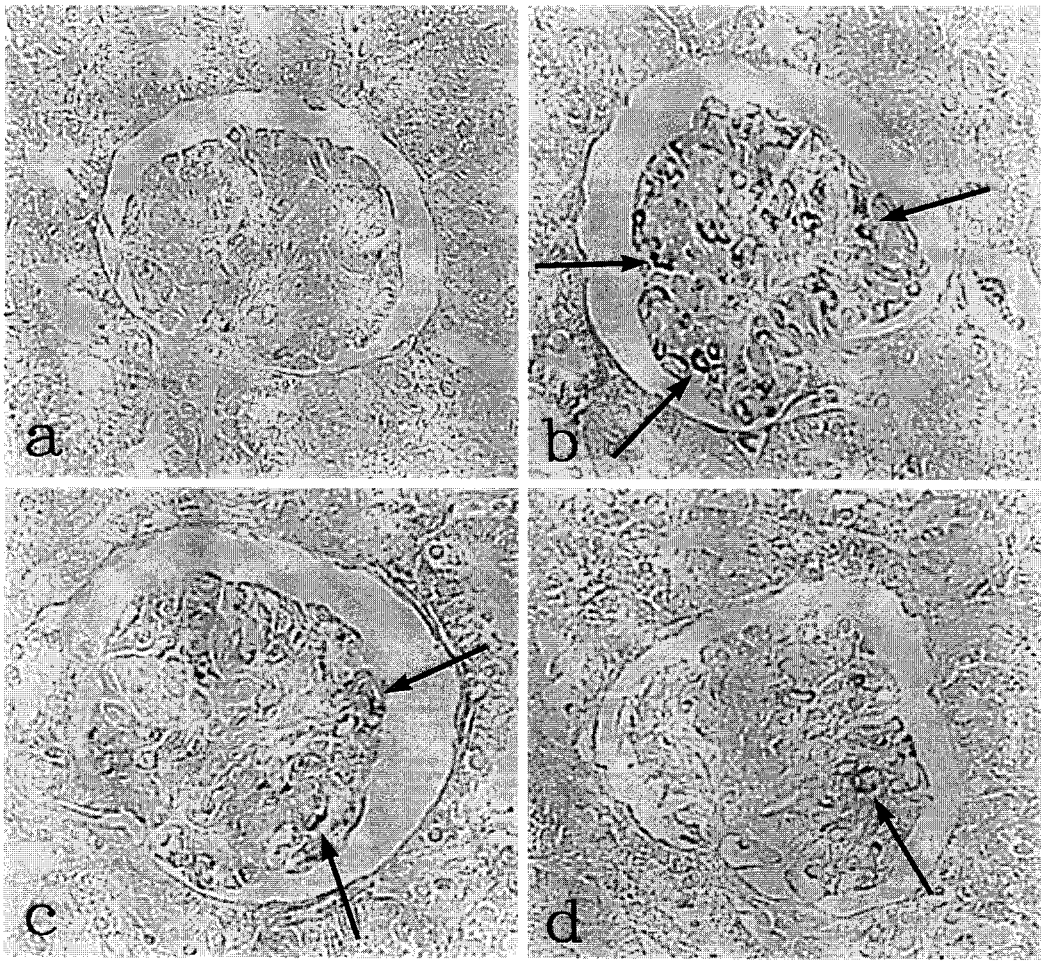

Fig. 4. Photographs of glomeruli immunohistochemically stained with anti-ICAM-1 monoclonal antibodies. Glomeruli were obtained 5 days after the anti-glomerular basement membrane serum injection. Normal (a); nephritic control (b); group given butein (200 mg/kg) (c); group given dexamethasone (0.1 mg/kg) (d). (→) indicates glomerular endothelial and mesangial localization of ICAM-1. Original magnification is ×400.

