Combination treatment of dipeptidyl peptidase IV inhibitor (sitagliptin) and angiotensin-II type 1 receptor blocker (losartan) suppresses progression in a nondiabetic rat model of steatohepatitis

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Running title: Effects of DPP4-I + ARB combination on NASH

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Abstract:

Aim: Dipeptidyl peptidase-4 (DPP4) inhibitors (DPP4-I) are oral glucose-lowering drugs for type 2 diabetes mellitus. Previously, we demonstrated that DPP4-I (sitagliptin) exerted suppressive effects on experimental liver fibrosis in rats. Blockade of the renin–angiotensin system by angiotensin-II type 1 receptor blocker (ARB: losartan), commonly used in the management of hypertension, has been shown to significantly alleviate hepatic fibrogenesis and carcinogenesis. We aimed to elucidate the effects and possible mechanisms of a sitagliptin+losartan combination on the progression of nondiabetic nonalcoholic steatohepatitis (NASH) in a rat model.

Methods: To induce NASH, Fischer 344 rats were fed a choline-deficient L-amino acid-defined (CDAA) diet for 12 weeks. We elucidated the chemopreventive effects of sitagliptin+losartan, especially in conjunction with hepatic stellate cell (HSC) activation, angiogenesis, and oxidative stress, all known to play important roles in the progression of NASH.

Results: Sitagliptin+losartan suppressed CDAA diet-induced hepatic fibrogenesis and carcinogenesis. The combination treatment exerted a greater inhibitory effect than monotherapy. These inhibitory effects occurred almost concurrently with the suppression of HSC activation, neovascularization, and oxidative stress. In vitro studies showed that sitagliptin+losartan inhibited angiotensin II-induced the proliferation and expression of transforming growth factor-β1 and α1 (I)-procollagen mRNA of activated HSC and in vitro angiogenesis, in parallel with the suppression observed in in vivo studies.

Conclusions: The widely and safely used sitagliptin+losartan combination

treatment in clinical practice could be an effective strategy against NASH.

Keywords: nonalcoholic steatohepatitis, hepatic fibrogenesis, hepatocarcinogenesis, sitagliptin, losartan

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease (CLD) in developed countries and is recognized as the hepatic manifestation of metabolic disorders, including type-2 diabetes mellitus (T2DM) and hypertension.[1] Approximately 20%–30% NAFLD patients develop nonalcoholic steatohepatitis (NASH), but only some further develop fibrosis, cirrhosis, and eventually, hepatocellular carcinoma (HCC).[2] Dipeptidyl peptidase-4 inhibitors (DPP4-Is: sitagliptin) are commonly used in T2DM treatment. DPP4/CD26 is a 110-kDa cell-surface glycoprotein (serine protease family) highly expressed in endothelial cells (ECs), epithelial cells, and T lymphocytes. DPP4 is identified as a multifunctional protein involved in different biological processes, including inflammation, malignant transformation, and tumor immunity.[3, 4] However, the physiological functions of DPP4 in NASH pathogenesis are incompletely understood.

The renin–angiotensin system (RAS) is crucial in CLD.[5] We have demonstrated that angiotensin-II (AT-II) signal transduction blockade through AT-II type-1 receptor (AT1R) inhibits hepatic fibrogenesis along with the suppression of hepatic stellate-cell (Ac-HSC) activation.[6] RAS blockade by clinically relevant doses of AT1R blocker (ARB: losartan) inhibits hepatocarcinogenesis[7] and HCC growth,[8] while inhibiting vascular endothelial growth factor (VEGF)-mediated neovascularization. Although monotherapy is desirable, liver fibrosis and carcinogenesis are often inadequately prevented,[9] and combination therapies exert more potent inhibitory effects on NASH progression.[7,10] Varying pharmacotherapeutic approaches in combination with losartan monotherapy are

required for effective NASH treatment. We aimed to elucidate the effect of sitagliptin + losartan combination therapy on NASH progression in rats induced by a choline-deficient L-amino acid-defined (CDAA) diet. We also investigated possible mechanisms related to this therapeutic effect.

Methods

Animals and reagents

Fifty 6-week-old male Fischer 344 (F344) rats (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were housed in stainless steel mesh cages under controlled conditions: temperature 23° ± 3°C; relative humidity 50% ± 20%; 10–15 air/h; and 12-h day/night cycle. Animals had *ad libitum* tap water access. Sitagliptin and losartan were purchased from Merck Ltd. (Tokyo, Japan). AT-II and vascular endothelial growth factor (VEGF) were purchased from WAKO Pure Chemical Industries, Ltd. (Tokyo, Japan) and Sigma-Aldrich Corp (Tokyo, Japan), respectively. Conventional chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan). CDAA diet and choline-sufficient L-amino acid-defined (CSAA) diet were purchased from CLEA Japan Inc. (Tokyo, Japan).

Animal treatment

All experiments were conducted over 12 weeks. Rats were randomly divided into five groups (n = 10 in each experimental group). Rats in group (G)1 were designated the negative control group, fed a CSAA diet, and given distilled water as a vehicle. Rats in G2 5 were fed a CDAA diet. Rats in G2 were given phosphate-buffered saline (PBS) through daily oral gavage. Clinically-equivalent

doses of sitagliptin (150 mg/kg/day)[11] and losartan (30 mg/kg/day)[12,13] were given to G3 and G4 rats, respectively, through oral gavage. Rats in G5 were given sitagliptin+losartan. Food intake was similar among groups. At end of the study period, rats were anesthetized with diethyl ether and several indices were examined. All procedures were performed according to standard protocols, in compliance with standard recommendations.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was extracted from powdered frozen liver and intestinal tissues using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Total RNA (1 μg) from each sample underwent RT to complementary DNA (cDNA) using the High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. mRNA expression levels, including those of transforming growth factor (TGF)-β 1, α1(I)-procollagen, and CD31 from liver tissue and isolated Ac-HSCs were quantified by real-time RT-PCR using SYBR Green on a Step One Plus sequence-detection system (Applied Biosystems Inc.). PCR procedures were as follows: samples were heated for 20 s at 95°C and were then subjected to 40 cycles of denaturing for 3 s at 95°C and annealing for 30 s at 60°C. In this experiment, beta-actin (β-actin) was used as an endogenous control. Primer sequences used were as follows: TGF-β1, forward 5′-CGGCAGCTGTACATTGACTT-3′ and reverse 5′-AGCGCACGATCATGTTGGAC-3′; α1(I)-procollagen, forward 5′-AGCTCCTGGGCCTATCTGATGA-3′; CD31, forward, 5′-

GGCGTCCTGTCCGGAATC-3′ and reverse 5′-AGAACTCCTGCACAGTGACGTATT-3′; β-actin, forward 5′-GGAGATTACTGCCCTGGCTCCTA-3′ and reverse 5′-GACTCATCGTACTCCTGC TTGCTG-3′.

Histological and immunohistochemical analyses

In all experimental groups, 5-μm-thick sections of formalin-fixed paraffin-embedded liver specimens were routinely processed for Sirius Red staining to evaluate hepatic fibrosis. Immunohistochemical staining of enzyme-altered preneoplastic lesions [i.e., placental forms of glutathione S-transferase (GST-P) (MBL Co. Ltd., Nagoya, Japan)] was performed with α-smooth muscle actin (α-SMA) (DAKO, Kyoto, Japan) and 8-hydroxydeoxyguanosine (8-OHdG) (NIKKEN SEIL, Tokyo, Japan), as described previously.[14,15] Stained sections were analyzed using Image J Software (National Institutes of Health). Six microscopic visual fields (magnification ×40)/specimen from the 10 rats were used for Image J analysis, as described previously.[16] Histological features were semiquantitatively assessed in accordance with the NAFLD activity scoring system, as described previously.[17]

Measurement of VEGF and malondialdehyde (MDA)

After equalization of the protein contents of 200 mg of frozen liver samples,[18] hepatic VEGF and MDA levels were determined using enzyme-linked immunosorbent assay (ELISA) (R&D Systems) and MDA assay kits (NWLSS, Vancouver, WA), respectively, according to the manufacturers' instructions.

In vitro assays

HSCs were isolated from F344 rats by sequential hepatic digestion with pronase and collagenase, as described previously.[19] Freshly isolated HSCs were plated at a density of 5×10^5 cells/ml on uncoated plastic dishes. After a 5-day culture, HSCs became similar to myofibroblasts with decreased lipid vesicles and increased α-SMA expression. After activation of HSCs by a 7-day plastic culture, all cells became uniformly distributed and α-SMA-positive. The human HepG2 HCC cell line and human umbilical vein ECs (HUVECs) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). *In vitro* proliferation was examined using a colorimetric assay according to the cleavage of WST-1 (a tetrazolium salt) by mitochondrial dehydrogenases to form formazan in viable cells (Roche Applied Science, Laval, Canada). In brief, Ac-HSC, HepG2 cells, or HUVECs were seeded in 96-well plates comprising 100 μl of FBS-free media and were incubated at 37°C for 48 h in the presence or absence of AT-II (1 μ M), sitagliptin (2 μ M), and/or losartan (10 μ M), followed by the addition of WST-1 (20 μl). After 1 h of incubation, the absorbance of each well was measured at 450 nm using a microplate reader at a 620-nm reference wavelength. *In vitro* angiogenesis was assessed as capillary-like structure formation in HUVECs co-cultured with human diploid fibroblasts, as described previously.[15] For the *in vitro* assessment of angiogenesis, we used the EC-tube formation assay, as described previously.[20] In brief, Matrigel (Collaborative Biochemical Products, Bedford, MA) was placed in 24-well tissue culture plates (150 ml/well) and was allowed to set at 37°C for 30 min. In total, 2.5×10^4 HUVECs were added to each well and

incubated at 37°C for 20 h under a 5% CO_2 atmosphere in the presence or absence of sitagliptin+losartan. Semiquantitative analysis of tubule formation was performed using the Fuji BAS 2000 image analyzer (Fuji, Tokyo, Japan).

