factor, nitric oxide synthase, and metalloproteinase [4, 5]. These properties enhance colon carcinogenesis and disease progression. The renin-angiotensin-activating system is recognized as an important molecular target for CRC prevention and treatment. Several inhibitors of the renin-angiotensin-activating system suppress cancer development, cancer cell growth, angiogenesis, and metastasis [4–9].

Diabetes mellitus is a common problem in countries adopting the Western lifestyle. The results of several epidemiological studies show an association between type 2 diabetes and the risk of colorectal, pancreatic, breast, liver, gastric, and endometrial cancer [10]. The risk of malignancies is increased at earlier stages in cases of abnormalities in glucose metabolism, and there is a linear relationship between cancer risk and plasma insulin levels [10]. With regard to CRCs, a meta-analysis of 15 studies, including 2,593,935 participants, showed that diabetes is associated with an increased risk of CRC (relative risk, 1.30; 95 % CI, 1.20–1.40). Diabetes is also associated with CRC mortality (relative risk, 1.26; 95 % CI, 1.05–1.50) [11]. High glycated hemoglobin (HbA1c) levels are also associated with an increased risk of CRC (odds ratio, 1.57; 95 % CI, 0.94–2.60) [12]. In several studies, it has been demonstrated that hyperinsulinemia, elevated levels of C-peptide, elevated body mass index, high levels of insulin growth factor-1, low levels of insulin growth factor binding protein-3, high leptin levels, and low adiponectin levels are all involved in carcinogenesis [13]. Increased blood concentrations of insulin and insulin-like growth factor are particularly important in enhancing the risk of CRC [14]. However, a detailed understanding of how diabetes might increase the risk of CRC is still lacking.

In the present study, we were prompted to investigate whether angiotensin activation, which is associated with diabetic hyperglycemia, occurs in CRC cells. Further, we investigated whether the angiotensin activation system enhances liver metastasis of CRCs and the therapeutic significance of anti-angiotensin and hypoglycemic agents on liver metastasis.

## Materials and methods

### Cell culture and reagents

HT29 human colon cancer cell line was purchased from Dainihon Pharmaceutical, Tokyo, Japan, CT26 mouse colon cancer cells was kindly provided from Professor Isaiah J. Fidler (M.D. Anderson Cancer Center, USA) [15]. Cells were maintained in Dulbecco's modified essential medium (Sigma Chemical, St. Louis, MO) containing 10 % fetal bovine serum (Sigma) under the conditions of 5 % $CO_2$ in air at 37 °C. The glucose concentration was 100 mg/dl in the regular medium. Angiotensin II (Abgent, San Diego, CA), angiotensinogen (Calbiochem, Darmstadt, Germany), D-glucose (Sigma), renin inhibitor peptide (Abgent), chymase inhibitor (MP Biomedicals, Solon, OH), and ACE inhibitor (Wako Pure Chemical, Osaka, Japan) were purchased.

### Immunoblot analysis

Whole-cell lysates were prepared as described previously [16]. Antibodies for A-II (Serotec, Oxford, UK), angiotensinogen (R&D Systems, Minneapolis, MN), ATR1 (Assay Designs, Ann Arbor, MI), renin (AnaSpec, San Jose, CA), chymase (Spring Bioscience, Fremont, CA, USA), ACE (Life Span Biosciences, Salt Lake City, UT), and cathepsin D (Santa-Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. An anti-tubulin antibody was used as a loading control (Oncogene Research Products, Cambridge, MA). The immune complex was visualized using Enhanced Chemiluminescence Western-blot detection system (Amersham, Aylesbury, UK).

### Cell growth, apoptosis, and in vitro invasion assay

Cells were seeded at a density of 10,000 cells per well in 12-well tissue culture plates. Cell growth was assessed by cell counting using autocytometer (Sysmecs, Kobe, Japan). Apoptosis induced by sodium nitroprusside ($10^{-2}$ M, Wako) were assessed by staining with Hoechst 33258 fluorescent dye (Wako). The number of apoptotic cells was determined by examining 1,000 cells [17]. A modified Boyden chamber assay was performed to examine the in vitro invasion of colon cancer cells [16]. The experiments were performed three times.

### Animal model

BALB/c nu/nu athymic mice (male, 4 weeks old) and BALB/c mice (male, 4 weeks old), purchased from Japan SLC (Shizuoka, Japan) were used as a metastasis model. The mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University. Single-cell suspensions of CRC cells ($1 \times 10^6$) in Hanks balanced saline solution were injected into the mouse spleen. The mice were sacrificed to count the number and size of metastatic foci in the liver [18]. In each group, six mice were treated and the experiments were repeated three times.

Mice were fed with or without 10 % glucose drinking (Otsuka Pharmaceutical, Tokushima, Japan). Mouse blood sugar and serum A-II, were measured by Medisafe Reader

(Terumo, Tokyo, Japan) and A-II ELISA kits (Phoenix Pharmaceuticals, Belmont, CA), respectively, according to provider's instructions.

### Antisense S-oligodeoxynucleotide (S-ODN)

Mouse angiotensinogen (ATG, GenBank NM_007428.3) antisense S-ODN comprising nucleotides 1–18 (5′- atg cac aga tcg gag atg -3′) was conjugated with cholesterol (Japan BioService, Asaka, Japan). Cholesterol-conjugated ATG antisense S-ODN (25 μg/kg body weight) was intraperitoneally injected once a week for 4 weeks (Fig. 5a) [18].