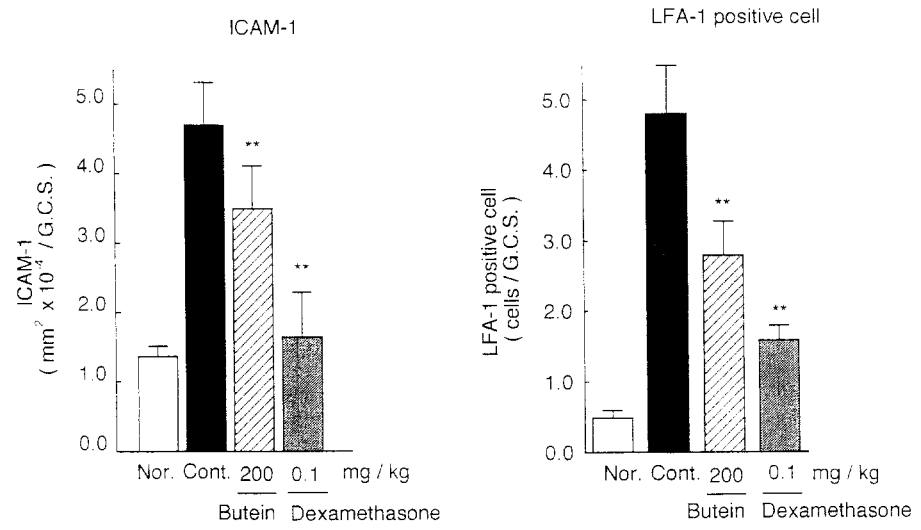

Fig. 5. Effect of butein on ICAM-1 expression in glomeruli of rats with original-type anti-glomerular basement membrane antibody-associated nephritis. Test drugs were given p.o. daily during the period from the day after the anti-glomerular basement membrane serum injection to 5 days. ICAM-1 expression was analyzed 5 days after the anti-glomerular basement membrane serum injection. Each column denotes the mean ± S.D. for 5 rats. Nor. normal; Cont. nephritic control. * $P < 0.05$, ** $P < 0.01$, compared to nephritic control.

3. Results

3.1. Effect of butein on anti-glomerular basement membrane antibody-associated glomerulonephritis

3.1.1. Urinary protein excretion (Fig. 1)

At 1 day following injection of the anti-glomerular basement membrane antibody, the nephritic control rats had severe proteinuria (nephritic control: 265.5 ± 79.8 mg/day, versus normal: 2.2 ± 0.4 mg/day) and the urinary protein excretion in the nephritic control was 122.1 mg/day on 15 days. Butein at 200 mg/kg per day p.o. markedly suppressed the urinary protein excretion by 37%–60%. Butein at 2 or 20 mg/kg per day p.o. also suppressed proteinuria at 11 days by 23%. Cyclosporin A at 20 mg/kg per day p.o. markedly suppressed protein excretion into urine at 11 days by 61%.

3.1.2. Histophatological alteration (Figs. 2 and 3)

Histopathological observations of the glomeruli on day 15 indicated that the number of nuclei and the incidence of adhesion in glomeruli had significantly increased in the nephritic control rats, as compared to normal animals. Hypercellularity and adhesion were significantly sup-