Statistical analyses

Student's *t*-test or one-way analysis of variance, followed by Bonferroni's multiple-comparison test, was performed. Barlett's test was performed to determine the homology of variance. Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA). All tests were two-tailed, and *P*-values of <0.05 were considered statistically significant.

Results

General findings

Data from all experimental groups are shown in Table.1. The final body weight and relative liver weight in CDAA-fed rats (G2–G5) were lesser than those in CSAA-fed rats (G1). The relative liver weights in G2–G5 were greater than those in G1. The serum alanine aminotransferase (ALT) level was significantly higher in G2–G5 than in G1. The ALT level was significantly lower in G3 and G5 and marginally lower in G4 than in G2. No significant differences in albumin, total bilirubin, glucose, and insulin levels were observed among the groups.

Effects of sitagliptin+losartan on hepatic fibrogenesis

First, we examined the effects of clinically comparable doses of sitagliptin+losartan on liver fibrosis induced by a CDAA diet for 12 weeks. As

shown in Fig. 1, hepatic fibrogenesis was significantly suppressed by sitagliptin (G3) and losartan (G4) compared with that in the CDAA group (G2). Sitagliptin+losartan (G5) exerted a stronger inhibitory effect than either monotherapy. There was no fibrosis development in CSAA-treated rats (G1). α-SMA immunochemistry was performed to evaluate the suppressive effect of sitagliptin+losartan on HSC activation during hepatic fibrogenesis. A significant decrease in α-SMA-positive Ac-HSC numbers was observed with sitagliptin+losartan (Fig. 2A). Computer-assisted semiquantitative analysis of α-SMA immunohistochemistry revealed a decrease in the α-SMA staining area along with hepatic fibrogenesis inhibition (Fig. 2B). We found that sitagliptin+losartan markedly suppressed hepatic mRNA expression of both TGF-β1 and α1 (I)-procollagen compared with that in G2 rats (Figs. 3A and 3B, respectively). Similar to the effect on liver fibrosis, sitagliptin+losartan showed more potent inhibitory effects on hepatic expression of TGFβ1 and α1 (I)-procollagen than either monotherapy. These suppressive effects were consistent with a reduction in the hepatic fibrosis area.

In vitro effects of sitagliptin+losartan on Ac-HSC

Next, we examined the direct effect of sitagliptin+losartan on AT-II-induced activated Ac-HSC proliferation *in vitro*. Primary HSCs incubated with AT-II for 48 h showed significant increments in cell proliferation. Either monotherapy significantly attenuated this stimulating effect, and sitagliptin+losartan exerted a stronger inhibitory effect on AT-II-induced Ac-HSC proliferation (Fig. 4A). We further examined the mRNA expression of TGF-β1 and α1 (I)-procollagen by Ac-

HSCs. Similar to the relationship of hepatic α-SMA-positive cells, the suppressive effect of sitagliptin+losartan on TGF-β1 and α1 (I)-procollagen mRNA expression *in vitro* paralleled the suppression of Ac-HSC proliferation (Figs. 4B and 4C).

Effects of sitagliptin+losartan on preneoplastic lesion development

We subsequently investigated the effects of sitagliptin+losartan on GST-P-positive preneoplastic lesions in conjunction with neovascularization (Fig. 5A). GST-P-positive preneoplastic lesion size and number were significantly decreased after sitagliptin+losartan treatment (Figs. 5B and 5C). Similar to the changes in liver fibrosis, sitagliptin+losartan exerted a stronger inhibitory effect than either monotherapy. No GST-P-positive lesions developed in G1 rats. To elucidate the association between the inhibitory effects of sitagliptin+losartan on the development of preneoplastic lesions and suppression of neovascularization, the effect of sitagliptin+losartan on hepatic CD31 mRNA expression was determined using real-time PCR. Sitagliptin+losartan significantly attenuated CD31 mRNA expression, along with the inhibition of GST-P-positive preneoplastic lesions (Fig. 6A). To elucidate whether the suppressive effects on neovascularization were accompanied by VEGF inhibition, we further examined the effect of sitagliptin+losartan on hepatic VEGF protein expression. As shown in Fig. 6B, sitagliptin+losartan exerted a greater inhibitory effect on hepatic VEGF expression than either monotherapy; this was accompanied by the suppression of CD31 mRNA expression.

Effect of sitagliptin+losartan on HCC cells in vitro

To elucidate the possible mechanism of the inhibitory effect of sitagliptin+losartan, we explored the effect of these agents on HCC cell line proliferation. AT-II- and VEGF-induced HepG2-cell proliferations were not affected by clinically equivalent doses of sitagliptin+losartan (Fig. 7A).

Effect of sitagliptin+losartan on EC-tube formation in vitro

We also examined the effects of sitagliptin+losartan on EC proliferation. Similar to the effect on HepG2 cell, AT-II-induced EC cell proliferation was not affected by sitagliptin and/or losartan (Fig. 7B). To elucidate whether sitagliptin+losartan directly affected angiogenesis, we further examined the effect of both agents on EC-tube formation *in vitro*. AT-II significantly promoted EC-tube formation, and sitagliptin and losartan individually suppressed AT-II-induced EC-tube formation. Sitagliptin+losartan exerted more potent suppression than either monotherapy (Fig. 8A). Semiquantitative analysis confirmed that the total length of tubules formed in the sitagliptin+losartan culture was almost similar to that formed in the untreated control culture (Fig. 8B).

Effects of sitagliptin+losartan on hepatic oxidative stress

Because reactive oxygen species (ROS) plays a critical role in NASH progression, we elucidated the effect of sitagliptin+losartan on markers of lipid peroxidation and oxidative DNA damage, particularly MDA and 8-OHdG, respectively. Compared with G2, the MDA content was significantly decreased in the sitagliptin (G3) and losartan (G4) groups (Fig. 9A). The number of hepatic 8-OHdG-immunopositive cells was also lower in G3 and G4 than in G2 (Fig. 9B). The

inhibitory effects of both agents on MDA and 8-OHdG were of similar magnitude, and sitagliptin+losartan exerted a much stronger inhibitory effect than either monotherapy.

Changes in NAFLD activity score

Regarding the microscopic observation of steatosis, inflammation, and ballooning, there was significant histological improvement in these three parameters in G3 and G5. Losartan marginally improved steatosis and inflammation (Table 2). These findings were associated with improved ALT levels, suggesting that sitagliptin exerted cytoprotective effects on hepatocytes.

Discussion

In the present study, we demonstrated that sitagliptin+losartan markedly attenuated CDAA-diet-induced hepatic fibrogenesis and carcinogenesis almost parallel with Ac-HSC, VEGF-mediated neovascularization, and ROS. Sitagliptin + losartan demonstrated greater antifibrotic and anticarcinogenic effects than either monotherapy. Although we observed that either monotherapy exerted significant inhibitory effect on liver fibrogenesis and carcinogenesis progression in a rat model of steatohepatitis, it may be difficult to completely suppress the cumulative development of liver fibrosis and HCC with a single agent in clinical practice.[21] We demonstrated that sitagliptin at a clinically equivalent dose successfully inhibited liver fibrosis via the suppression of several intracellular signaling pathways in As-HSC.[11] We also previously demonstrated that AT-II signal transduction blockade through AT1R inhibited liver fibrosis[22] and

preneoplastic lesion[9] development in rats. The use of a single pharmocotherapeutic agent to inhibit cumulative development of CLD has proved challenging in experimental[23] and clinical practices.[21] Accordingly, sitagliptin+losartan may have utility in NASH treatment. The beneficial effects of sitagliptin+losartan on inflammatory activity and oxidative stress in diabetic mice have been reported.[24] For future clinical applications, sitagliptin+losartan will likely be required to yield a substantial therapeutic benefit in slowing NASH progression.

In the present study, the inhibitory effect of sitagliptin+losartan on hepatocarcinogenesis was mainly mediated through the inhibition of EC-tube formation and was not because of the direct action of *in vitro* EC- or HCC-cell proliferation. The inhibition of EC-tube formation was mediated by the suppression of vascular endothelial (VE)–cadherin tyrosine phosphorylation and inhibition of Akt activation.[25] In contrast, *in vitro* studies revealed that sitagliptin enhanced retinal vascular permeability through VE–cadherin phosphorylation.[26] Recent evidence indicates a paradoxical impairment of angiogenesis, endothelial function, and circulating numbers of endothelial progenitor cells in DPP4-deficient rats after critical limb ischemia.[27] Moreover, sitagliptin reportedly accelerates endothelial regeneration after acute arterial injury by enhanced recruitment of circulating progenitor cells.[28] This discrepancy may partly be because of varying DPP4 activity among different organs and tissues. Collectively, sitagliptin+losartan-mediated chemoprevention of experimental hepatocarcinogenesis could be achieved, at least in part, through the suppression of VEGF-mediated neovascularization.