### Surgical specimens

Pathological diagnosis and clinical data were reviewed in 121 patients with CRCs that were diagnosed in Department of Molecular Pathology, Nara Medical University from 2008 to 2010. Out of 121 cases, 63 were stage B (cases invaded into the muscularis propria layer or above), 43 were stage C (any cases with lymph node metastasis), and 15 were stage D (any case with or without lymph node metastases but with distant metastases; all cases metastasized to the liver). In these cases, fresh tissues were also obtained from primary tumors for examining A-II, renin, and chymase concentrations. The tissues were frozen by liquid nitrogen and kept at −80 °C. Frozen tumor tissues were sonicated in lysis buffer, which supernatants were used for A-II, renin, and chymase were determined using ELISA kits (Phoenix, DRG International. East Mountainside, NJ, and Uscn Life Science, Wuhan, China, respectively) [18].

Diagnosis of diabetes mellitus was done according to the Classification and Diagnostic Criteria of Diabetes Mellitus by Japan Diabetes Society in 1999 [19]. The diagnosis was based on the diabetic type glycemia, which is defined when fasting plasma glucose is 126 mg/dl or higher, and/or plasma glucose 2 h after 75 g glucose load is 200 mg/dl or higher. Casual plasma glucose higher than 200 mg/dl is also regarded as indicating diabetic type. HbA1c levels at the disease diagnosis were analyzed in the study.

Because written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection. The procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

### Immunohistochemistry

Consecutive 4-μm sections were immunohistochemically stained using the immunoperoxidase technique described previously [18]. Antibodies to A-II (Serotec), renin (AnaSpec), chymase (Spring Bioscience) were used as a primary antibody with a concentration of 0.5 μg/ml. The specimens were color-developed with diamine benzidine (DAKO) and counter stained with Meyer's hematoxylin (Sigma).

### Short interferent RNA

FlexiTube short interferent RNAs (siRNAs) for renin and chymase were purchased from Qiagen Genomics (Bothell, WA). AllStars Negative Control siRNA was used for control (Qiagen). Cells were transfected with 50 nM siRNA for each gene using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### Statistical analysis

Statistical analyses of experimental data were performed using Mann–Whitney $U$ test, Kruskal–Wallis test with Dunn's multiple comparison test (nonparametric ANOVA), unpaired $t$ test with Welch correction, and Chi-squared test. Nonparametric correlation was examined by Spearman rank correlation test. Statistical significance was defined as a two-sided $P$ value of $<0.05$.

## Results

### Effects of A-II and ATG on CRC cells

Effects of A-II on cell growth, invasion, and apoptosis were examined in the CRC cell lines HT29 and CT26 (Fig. 1a–d). A-II enhanced cell growth and in vitro invasion into type IV collagen in the 2 cell lines in a dose-dependent manner. In contrast, apoptosis was decreased by A-II in a dose-dependent manner. Effects of ATG on cell growth, invasion, and apoptosis were examined in HT29 and CT26 cells (Fig. 1e–h). ATG enhanced cell growth and in vitro invasion in HT29 cells in a dose-dependent manner, but was unable to produce these changes in CT26 cells. In addition, apoptosis was decreased by ATG in HT29 cells, but not in CT26 cells, in a dose-dependent manner.

### Expression of angiotensin-associated genes in CRC cells

The expression of angiotensin-associated genes was examined in HT29 and CT26 cells (Fig. 2a). Both cell lines expressed ATR1, but did not express ATG or A-I converting enzyme (ACE). They did, however, express chymase, which possesses an ACE-like activity. Renin was expressed in only HT29 cells but not in CT26 cells. Cathepsin D, which possesses a renin-like activity, was not expressed in either of the two cell lines.