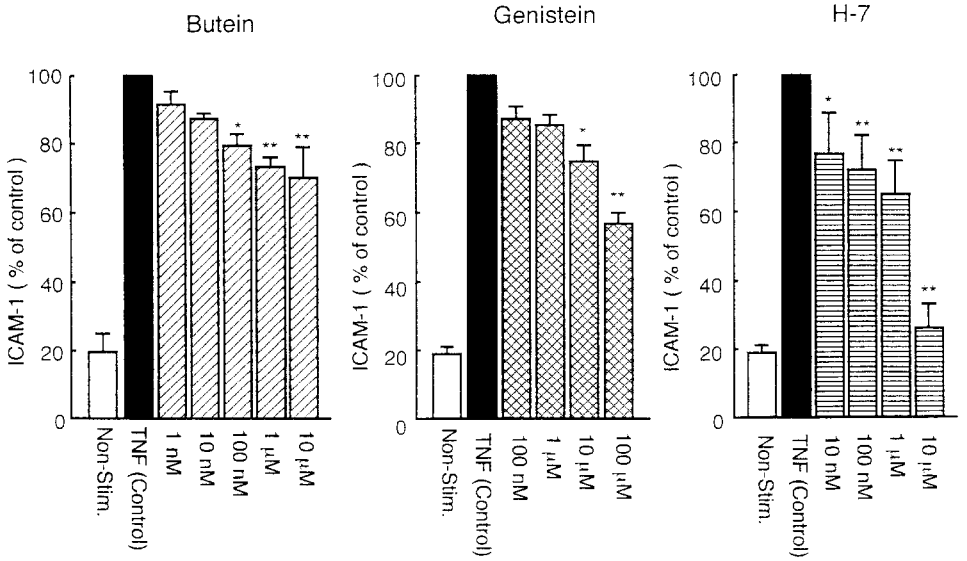

Fig. 6. Effect of butein on endothelial ICAM-1 expression induced by TNF-α. Human umbilical vein endothelial cells were stimulated with TNF-α (100 units/ml, 12 h) in the absence or presence of butein, genistein, or cyclosporin A. ICAM-1 expression was determined by cell ELISA. Results are presented as a percentage of ICAM-1 to expression induced by TNF-α in the absence of each drug and represent the means ± S.E. of 4–10 experiments. * $P < 0.05$, ** $P < 0.01$, compared to TNF-α stimulation (control).

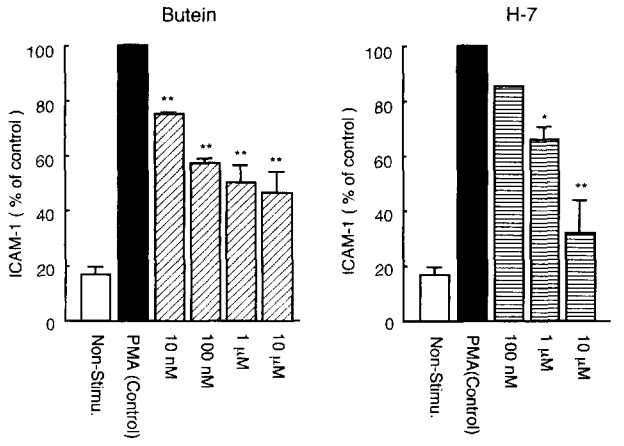

Fig. 7. Effect of butein on endothelial ICAM-1 expression induced by phorbol 12-myristate 13-acetate (PMA). Human umbilical vein endothelial cells were stimulated with PMA (100 ng/ml, 4 h) in the absence or presence of butein or H-7. ICAM-1 expression was determined by cell ELISA. Results are presented as a percentage of ICAM-1 expression induced by PMA in the absence of each drug and represent the means ± S.E. of 5–10 experiments. * $P < 0.05$, ** $P < 0.01$, compared to PMA stimulation (control).

pressed in the butein-treated groups at day 15. Cyclosporin A also suppressed these parameters.

3.2. Effect of butein on up-regulation of ICAM-1 and accumulation of LFA-1 positive cells in nephritic glomeruli (Figs. 4 and 5)

At 5 days following the anti-glomerular basement membrane serum injection, ICAM-1 expression and accumulation of LFA-1-positive cells in glomeruli was markedly greater in nephritic control rats than in normal animals. Butein at 200 mg/kg per day p.o. inhibited the up-regulation of ICAM-1 and the accumulation of LFA-1-positive cells at 5 days by 36% and 49%, respectively. Dexamethasone completely suppressed the up-regulation of ICAM-1 and markedly inhibited the accumulation of LFA-1-positive cells at 5 days.

3.3. Effect of butein on ICAM-1 expression on the surface of human umbilical vein endothelial cells in response to TNF-α or PMA (Figs. 6 and 7)

Endothelial ICAM-1 expression induced by TNF-α (100 U/ml) increased by 4 h and increased about 4-fold compared to the control level by 12–20 h. Treatment with PMA (100 ng/ml) resulted in the induction of ICAM-1, which peaked at 4 h and then diminished over the course of 72 h to levels approaching basal expression on unstimulated human umbilical vein endothelial cells (data not shown). Moreover, anti-TNF polyclonal antibody excluded ICAM-1 expression on human umbilical vein endothelial cells in response to TNF-α, but not to anti-IL-1 polyclonal antibody (data not shown).