DPP4 plays a pivotal role in adaptive immunity, as represented by cytokine production, lymphocyte trafficking of toll-like receptor activation,[29] and immune cell activation through the intracellular signaling pathway, including mitogen-activated protein kinase.[30] Sitagliptin protects the biologically active form of CXCL10, which represents an angiostatic chemokine, and results in the reduction of melanoma growth by modulation of the tumor microenvironment and enhancement of antitumor immunity.[4] Taken together, it is possible that the effects of sitagliptin+losartan were not simply because of VEGF-mediated hepatic neovascularization suppression, i.e., the coordinated effects of sitagliptin and losartan on different pathways were involved. Further studies are warranted to elucidate the exact mechanism underlying the enhancement of the anticarcinogenic properties of sitagliptin in NASH progression.

However, the exact mechanism underlying the effects of sitagliptin+losartan remains unclear at this time. NASH is considered a disorder of different pathogeneses framed according to the multiple parallel hit theory, which states that inflammation occurs as a consequence of discrete entities, including oxidative stress and lipid peroxidation.[31] Increased ROS production reportedly occurs very early in the histological spectrum of NAFLD.[32] A CDAA diet triggered severe fibrosis and enzyme-altered preneoplastic foci at 12 weeks through the generation of oxidative stress in the rat liver due to strong N2-guanine tRNA methyltransferase II activity[33, 34] and relative resistance to DNA hypomethylation. [35] In contrast, no discernible preneoplastic lesion was found at 22 weeks after CDAA diet feeding because of the lack of a sensitive marker for small tumor foci in mice, as typified by the glutathione S-transferase placental

form in rats.[36, 37] Preneoplastic foci of altered hepatocytes were observed at 65 weeks in mice fed CDAA diet.[38] Therefore, we focused on the role of ROS in NASH progression in rats. Consistent with our study, another study showed that sitagliptin decreases 1,2-dimethylhydrazine-induced colon carcinogenesis, along with the inhibition of ROS production in rats.[39] Sitagliptin reportedly inhibits methionine/choline-deficient diet-induced steatohepatitis by downregulating hepatic expression of p450 2E1 and 4HNE, both of which are referred to as biomarkers of oxidative stress.[40] The mechanism by which sitagliptin inhibits oxidative stress is unclear, but one possibility is a decrease in myeloperoxidase activity that catalyzes the conversion of nitrite into the reactive nitrite free radical, as demonstrated in the striatum of parkinsonian rats.[41] Losartan suppresses AT-II-induced ROS production via AT1R and NADPH oxidase-2.[42] The direct interaction among sitagliptin+losartan, angiogenesis, and ROS during hepatocarcinogenesis is currently being assessed in our laboratory. Nevertheless, our present findings led us to surmise that sitagliptin+losartan can potentially exert synergistic effects on the prevention of hepatocarcinogenesis by the suppression of angiogenesis and concomitant reduction of oxidative stress.

In summary, we have shown that sitagliptin+losartan is beneficial in hepatic fibrogenesis and carcinogenesis almost concurrently with the suppression of Ac-HSC, neovascularization, and oxidative stress. Notably, the inhibitory effects of both agents on NASH progression could be achieved at clinically comparable and low doses. Because both agents are widely and safely used in clinical practice, sitagliptin+losartan may represent a potential future therapeutic strategy against CLD progression.

Conflict of Interest

The authors declare no conflicts of interest.

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Figure legends

Fig. 1 (A) Photomicrographs of hepatic sections with Sirius Red staining. Extensive liver fibrosis with accumulation of lipid droplets is observed in the CDAA group (G2). Monotherapy with either sitagliptin (G3) or losartan (G4) demonstrates a significant inhibitory effect. Sitagliptin+losartan (G5) has a more potent inhibitory effect than either monotherapy. No fibrosis was observed in the CSAA group (G1). (B) Semiquantitative analysis confirms histological findings.

Fig. 2 (A) Immunohistochemical analysis of α-SMA expression.
Compared with G2, monotherapy with either sitagliptin (G3) or losartan (G4) results in a marked inhibitory effect on hepatic α-SMA expression. Sitagliptin+losartan (G5) exerts a stronger inhibitory effect. No α-SMA-positive cells are observed in hepatic sections from G1 rats. (B) Cells stained by α-SMA immunohistochemistry were measured by an image analysis software.
Values represent mean ± SD (bars; n = 10).
*$P < 0.05$, **$P < 0.01$

Fig. 3 Effects of sitagliptin+losartan on hepatic mRNA expression of TGF-β1 and α1(I)-procollagen.
(A) TGF-β1 and (B) α1(I)-procollagen mRNA levels are measured by real-time PCR as described in Methods. Compared with monotherapy groups (G3, G4), Sitagliptin+losartan (G5) shows a significant inhibitory effect on hepatic TGF-β1 and α1(I)-procollagen mRNA expression. These inhibitory effects closely match the changes in fibrosis development and α-SMA.

Data represent mean ± SD (bars; n = 10).

*P < 0.05, **P < 0.01

Fig. 4 Effects of sitagliptin+losartan on AT-II-induced Ac-HSC proliferation and TGF-β1 and α1(I)-procollagen mRNA expression.

(A) AT-II-induced Ac-HSC proliferation is attenuated by either monotherapy. Sitagliptin+losartan exerts a stronger suppressive effect than either monotherapy. Compared with monotherapy groups (G3, G4), sitagliptin+losartan (G5) shows a significant inhibitory effect on AT-II-induced TGF-β1 (B) and α1(I)-procollagen (C) mRNA expression in Ac-HSC. These inhibitory effects closely match the changes in Ac-HSC proliferation.

Bars represent mean ± SD (bars; n = 8).

*P < 0.05, **P < 0.01

Fig. 5 (A) Representative photomicrographs of GST-P-positive hepatic preneoplastic lesions. (B) Semiquantitative analysis reveals that monotherapy with either sitagliptin (G3) or losartan (G4) exerts significant inhibitory effects on the size and number of preneoplastic foci compared with that in CDAA-fed rats (G2). Sitagliptin+losartan (G5) exerts a stronger inhibitory effect. No preneoplastic lesions are observed in CSAA-fed rats (G1).

Values are presented as mean ± SD (bars; n = 10).

*P < 0.05, **P < 0.01

Fig. 6 Effects of sitagliptin+losartan on hepatic CD31 mRNA expression and

hepatic VEGF protein expression.

(A) Hepatic CD31 mRNA expression significantly increases in the CDAA group (G2) compared with the CSAA group (G1). Monotherapy with either sitagliptin or losartan markedly inhibits both CD31 expression compared with that in G2. Sitagliptin+losartan (G5) exerts a stronger inhibitory effect compared with either agent (G3, G4). (B) Similar to the changes in CD31, the CDAA increase in hepatic VEGF protein expression is significantly suppressed in G3 and G4, and a stronger suppressive effect is observed in G5. HPF, high-power field

Values are presented as mean ± SD (bars; n=10).

*$P < 0.05$, **$P < 0.01$

Fig. 7 Effects of sitagliptin+losartan on cell proliferation of (A) HCC cells and (B) endothelial cells.

AT-II and VEGF-induced HepG2-cell and EC proliferation is not affected by clinically equivalent doses of either sitagliptin (2 μM) or losartan (10 μM).

Values are presented as mean ± SD (bars; n = 8).

*$P < 0.05$, **$P < 0.01$

Fig. 8 Characteristics and index of *in vitro* endothelial cell (EC)-tube formation.

(A) AT-II treatment (1 μM; b) increased EC-tube formation compared with that in the PBS-treated control group (a). Monotherapy with either sitagliptin (2 μM; c) or losartan (10 μM; d) suppresses AT-II-induced EC-tube formation and treatment with sitagliptin+losartan (e) exerts stronger suppression than either monotherapy. (B) Semiquantitative analysis by an image-analyzing system confirms the

abovementioned findings.

Values are presented as mean ± SD (bars; n = 10).