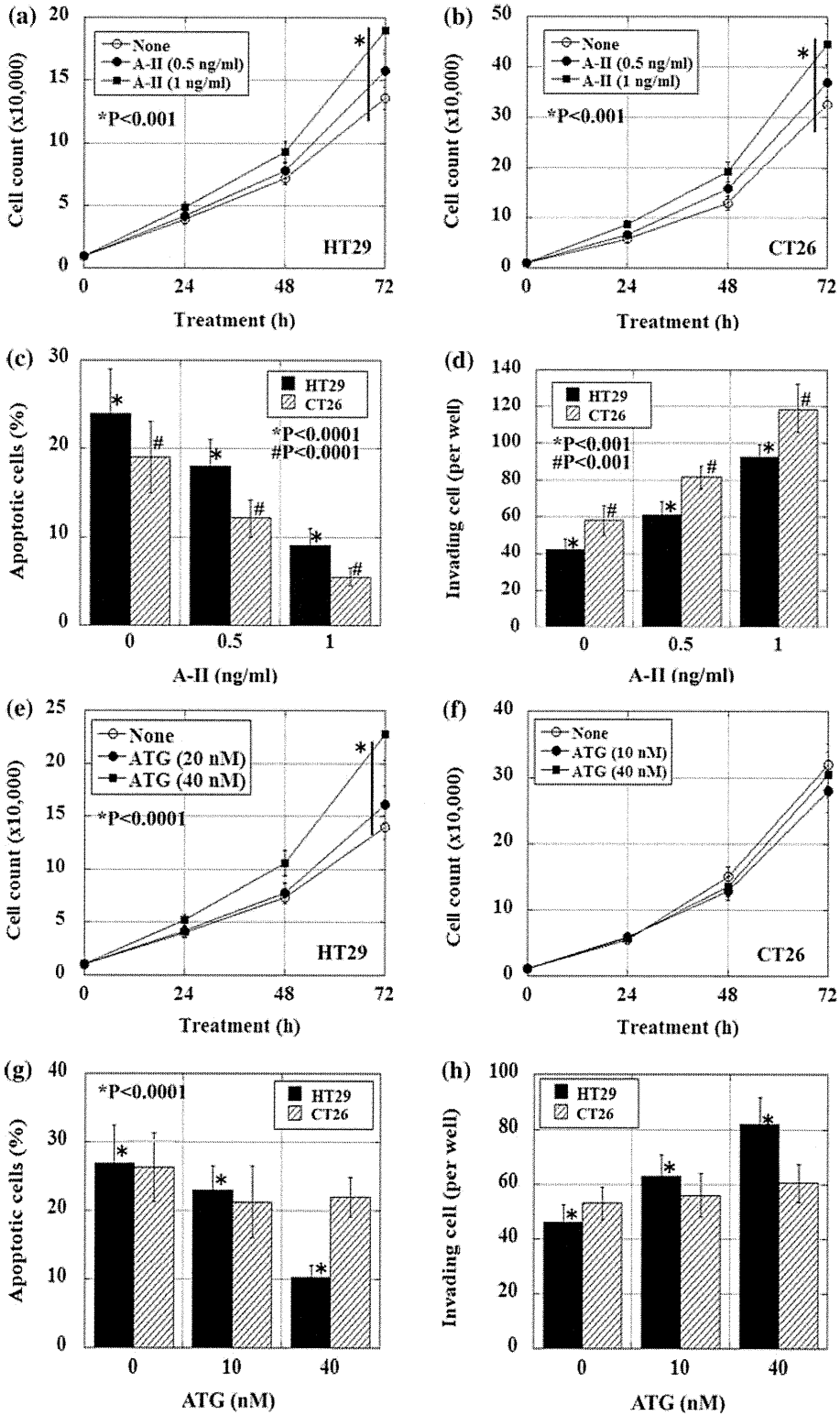


**Fig. 1** Effects of angiotensin II and angiotensinogen on cell growth, apoptosis, and invasion of colon cancer cells. HT29 and CT26 cells were treated with angiotensin II (A-II) (0.5 or 1.0 ng/ml) (**a**–**d**) or angiotensinogen (ATG) (10 or 40 nM) (**e**–**h**). **a**, **b**, **e**, **f** Cell growth, **c**, **g** apoptosis, and **d**, **h** in vitro invasion. *Error bar*, SD

Next, we examined the expression of renin in HT29 and CT26 cells in association with changing glucose concentration (Fig. 2b). When the medium contained 100 mg/dl glucose, renin protein was detected in only HT29 cells but not in CT26 cells. When the medium contained glucose at 200 mg/dl or more, the expression of renin increased with increasing glucose concentration in a dose-dependent manner.

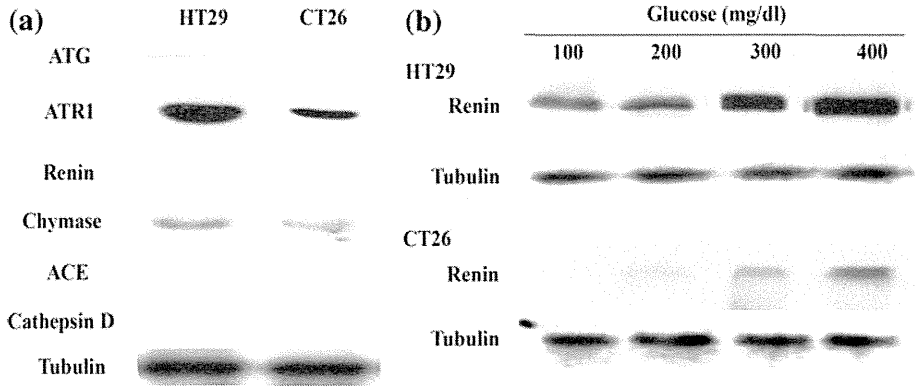


**Fig. 2** Expression of angiotensin-related proteins in colon cancer cells. **a** Protein levels of angiotensinogen (ATG), angiotensin II type 1 receptor (ATR1), renin, chymase, angiotensin converting enzyme (ACE), and cathepsin D were examined by immunoblotting. **b** Protein levels of renin in HT29 and CT26 cells treated with various concentrations of glucose in the medium. Tubulin was used as the loading control