As shown in Figs. 6 and 7, butein at 0.1–10 μM prevented ICAM-1 expression on human umbilical vein endothelial cells in response to TNF-α (12 h) by 25–35% at a nontoxic concentration of butein. Genistein and H-7 inhibited TNF-α-induced ICAM-1 expression on the surface of human umbilical vein endothelial cells by 27% and 90%, respectively. Cyclosporin A did not inhibit the up-regulation of ICAM-1 expression on human umbilical vein endothelial cells in response to TNF-α (12 h) (data not shown). Furthermore, butein markedly suppressed up-regulation of ICAM-1 on the surface of human umbilical vein endothelial cells stimulated by PMA (4 h). H-7 also prevented ICAM-1 expression of the human umbilical vein endothelial cells in response to PMA (4 h). However, butein failed to suppress the up-regulation of VCAM-1 and ELAM-1 expression on the surface of human umbilical vein endothelial cell in response to TNF-α or PMA (data not shown).

3.4. Effect of butein on polymorphonuclear leukocyte adhesion to human umbilical vein endothelial cells stimulated by TNF-α (Figs. 8–10)

Polymorphonuclear leukocyte adhesion to TNF-α activated human umbilical vein endothelial cells increased 5-fold compared to the adhesion to non-stimulated human umbilical vein endothelial cells. When human umbilical vein endothelial cells were simultaneously treated with butein and TNF-α, butein at 1 and 10 μM inhibited the adhesion of polymorphonuclear leukocytes to human umbilical vein endothelial cells by 65% and 98%, respectively

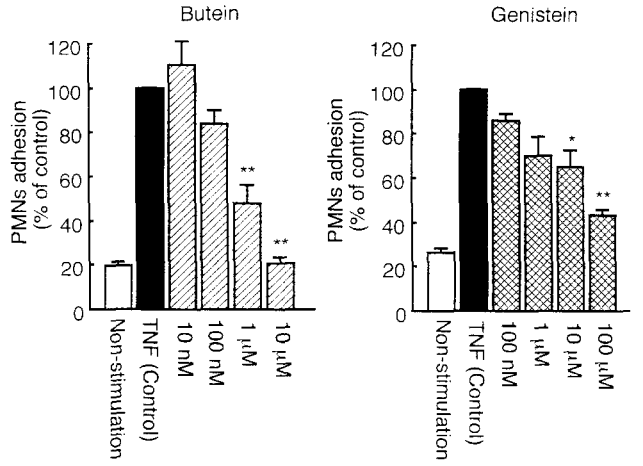

Fig. 8. Effects of butein, H-7 and cyclosporin A on the adhesion of polymorphonuclear leukocytes to TNF-α-stimulated human umbilical vein endothelial cells. Human umbilical vein endothelial cell monolayers were incubated with medium alone, TNF-α (100 units/ml) or TNFα + the drug for 12 h; the medium was exchanged, ^{51}Cr-labeled polymorphonuclear leukocytes were added to activated or inactivated human umbilical vein endothelial cells and they were incubated for 20 min at 37°C in 5% CO_2. Data shown represent the means ± S.E. of 3–6 experiments. * $P < 0.05$, ** $P < 0.01$, compared to TNF-α stimulation (control).

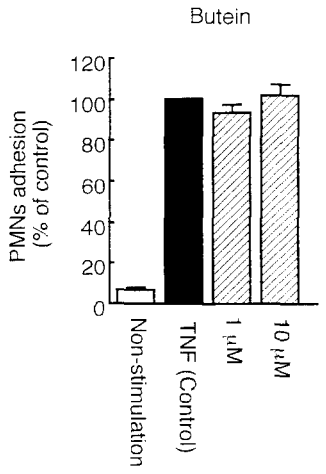

Fig. 9. Effects of butein on the function of adhesion molecules on the surface of human umbilical vein endothelial cells. Human umbilical vein endothelial cell monolayers were incubated with medium alone or TNF-α (100 units/ml) for 20 h; the medium was exchanged, human umbilical vein endothelial cell monolayer treated with medium alone or butein for 1 h, ^{51}Cr-labeled polymorphonuclear leukocytes were added to activated or inactivated human umbilical vein endothelial cells and they were incubated for 20 min at 37°C in 5% CO_2. Data shown represent the means ± S.E. of 2–4 experiments.