*$P < 0.05$, **$P < 0.01$

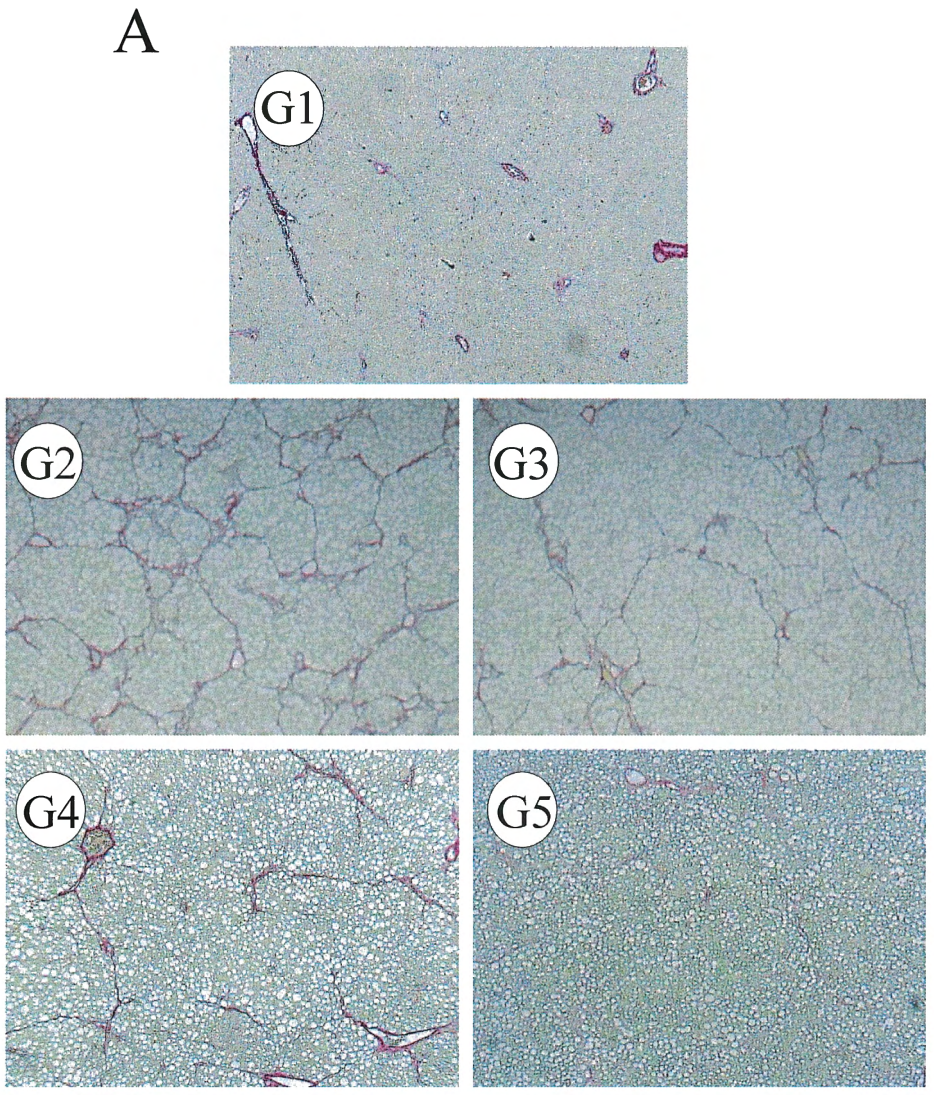
Fig.9 Effects of sitagliptin+losartan on hepatic reactive oxygen species (ROS) production.

A: Hepatic 8-hydroxydeoxyguanosine (8-OHdG)-immunopositive cell numbers significantly increase in the CDAA-fed group (G2) compared with the CSAA-fed control group (G1) and decrease in both the sitagliptin-(G3) and losartan-treated (G4) groups compared with G2. Sitagliptin+losartan (G5) exerts a stronger inhibitory effect compared with G3 and G4. B: Similar to the changes in 8-OHdG, hepatic lipid peroxidation, as measured by malondealdehyde (MDA), is markedly suppressed in G3 and G4, and a stronger suppressive effect is observed in G5.

Values are presented as mean ± SD (bars; n = 10).

*$P < 0.05$, **$P < 0.01$

Fig.1


A
G1
G2
G3
G4
G5

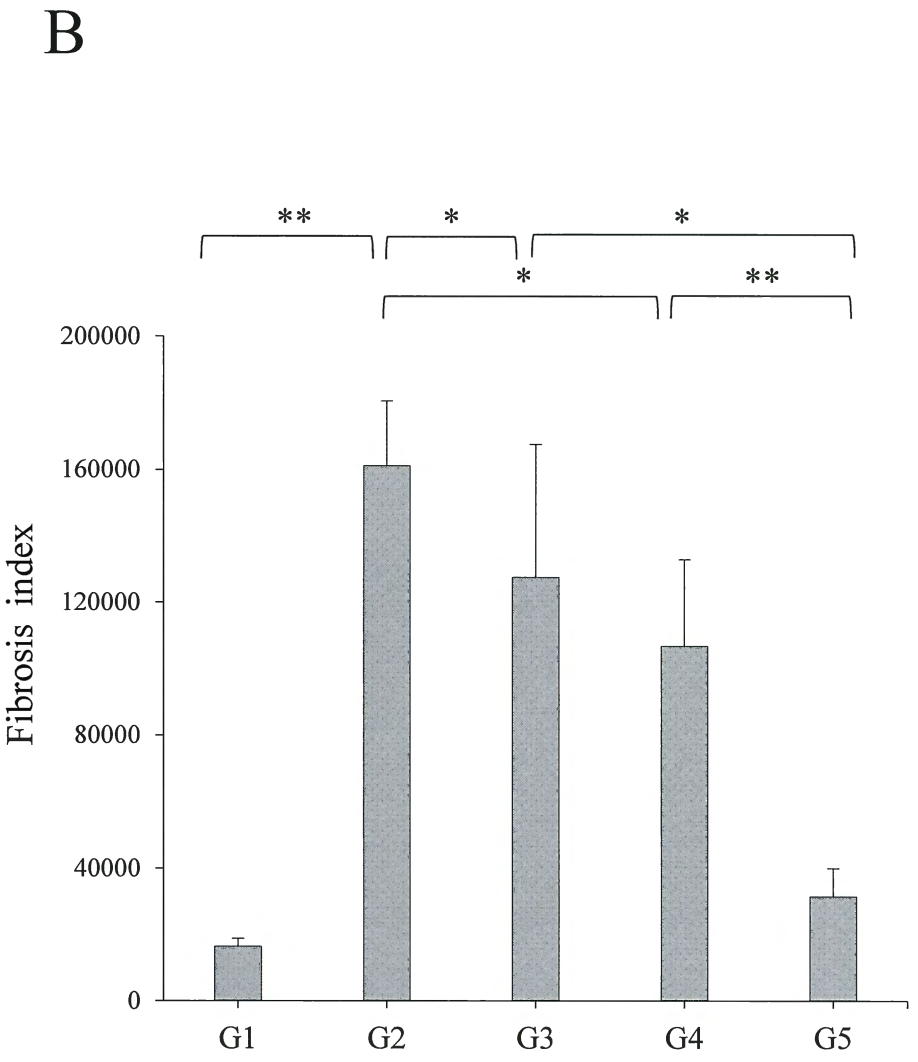

B
**
*
*
*
**
200000
160000
120000
80000
40000
0
Fibrosis index
G1
G2
G3
G4
G5

Fig.2

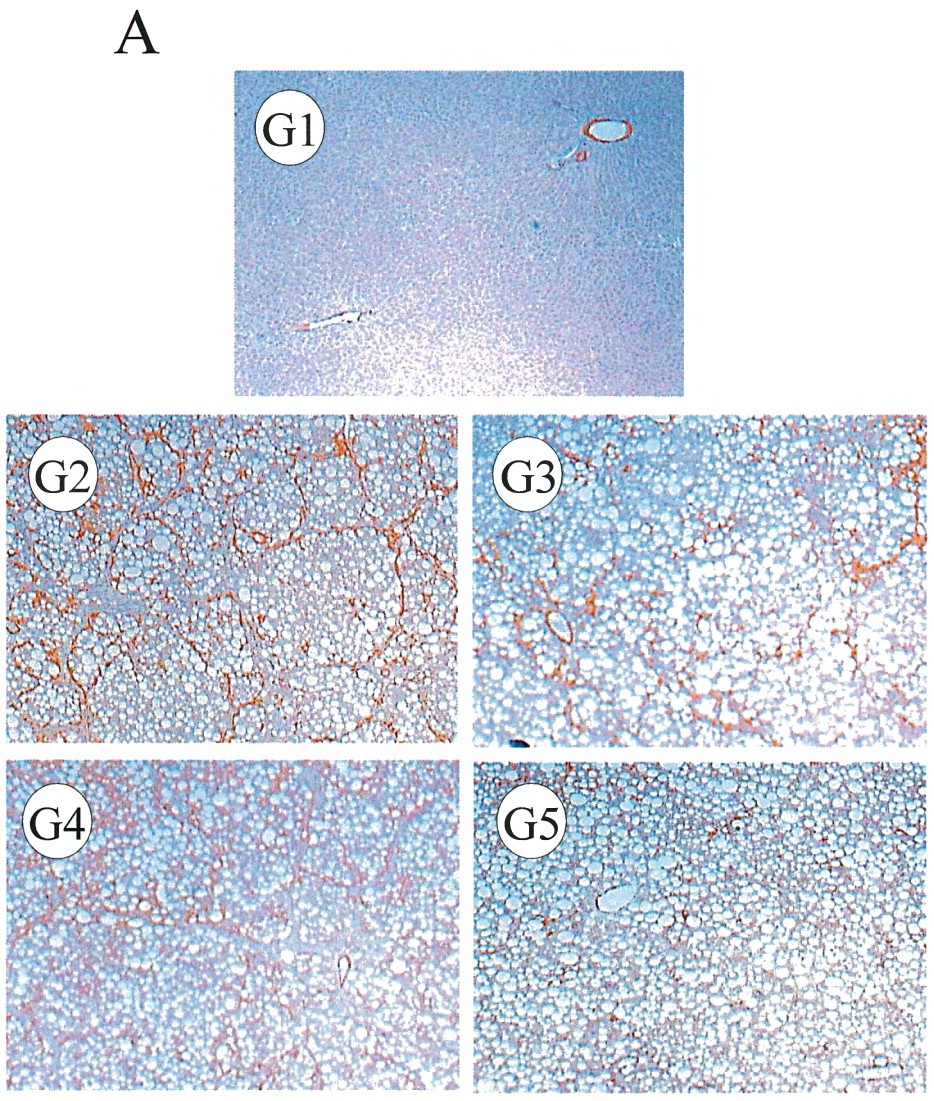

A
G1
G2
G3
G4
G5
B
**
**
**
**
**
αSMA index
140000
120000
100000
80000
60000
40000
20000
0
G1
G2
G3
G4
G5