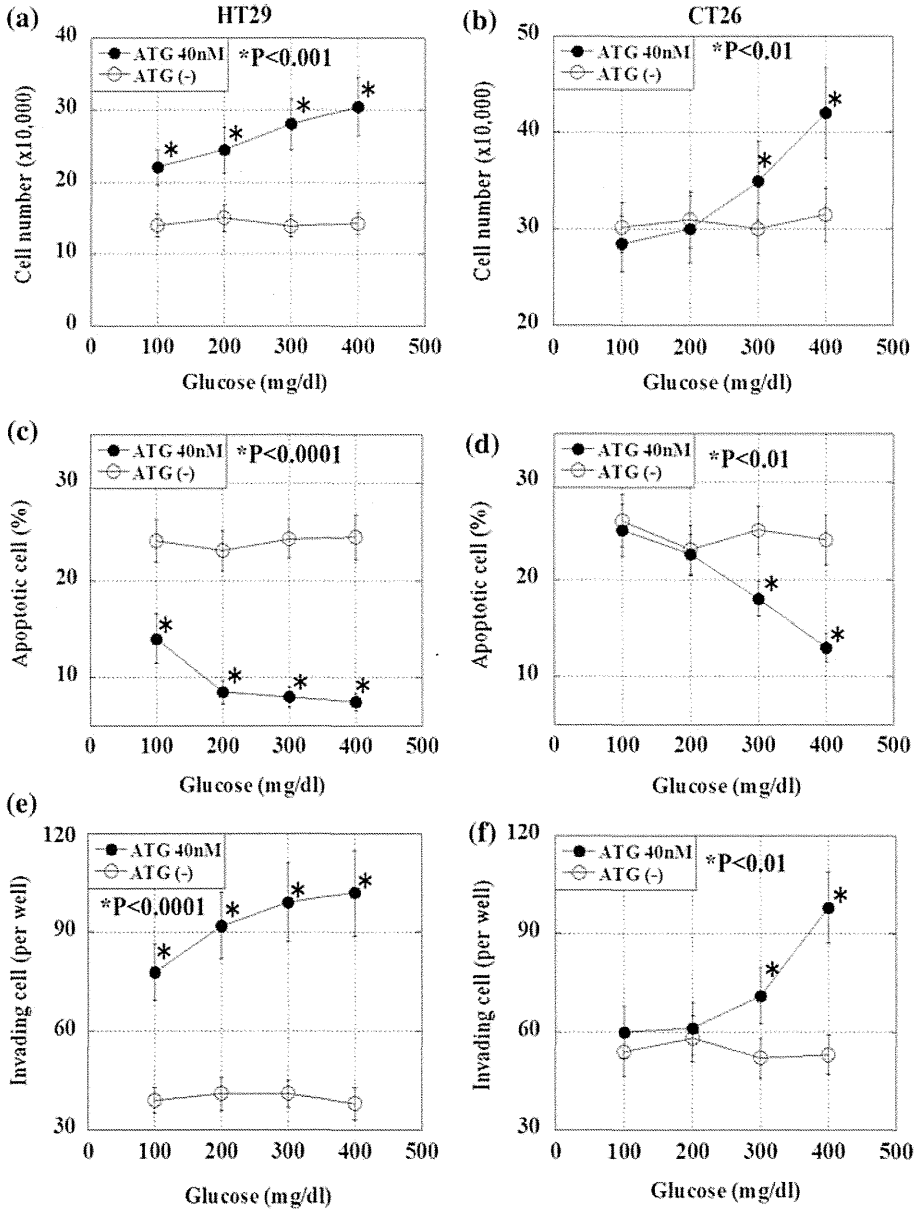


**Fig. 3** Effects of hyperglycemic conditions on the activation of angiotensin in colon cancer cells. Effects of glucose on angiotensinogen (AGT) activation in HT29 and CT26 cells treated with various concentrations of glucose for 48 h. **a**, **b** Cell growth, **c**, **d** apoptosis, and **e**, **f** in vitro invasion. *Error bar*, SD

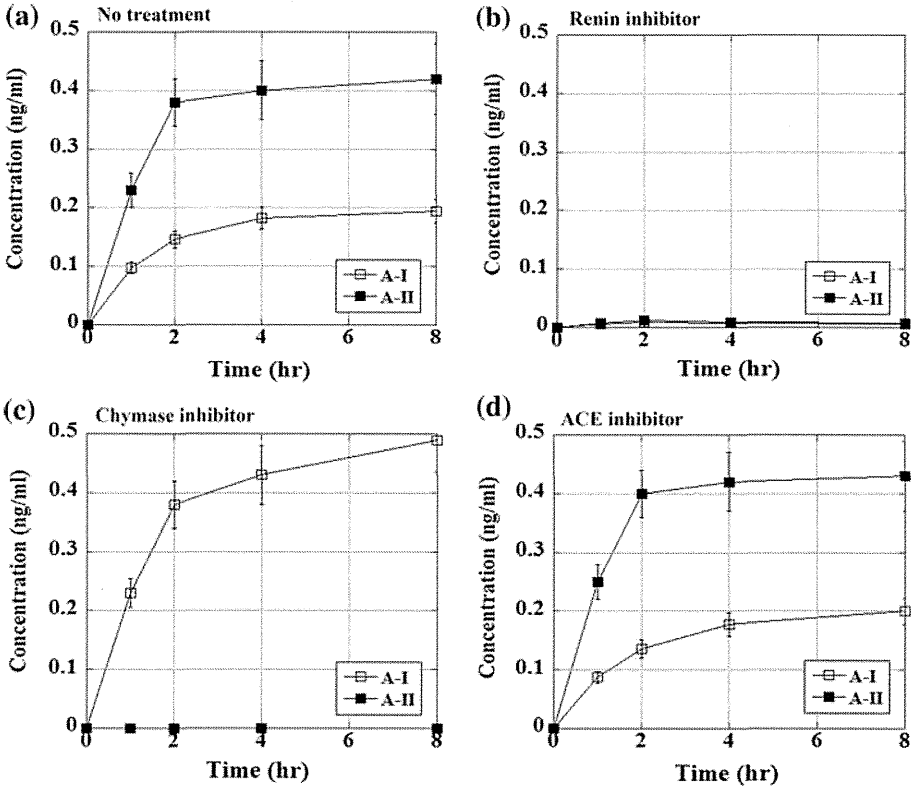


**Fig. 4** Effects of inhibitors of renin, chymase, and angiotensin converting enzyme on the generation of angiotensin I and II from angiotensinogen in HT29 human colon cancer cells. HT29 cells were treated concurrently with angiotensinogen (AGT) and inhibitors of renin, chymase, and angiotensin converting enzyme (ACE). Concentrations of angiotensin I (A-I) and -II (A-II) were examined by enzyme-linked immunosorbent assay. *Error bar*, SD

Pro-tumoral potential is enhanced by hyperglycemic conditions in CRC cells

In HT29 cells, ATG (40 nM) provided significantly pronounced effects of growth, invasion, and apoptosis inhibition in comparison with those found in Fig. 1 (Fig. 3). The effects were further increased in according to the glucose concentration of the culture medium. In CT26 cells, ATG did not affect the growth, invasion, and apoptosis in the regular glucose concentration (100 mg/dl); however, the high glucose concentration (300 and 400 mg/dl) provided pronounced effect of growth, invasion, and apoptosis inhibition. These findings suggest that in CRC cells, renin expression induced under hyperglycemic conditions may require ATG activation.