(Fig. 8). The effect of butein was dose-dependent. Genistein also inhibited the adhesion of polymorphonuclear leukocytes to activated human umbilical vein endothelial cells (Fig. 8). However, cyclosporin A failed to inhibit the adhesion of polymorphonuclear leukocytes to human umbilical vein endothelial cells stimulated by TNF-α (data not shown). When TNF-α-activated human umbilical vein endothelial cells were treated with butein, the adhesion of polymorphonuclear leukocytes was not affected by butein (Fig. 9).

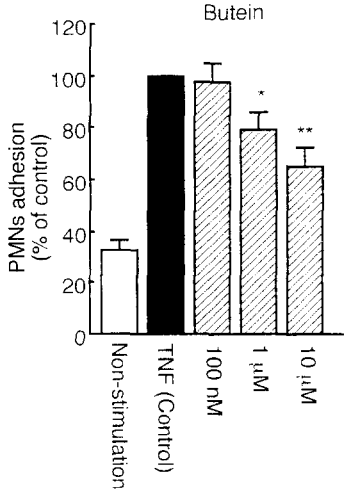

Fig. 10. Effects of butein on adhesion molecules on the surface of polymorphonuclear leukocytes. Human umbilical vein endothelial cell monolayers were incubated with medium alone, TNF-α (100 units/ml) for 12 h; the medium was exchanged, ^{51}Cr-labeled polymorphonuclear leukocytes treated with or without butein were added to activated or inactivated human umbilical vein endothelial cells and they were incubated for 20 min at 37°C in 5% CO_2. Data shown represent the means ± S.E. of 6 experiments. * $P < 0.05$, ** $P < 0.01$, compared to TNF-α stimulation (control).

When polymorphonuclear leukocytes were treated with butein, it (1 and 10 μM) inhibited the adhesion of polymorphonuclear leukocytes to TNF-α-activated human umbilical vein endothelial cells by 20% and 35% (Fig. 10).

4. Discussion

Our previous study demonstrated that butein suppressed the accumulation of CD4-positive cells and CD8-positive cells in glomeruli of rats with crescentic-type anti-glomerular basement membrane antibody-associated glomerulonephritis (Hayashi et al., 1996). In the present experiment, butein inhibited urinary protein excretion (Fig. 1) and histological alterations including hypercellularity (Figs. 2 and 3) and reduced the extent of ICAM-1 expression and accumulation of LFA-1-positive cells in nephritic glomeruli (Figs. 4 and 5) in anti-glomerular basement membrane antibody-associated glomerulonephritis. Butein suppressed up-regulation of ICAM-1 on the surface of human umbilical vein endothelial cells in response to TNF-α or PMA (Figs. 6 and 7), but not VCAM-1 and ELAM-1 expression. Moreover, butein inhibited the adhesion of polymorphonuclear leukocytes to human umbilical vein endothelial cells treated with TNF-α when either human umbilical vein endothelial cells or polymorphonuclear leukocytes were treated with butein (Figs. 8 and 10). However, butein did not inhibit the function of ICAM-1 (Fig. 9).