Fig.3

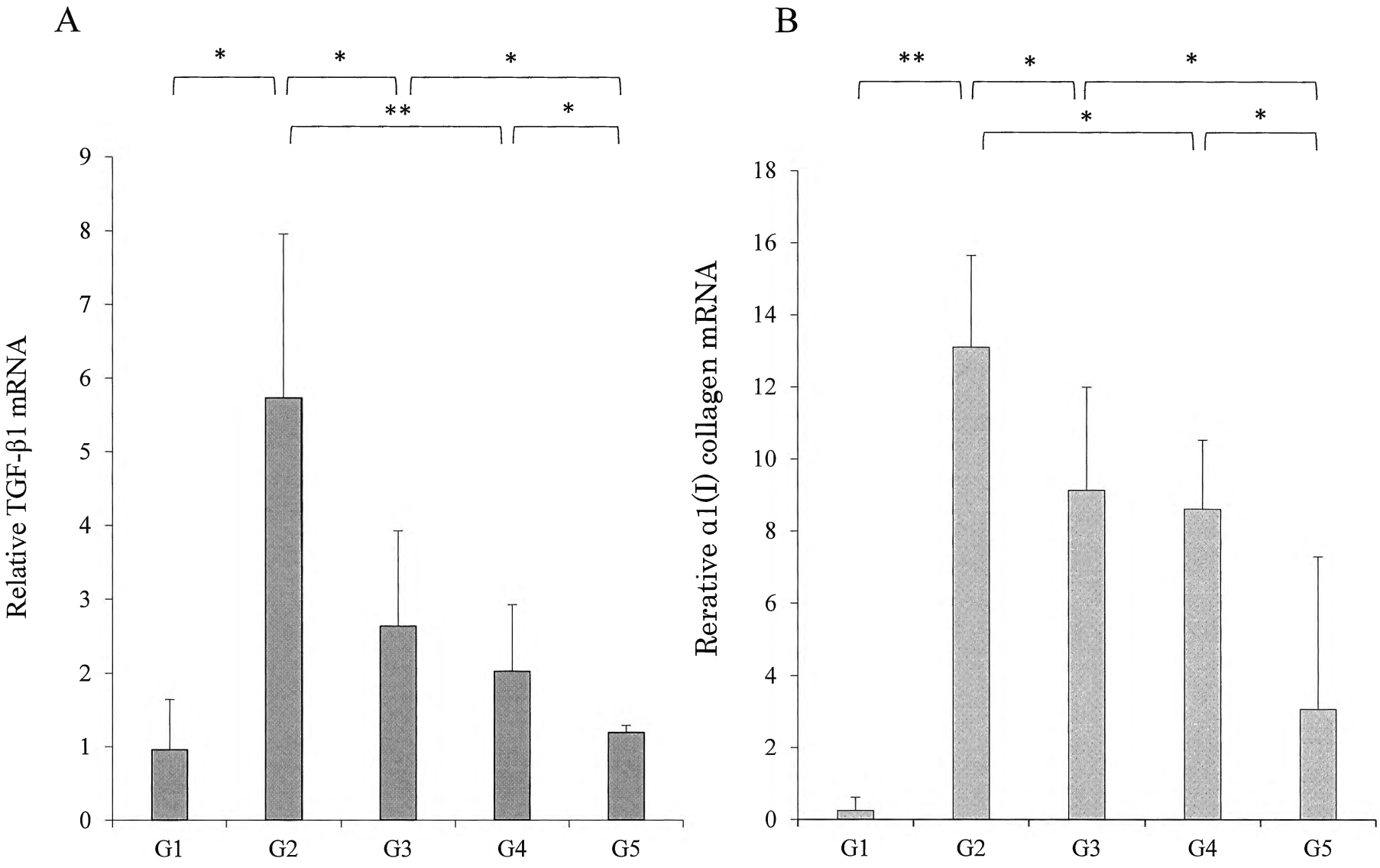

A
B
Relative TGF-β1 mRNA
Rerative α1(I) collagen mRNA
G1
G2
G3
G4
G5
0
1
2
3
4
5
6
7
8
9
10
12
14
16
18
*
**