Effects of inhibitors of renin, chymase, and ACE on A-I and A-II production in HT29 cells

To assess possible treatments targeting A-II, we compared the effects of inhibitors of renin, chymase, and ACE on the production of A-I and A-II in HT29 cells cultured in the regular medium (Fig. 4). Untreated HT29 cells showed production of A-I and A-II with plateau levels at 2 h after addition of ATG to the medium (Fig. 4a). A renin inhibitor peptide abrogated the production of both A-I and A-II (Fig. 4b). A chymase inhibitor peptide suppressed the production of A-II but not that of A-I (Fig. 4c). An ACE inhibitor did not affect the production of A-I or A-II in HT29 cells (Fig. 4d). Thus, A-II is produced from ATG by renin and chymase in HT29 cells.

Inhibition of ATG production suppresses liver metastasis in HT29 cells

Because ATG is produced in the liver, we examined the effects of angiotensin activation on liver metastasis of HT29 cells (Fig. 5). Hyperglycemic condition was also provided by 10 % glucose drink (Fig. 5a). Three groups were set for the experiments. In Group A, mice were not treated with ATG antisense *S*-oligodeoxynucleotide (ATG-AS) and glucose drink. In Group B, mice were provided with 10 % glucose drink. In Group C, mice were treated with 10 % glucose drink and ATG-AS. ATG expression in the mouse liver was suppressed by cholesterol-conjugated ATG antisense *S*-oligodeoxynucleotide (ATG-AS) (Fig. 5b). The blood sugar levels were higher in Group B and C than that in Group A (Fig. 5c). Since renin expression was upregulated according to the glucose concentration, we then examined the A-II concentration of the metastatic foci in the liver. In Group B, A-II concentration was higher than that in Group A. In contrast, A-II concentration was decreased in Group C in comparison with that in Group B (Fig. 5d). We examined the size, number, Ki-67 labeling as a proliferation marker, and microvessel