Leukocyte influx into nephritic glomeruli has been reported in experimental and human nephritis (Main et al., 1992; Holdsworth and Neale, 1984; Bolton et al., 1987). The inhibition of progression of glomerulonephritis by the administration of anti-macrophage sera or anti-ED-1 or CD8 monoclonal antibody suggests that monocytes or CD8-positive cells are one of the main factors in the progression of glomerular disease (Holdsworth et al., 1981; Kawasaki et al., 1992; Hattori et al., 1994). Moreover, Hattori et al. (1994) reported that ED-1-positive cells and CD8 positive cells contributed to the development of this nephritic model and that the immunosuppressive agents, cyclosporin A and azathioprine, markedly slowed the progress of this nephritis by inhibiting leukocyte accumulation in the nephritic glomeruli. Recent studies suggest a role for an adhesion molecule in mediating the migration of leukocytes in nephritic glomeruli, as assessed with anti-LFA-1 and ICAM-1 monoclonal antibodies in rats (Mulligan et al., 1993; Kawasaki et al., 1993). Therefore, the antinephritic action of butein may be due to the prevention of intraglomerular leukocyte infiltration through the inhibition of up-regulation of ICAM-1 expression on the surface of glomerular endothelial cells and the function of adhesion molecules on the surface of leukocytes.

In the in vitro studies, butein markedly inhibited the adhesion of polymorphonuclear leukocytes to human um-

bilical vein endothelial cells (Fig. 8) while inhibiting TNF-α-induced ICMA-1 expression on the surface of human umbilical vein endothelial cells by only about 40%. The significant inhibitory effect of butein on the adhesion of polymorphonuclear leukocytes to human umbilical vein endothelial cells is not completely explained by the result observed in the present experiment regarding ICAM-1 expression. Therefore, we carried out the next experiment to confirm the effect of butein on the function of adhesion molecules on the surface of polymorphonuclear leukocytes. When polymorphonuclear leukocytes were treated with butein, their adhesion to human umbilical vein endothelial cells was significantly suppressed. This result suggests that butein suppressed the accumulation of leukocytes in nephritic glomeruli through inhibition of the up-regulation of ICAM-1 expression in the nephritic glomeruli and inhibition of the function of adhesion molecules on the surface of leukocytes.

Recently, numerous investigations (VanArsdale and Ware, 1994; Hannum and Obeid, 1995) have clarified intracellular signal transduction after TNF-α stimulation. That ceramide activates protein kinase Cζ and phosphorylates EGF receptor in TNF signaling has aroused much interest (Lozano et al., 1994; Goldkorn et al., 1991; Hannum and Obeid, 1995). Furthermore, it is reported that TNF-α-induced expression of ICAM-1 linked to protein kinases C activation and protein kinase C activation can induce ICAM-1 mRNA expression in endothelial cells (Mattila et al., 1992; Myers et al., 1992). Staurosporin inhibits various kinases including protein kinase C and Src and suppresses TNF-α- or PMA-induced ICAM-1 expression on the surface of human umbilical vein endothelial cells (Lane et al., 1990). In this study, genistein, which inhibits tyrosine kinase, $pp60^{v\text{-}src}$ and EGF receptor-associated tyrosine kinase (Akiyama et al., 1989), also suppressed ICAM-1 expression on the surface of human umbilical vein endothelial cells stimulated by TNF-α (Fig. 6). These results suggest that there is a protein kinase C, Src and EGF receptor-associated tyrosine kinase-mediated pathway in the intracellular signal transduction for ICAM-1 expression mediated by TNF-α. Butein includes caffeic acid in its structure, which is analogous to that of genistein. It has been reported that butein suppresses the proliferation of human colon adenocarcinoma and HeLa cell (Yit and Das, 1994; Ramanathan et al., 1992). Moreover, caffeic acid derivatives inhibit lymphocyte tyrosine protein kinase, $p56^{lck}$, or FcϵR1-associated protein-tyrosine kinase, Sky, in in vitro studies (Li et al., 1991; Oliver et al., 1991). In the present experiments, butein suppressed the up-regulation of ICAM-1 expression on the surface of human umbilical vein endothelial cells stimulated by TNF-α or PMA. These results suggest that butein suppresses the up-regulation of ICAM-1 through the inhibition of TNF-α-mediated intracellular signal transduction.

To demonstrate this hypothesis, in further studies, we are going to investigate the effect of butein on TNF-α- or PMA-induced ICAM-1 mRNA expression and activity of kinases including protein kinase C.

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