Fig.4

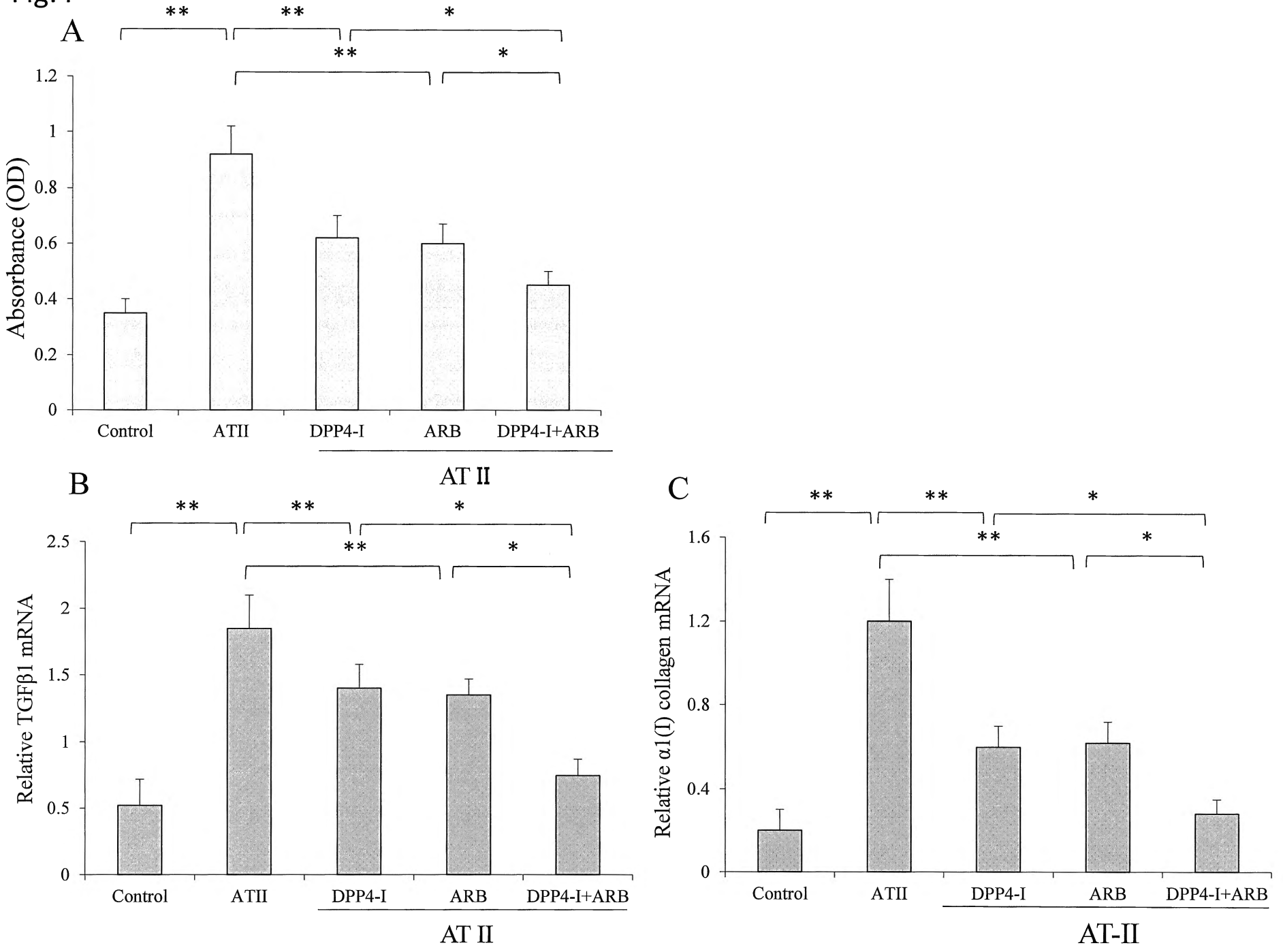
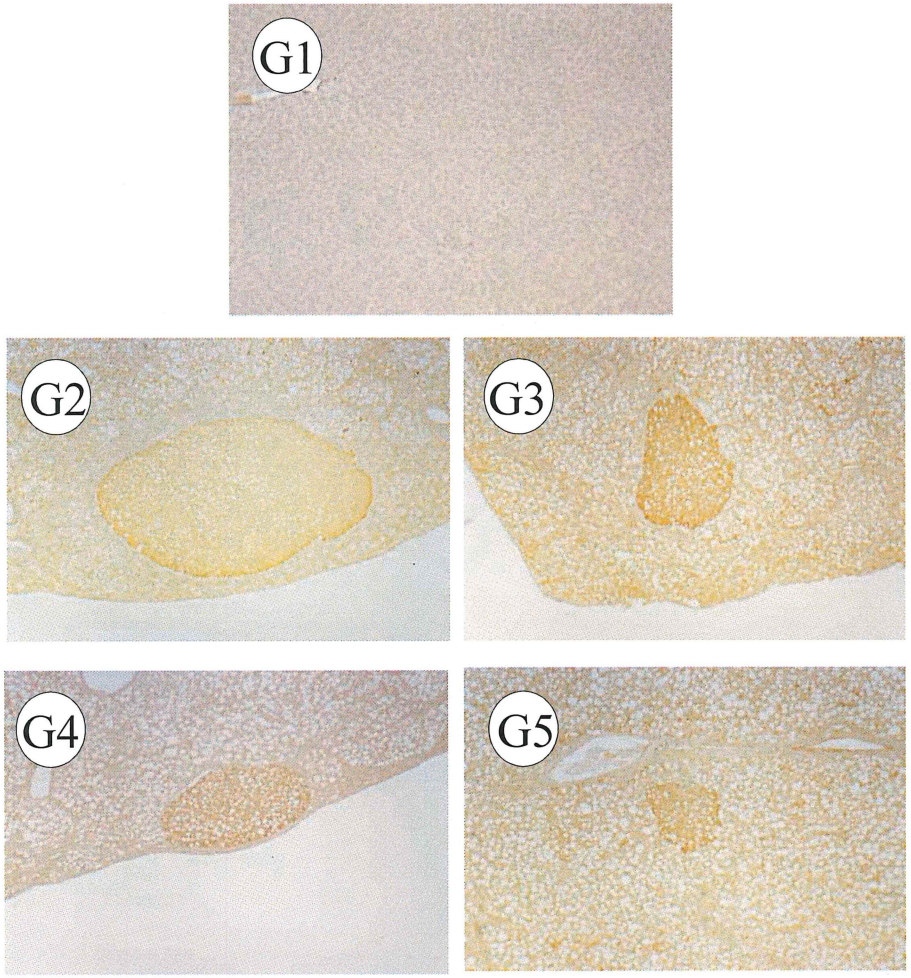
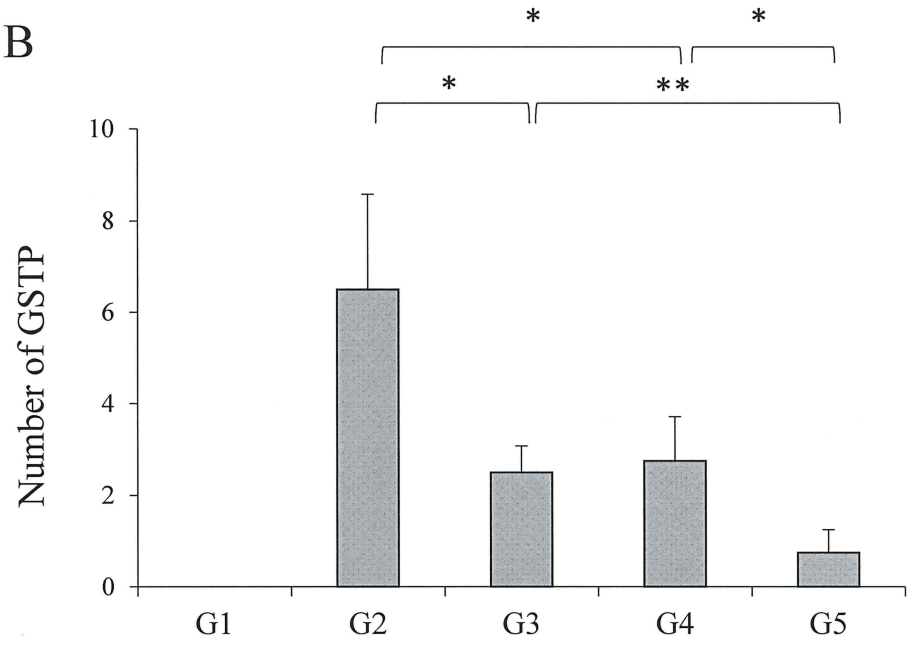

A
**
**
*
**
*
Absorbance (OD)
1.2
1
0.8
0.6
0.4
0.2
0
Control
ATII
DPP4-I
ARB
DPP4-I+ARB
AT II
B
**
**
*
**
*
Relative TGFβ1 mRNA
2.5
2
1.5
1
0.5
0
Control
ATII
DPP4-I
ARB
DPP4-I+ARB
AT II
C
**
**
*
**
*
Relative α1(I) collagen mRNA
1.6
1.2
0.8
0.4
0
Control
ATII
DPP4-I
ARB
DPP4-I+ARB
AT-II