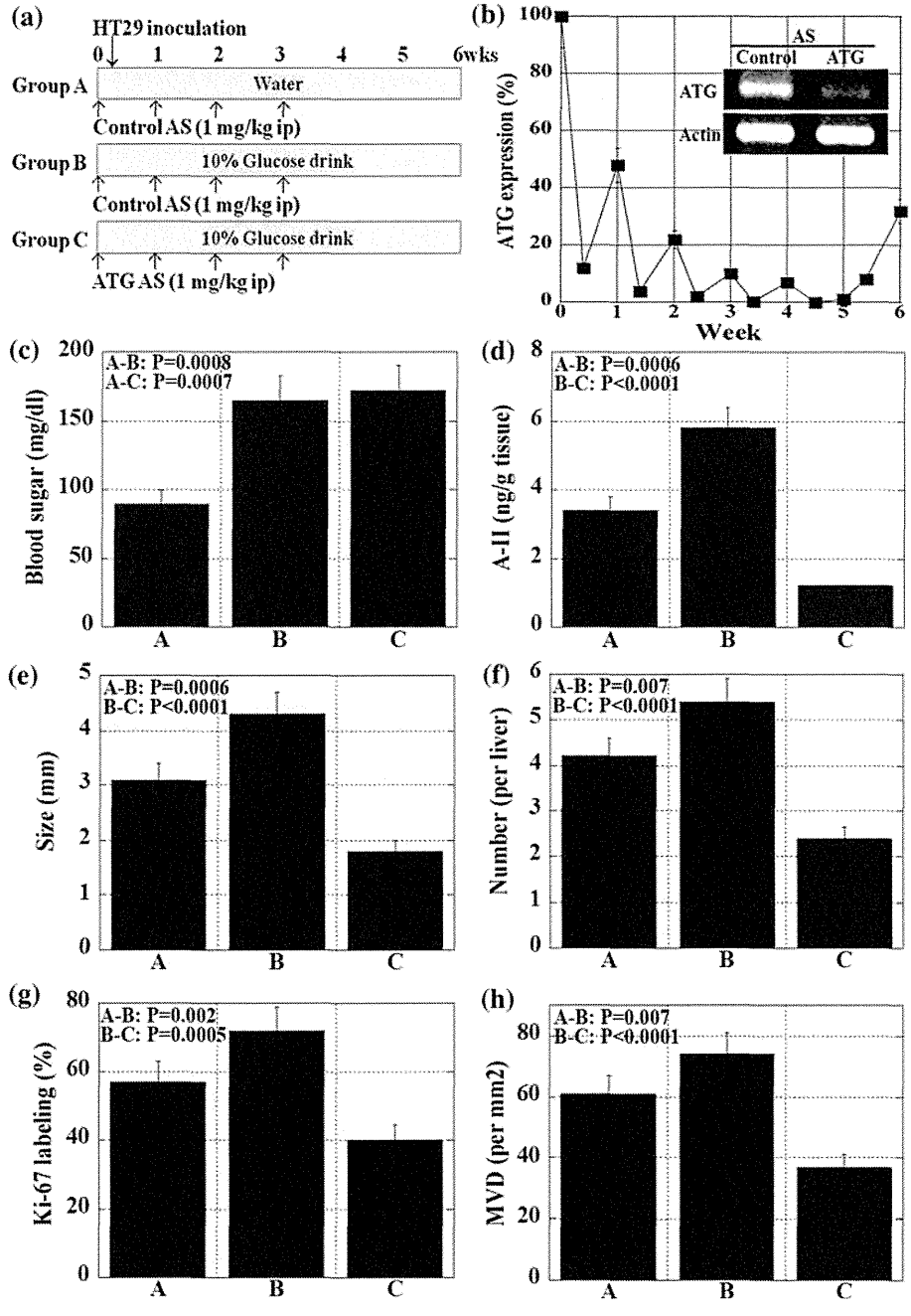


**Fig. 5** Effects of reduction of hepatic angiotensinogen production on liver metastasis of HT29 cells. **a** Protocol of a liver metastasis model. Nude mice fed with or without a drink containing 10 % glucose were administrated cholesterol-conjugated angiotensinogen (AGT) antisense S-oligodeoxynucleotide (ODN) or control S-ODN four times. HT29 cells ($1 \times 10^6$ cells) were inoculated in the spleen. In each group, six mice were examined. The experiments were repeated three times. **b** The time course of ATG mRNA expression was examined. *Bar*, SD. *Inset*: AGT mRNA expression in the liver of nude mice at week 6. Actin served as the loading control. **c** Blood sugar was examined in the aortic blood at the sacrifice. **d** Angiotensin II (A-II) concentration was examined in the liver metastatic foci by ELISA. **e**, **f** Tumor size and tumor multiplicity of the metastatic foci in the liver of nude mice at week 6. **g**, **h** Ki67-labeling index and microvessel density (MVD) of metastatic foci. AS: antisense S-ODN treatment, Control: mixed S-ODN treatment. *Error bar*, SD

density (MVD) as a marker of angiogensis in the metastatic foci (Fig. 5e–h). In Group B, the size, number, Ki-67 labeling, and MVD were pronounced than those in Group A, whereas ATG-downregulated Group C showed less pronounced parameters than those in Group B. Thus, the hyperglycemic condition enhanced liver metastasis of HT29 cells. In contrast, anti-angiotensin treatment by ATG-AS abrogated liver metastasis in comparison with that in hyperglycemic condition and even in control condition.

## Effect of knockdown of renin or chymase on liver metastasis

To confirm the significance of angiotensin activation system on the liver metastasis in CRC cells, we examined the effect of knockdown of renin or chymase on liver metastasis of HT29 cells (Fig. 6). As shown in Fig. 6a, HT29 cells treated with siRNA for renin or chymase were inoculated into the spleen of nude mice. The siRNA treated HT29 cells showed the reduction of expression of renin and

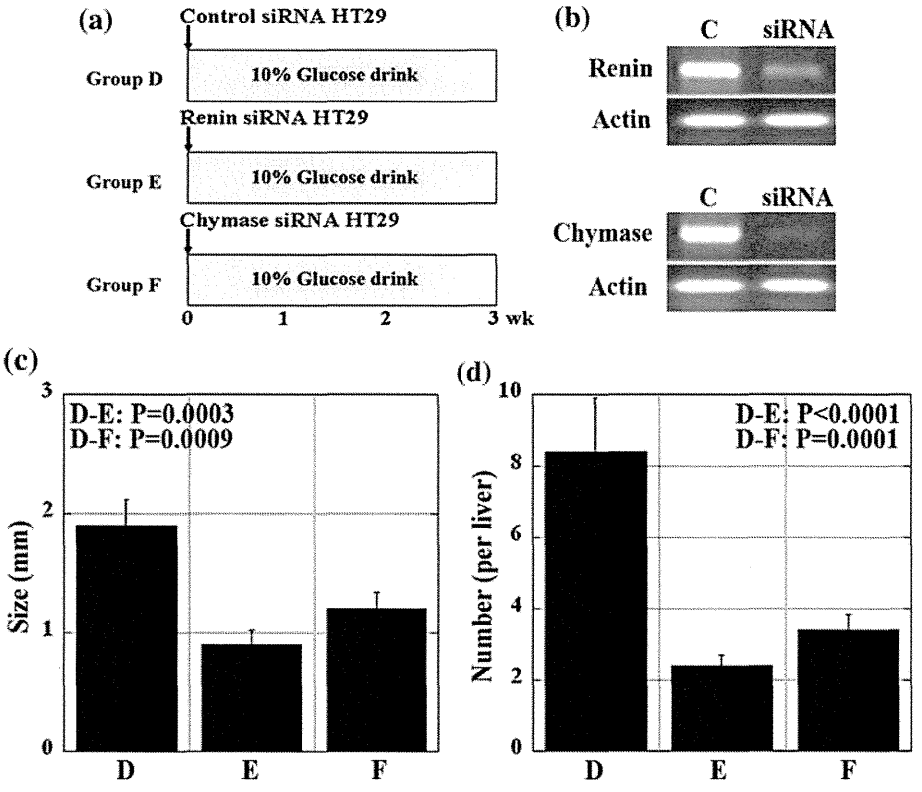


**Fig. 6** Effect of knockdown of renin or chymase on liver metastasis of HT29 cells (**a**) Protocol of the experiment. In each group, 8 nude mice were tested. **b** The expression of renin and chymase was examined by RT-PCR in HT29 cells treated with control siRNA (C) or siRNA to renin (*upper panel*) or chymase (*lower panel*). Actin was examined as an internal control. The size (**c**) and number (**d**) of the liver metastasis. *Bar*, SD

chymase to 12 and 3 %, respectively, of control siRNA treated cells (Fig. 6b). At week 3, knockdown of renin or chymase showed smaller and less numbered metastatic foci in the liver than those in control mice (Fig. 6c, d).