Fig.5

A


G1
G2
G3
G4
G5

B


*
*
*
**
Number of GSTP
10
8
6
4
2
0
G1
G2
G3
G4
G5

C

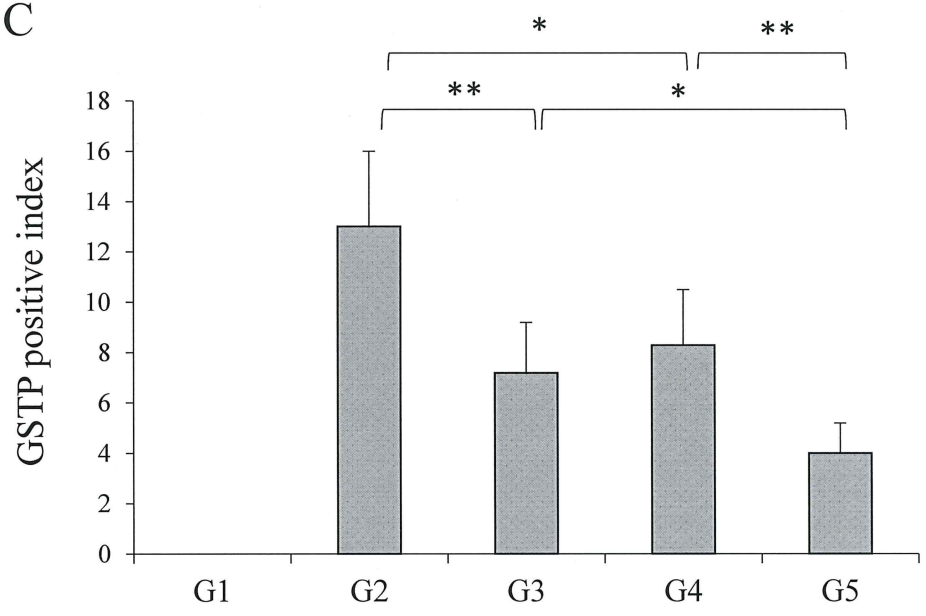

*
**
**
*
GSTP positive index
18
16
14
12
10
8
6
4
2
0
G1
G2
G3
G4
G5

Fig.6

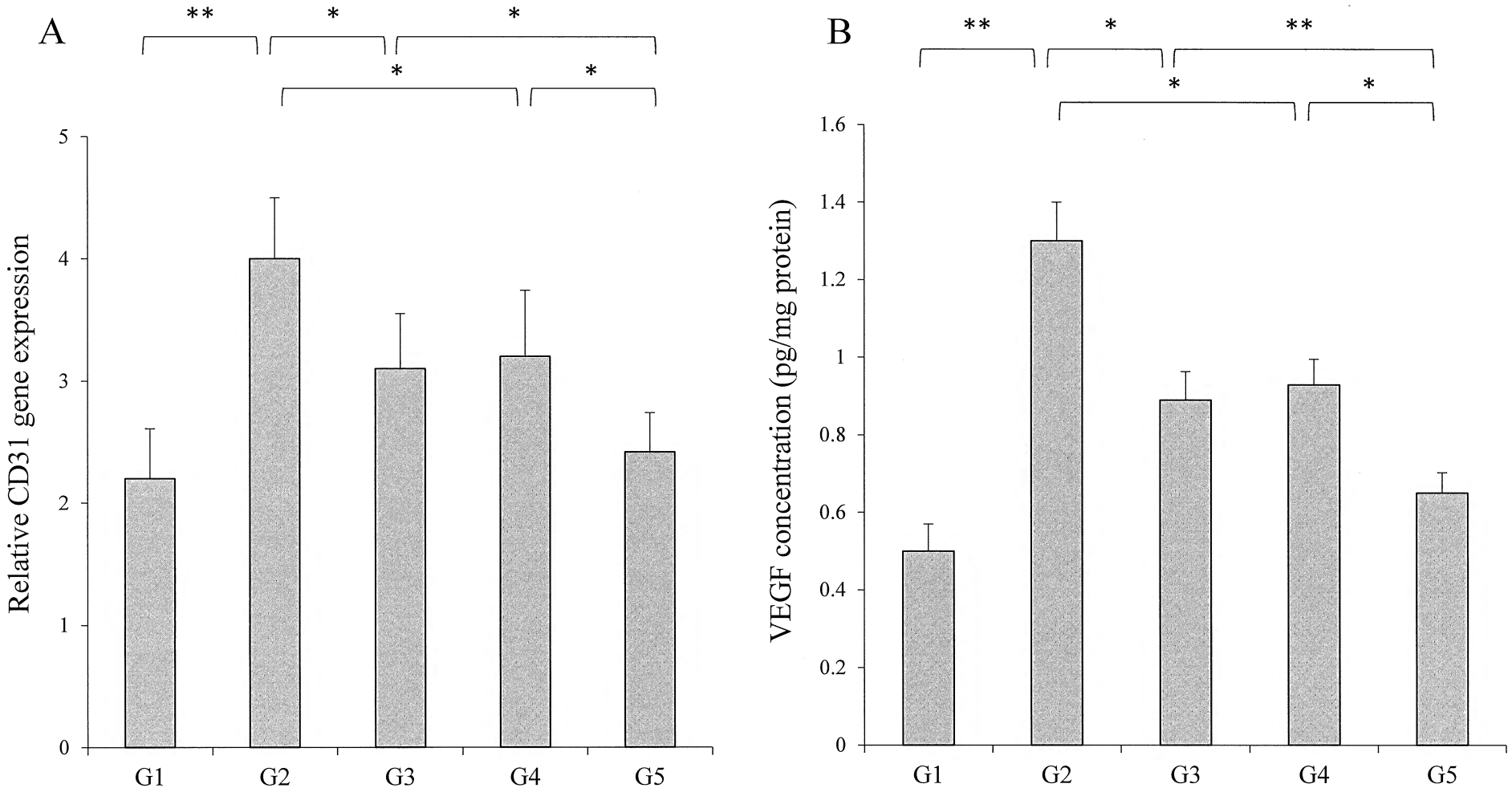

A
**
*
*
*
*
Relative CD31 gene expression
0
1
2
3
4
5
G1
G2
G3
G4
G5
B
**
*
**
*
*
VEGF concentration (pg/mg protein)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
G1
G2
G3
G4
G5

Fig.7

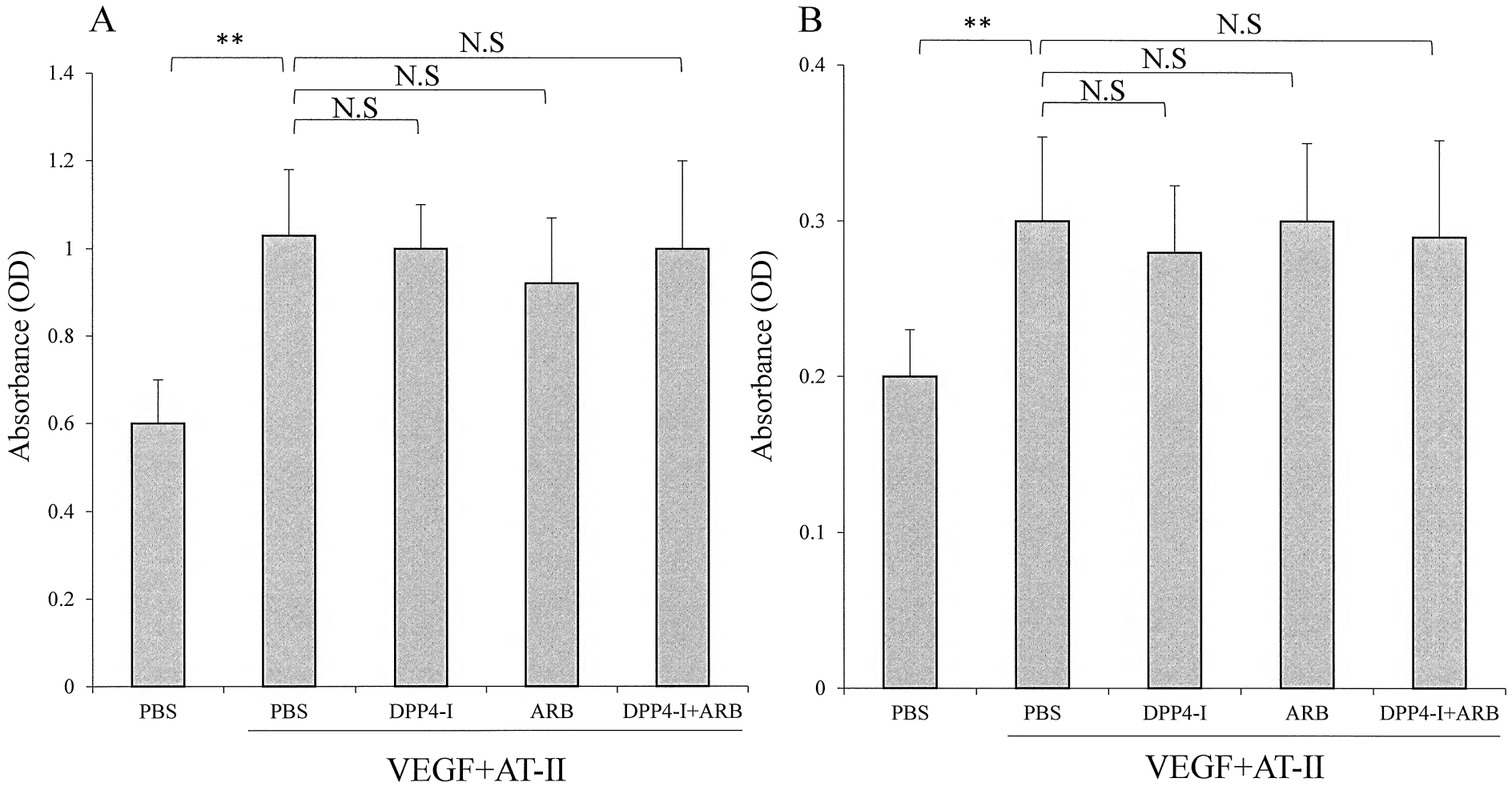
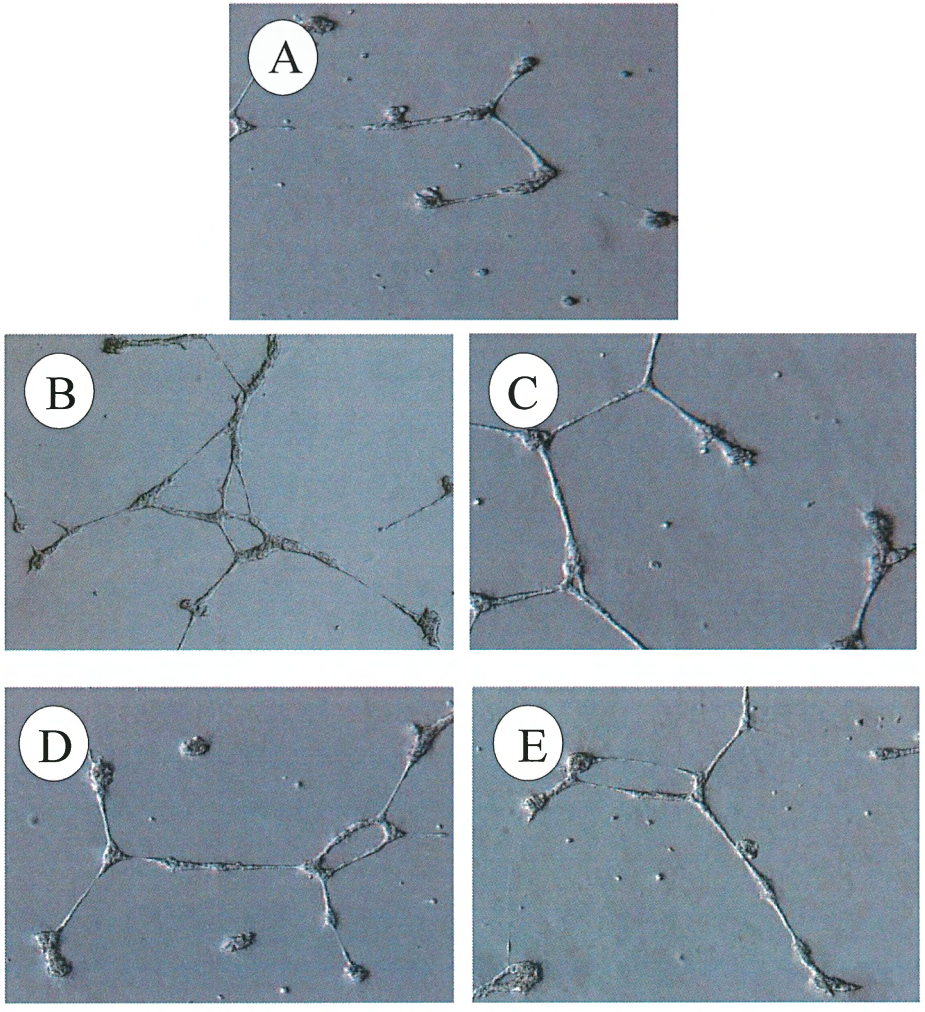

A
**
N.S
N.S
N.S
Absorbance (OD)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
PBS
DPP4-I
ARB
DPP4-I+ARB
VEGF+AT-II
B
**
N.S
N.S
N.S
Absorbance (OD)
0.4
0.3
0.2
0.1
0
PBS
PBS
DPP4-I
ARB
DPP4-I+ARB
VEGF+AT-II

Fig.8

A


A
B
C
D
E

B

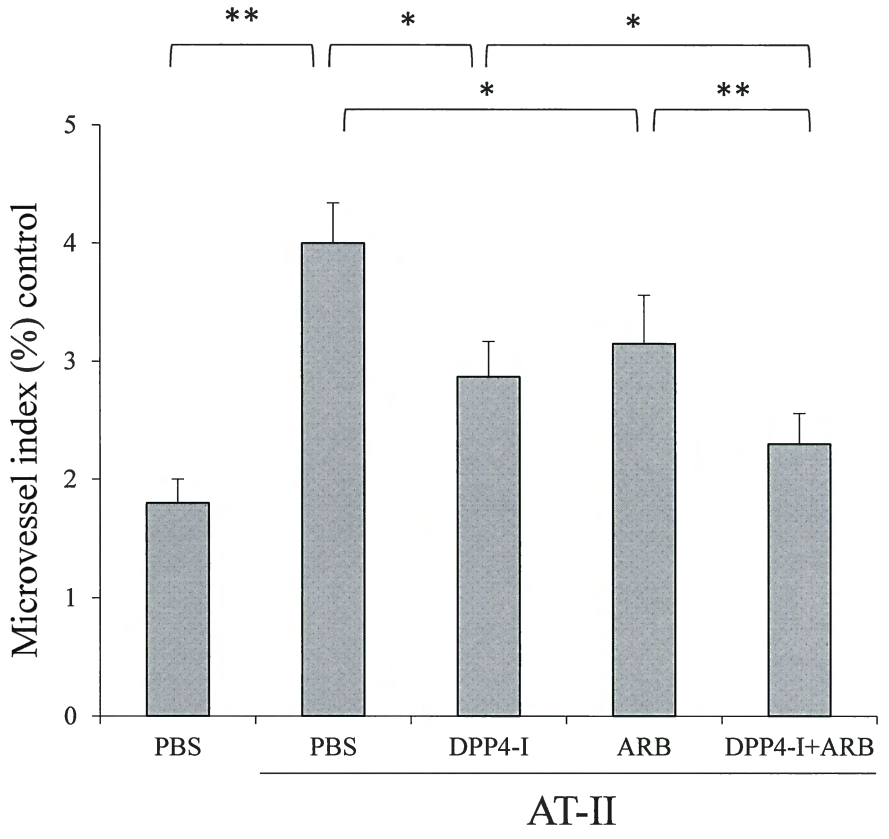

**
*
*
*
**
Microvessel index (%) control
0
1
2
3
4
5
PBS
PBS
DPP4-I
ARB
DPP4-I+ARB
AT-II

Fig.9

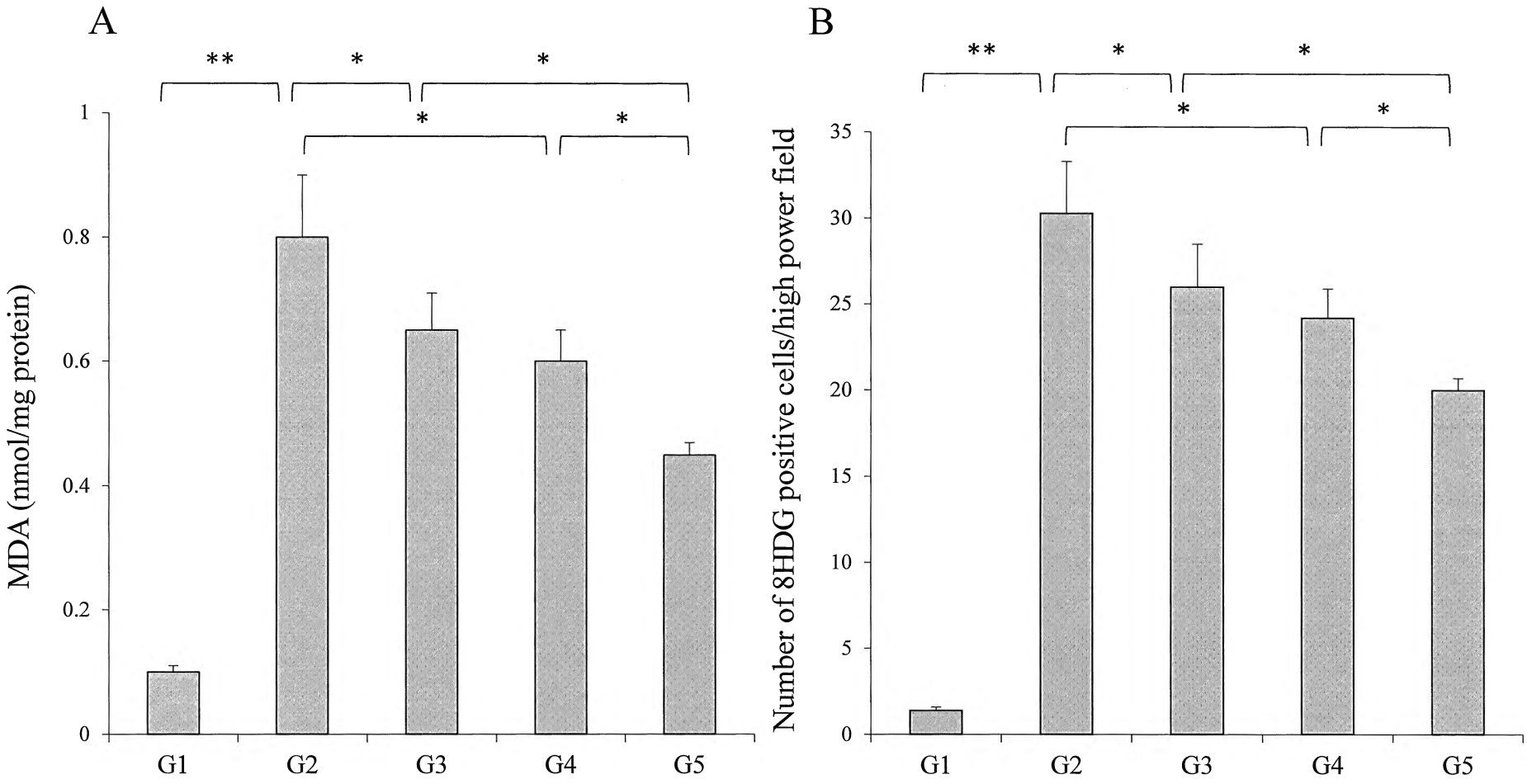

A
B
**
*
*
*
*
*
MDA (nmol/mg protein)
Number of 8HDG positive cells/high power field
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
35
G1
G2
G3
G4
G5

Table. 1 Characteristics of the experimental groups in the rat NASH model

	CSAA(G1)	CDAA(G2)	CDAA+Si (G3)	CDAA+ARB (G4)	CDAA+ Si+ARB (G5)
Number of rats	10	10	10	10	10
Body weight, g	325.2±16.4	239.0±8.6‡	226±10.5‡	244.3±8.3‡	213.7±11.4‡*
Liver/body weight ratio, g/100gBW	3.1±0.1	4.6±0.1‡	4.7±0.3‡	4.6±0.2‡	4.6±0.2‡
alanine aminotransferase, IU/l	57.5±10.3	255.2±10.6‡	220.6±12.6‡*	240.4±20.1‡	213.0±13.5‡*
Albumin, g/dl	4.0±0.1	3.9±0.3	4.0±0.3	4.0±0.5	4.2±0.4
Total bilirubin, mg/dl	0.12±0.02	0.11±0.03	0.09±0.03	0.11±0.05	0.13±0.03
Glucose, mg/dl	167.2±17.1	177.4±11.3	164.5±19.1	183.5±20.8	167.2 ± 17.1
Insulin, μU/mL	5.2 ±1.1	5.0 ± 0.9	4.8 ± 1.2	4.7 ± 1.3	5.1± 2.0

Data are mean±SD.G1-G5,Group1-5.
Statistically significant compared to G1 (‡ $p<0.01$) and G2(*$p<0.05$, ※$p<0.01$).
ALT, alanine aminotransferase; ALB, albumin; T-Bil, total bilirubin; CSAA, choline-sufficient L-amino acid defined; CDAA, choline-deficient L-amino acid-defined;Si, sitagliptin; AT-II, Angiotensin II; ARB, AT-II type1 receptor blocker.

Table. 2 NAFLD activity score

	G1	G2	G3	G4	G5
Steatosis	0	2.7±0.2	2.3±0.2†	2.6±0.2	2.1±0.2§
Ballooning	0	1.6±0.4	1.2±0.3†	1.4±0.2	1.1±0.5†
Inflammation	0	2.1±0.2	1.9±0.2†	1.9±0.4	1.8±0.2§

† $P<0.05$ compared to CDAA diet, G2
§ $P<0.01$ compared to CDAA diet, G2