## HbA1c and angiotensin-related factors in the primary tumors of CRC

Our results showed that high blood sugar induced A-II activation in CRC cells. We then examined the diabetic status and HbA1c levels in patients (Fig. 7). Among the 121 patients, 57 were or have been subsequently diagnosed as having diabetes according to the criteria [19]. In Fig. 7a, production of renin, chymase and A-II was examined by immunohistochemistry in the representative cases of diabetic stage D (liver metastasis-positive) and nondiabetic stage B. In the stage D case, cancer cells showed production of renin, chymase and A-II, whereas cancer cells in the stage B case showed only chymase immunoreactiveity. HbA1c concentration was higher in diabetic patients at each stage than in nondiabetic patients at the same stage (Fig. 7b; $P < 0.0001$ and $P < 0.01$). HbA1c concentration was also found to be associated with stage in diabetic patients ($P < 0.01$). We next examined the relationship between the angiotensin-activating ability of CRCs and disease progression (Fig. 7c). Because A-II is an activated product of ATG, examination of A-II in the CRC tumor tissues is a suitable marker of the angiotensin-activating ability of CRCs. We examined the A-II concentration in primary tumors. The tumoral A-II concentration was higher in stage C and D patients with diabetes than that in non-diabetic patients ($P < 0.05$). Tumoral A-II concentration was found to be strongly associated with pathological stage in diabetic patients ($P < 0.0001$).

Next, we examined renin-chymase expression in primary tumors (Fig. 7d, e). The tumoral renin concentration was higher in diabetic patients at each stage than in non-diabetic patients. Renin concentration was associated with stage only in diabetic patients (Fig. 7d). In contrast, tumoral chymase concentration was associated with stage in both diabetic and nondiabetic patients; however, chymase concentrations did not differ between diabetic and nondiabetic patients (Fig. 7e). Tumoral renin concentration correlated with HbA1c levels in the 121 patients examined (Spearman's $r = 0.9186$, $P < 0.0001$) (Fig. 7f). Finally, tumoral A-II concentration correlated with tumoral renin concentration across all patients (Spearman's $r = 0.7573$, $P < 0.0001$) (Fig. 7g). Thus, angiotensin activation is associated with the diabetic/hyperglycemic status of CRC patients and with liver metastasis.

## Discussion

In the present study, we examined the role of diabetic status on CRC progression, and found several important

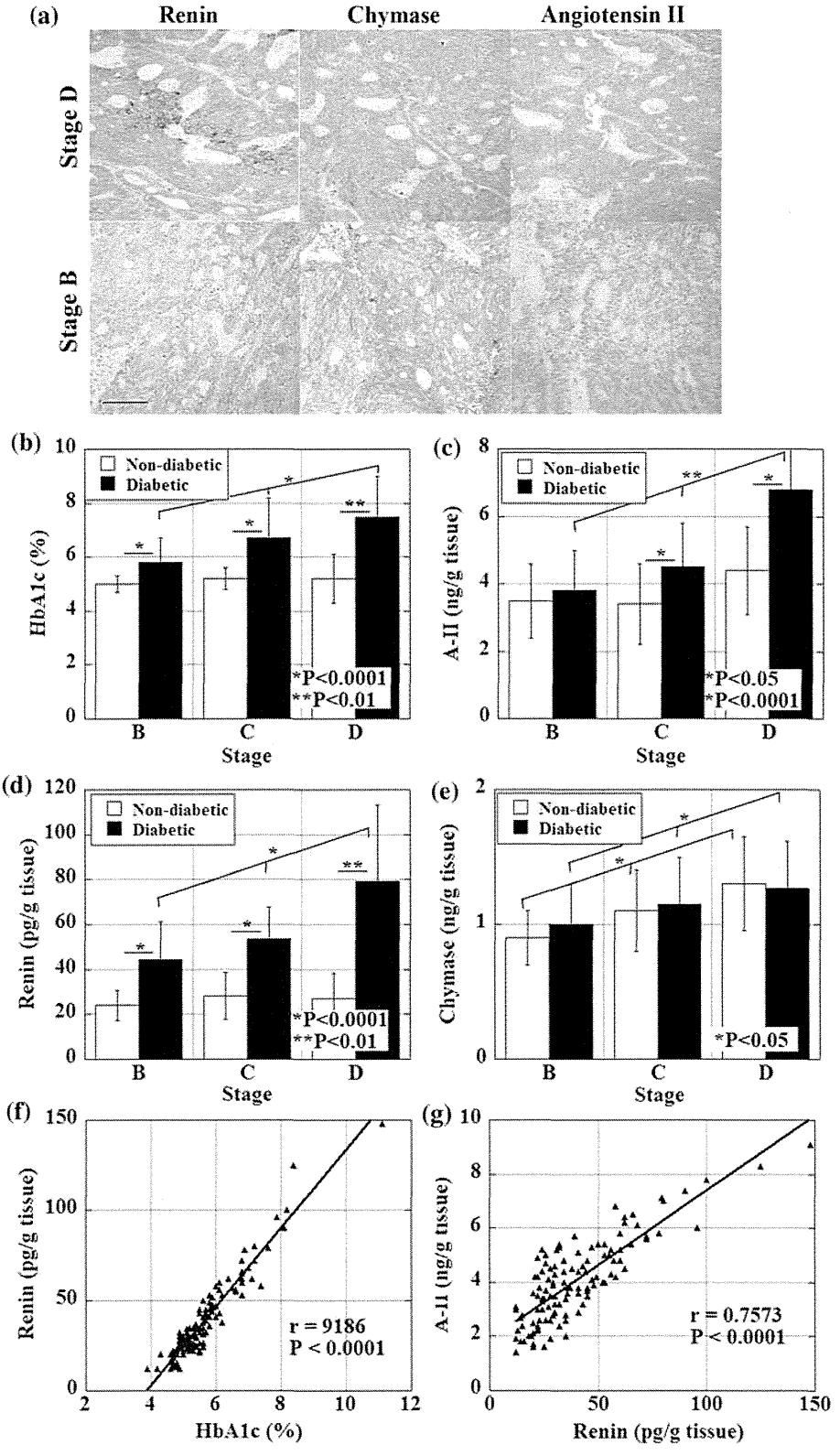


**Fig. 7** Expression of angiotensin-related factors and hemoglobin A1c in 121 human colon cancer patients. **a** Immunohistochemistry of renin, chymase, and angiotensin II in cases of diabetic stage D and nondiabetic stage B. *Bar*: 200 μm. **b–e** Relationship between stage and concentrations of hemoglobin (Hb)A1c (**b**), tumoral angiotensin II (A-II) (**c**), tumoral renin (**d**), or tumoral chymase (**e**) in diabetic and nondiabetic colon cancer patients. **f, g** Regression analysis of HbA1c levels and tumor renin concentration, and of tumor renin concentration and tumoral A-II concentration

results. CRC cells possess an angiotensin activation mechanism provided by the expression of renin and chymase. Cathepsin D is responsible for producing A-I from ATG in cardiac myocytes, fibroblasts, and vascular smooth muscle cells in place of renin [20]. However, the CRC cells in this study did not express cathepsin D.

Both HT29 and CT26 cells expressed renin, and this expression was associated with glucose concentration in a

dose-dependent manner. In cardiac fibroblasts, a high concentration of glucose significantly increases intracellular A-II levels by increasing the intracellular levels of renin [21]. Clinical data indeed show that diabetes was associated with high tumoral renin concentrations, high tumoral A-II concentrations, and liver metastasis.

We confirmed the pro-tumoral effects of A-II. A-II enhanced proliferation, invasion, survival, and liver metastasis of CRC cells. In CRC cases, A-II concentration in the primary tumors was higher in patients with liver metastasis than that in those without liver metastasis. These results suggest that liver metastasis may be enhanced in patients with diabetes by hyperglycemic status-associated angiotensin activation.

In the CRC cells, chymase, but not ACE, resulted in the conversion of A-I to A-II. Chymase, tonin, and cathepsin G all possess an ACE-like activity, which can be used as a substitute for ACE [22]. The major component of A-II activity in the human left cardiac ventricle consists of chymase, which is associated with hypoxia or ischemia [23]. Chymase expression in gastrointestinal cancer cells has not been reported; however, chymase-positive mast cell infiltration is associated with angiogenesis in gastric cancer [24, 25]. We confirmed that chymase is expressed in CRC cells as well as in infiltrating mast cells in human CRC cases (data not shown).

A-II is generated from AGT, which is produced in the liver [26]. We hypothesized that CRC cells possessing angiotensin-activating ability establish liver metastasis because these cells can produce abundant A-II from AGT in the liver. In the present study, we suppressed AGT production in the mouse liver by using pro-AGT antisense S-ODN, which significantly suppressed the liver metastasis of CRC cells. The results suggest that CRC cells with angiotensin-activating ability possess increased potential for liver metastasis. Moreover, the presence of a large amount of A-II in primary CRC tissues, which suggests the potential angiotensin-activating ability of CRC cells, was associated with a high frequency of liver metastasis. Hence, A-II concentration in primary CRC tissues is suggested as a good marker for liver metastasis.

Inhibitors of the renin-angiotensin system are widely used to treat hypertension. In the present study, some antiangiotensin agents, inhibitors of renin and chymase, suppressed liver metastasis of CRCs. ACE inhibitors and/or ARB have been reported to improve disease prognosis or progression in pancreatic and urogenital cancer [27, 28]. Antiangiotensin systemic therapy should therefore be tested for prevention of liver metastasis in cases of colon cancer.

Considering that hyperglycemia is associated with liver metastasis of colon cancer through renin upregulation, diabetic status is believed to be a risk factor for liver metastasis. Controlling blood sugar levels might therefore be important in preventing liver metastasis in colon cancer patients.

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ARTICLE

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# IL-1β-Mediated Up-Regulation of DEC1 in Human Gingiva Cells Via the Akt Pathway

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## ABSTRACT

Growing evidence indicates that inflammation is a contributing factor leading to cancer development. However, pathways involved in this progression are not well understood. The involvement of DEC1 in cancer prompted us to examine whether pro-inflammatory cytokine interleukin-1β (IL-1β) induces the expression of DEC1 in oral inflammation. We found that IL-1β up-regulated DEC1 and hypoxia-inducible factor-1α (HIF-1α) protein and elevated the HIF-1α-responsive gene vascular endothelial growth factor (VEGF) expression in human primary gingival cells. HIF-1α and DEC1 immunoreactivity were significantly higher in the cases of gingival inflammation. We demonstrate that IL-1β up-regulates DEC1 and HIF-1α protein through a classical inflammatory signaling pathway involving Akt. Our data strongly suggest that PI-3K–Akt is an upstream participant in IL-1β-mediated DEC1 and HIF-1α induction. This is supported by the following data: (1) IL-1β induces 473 serine phosphorylation of Akt; (2) IL-1β-mediated Akt activation occurs in a PI-3K-dependent manner, and specific inhibition of PI-3K prevents Akt phosphorylation; and (3) inhibition of Akt prevents IL-1β-mediated DEC1 and HIF-1α induction. Taken together, these results suggest that DEC1 is one of the important transcription factors in inflammation. J. Cell. Biochem. 113: 3246–3253, 2012.



**KEY WORDS:** IL-1β; DEC1; AKT; GINGIVAL EPITHELIUM

Two members of DEC/STRA/SHARP proteins are identified in each mammalian species studied with a sequence identity of >90% in the basic helix–loop–helix (bHLH) region and >40% in the total proteins, respectively [Fujimoto et al., 2001]. They exhibit an overlapping tissue distribution, and their expression is highly elevated in response to environmental stimuli [Boudjelal et al., 1997; Rossner et al., 1997; Shen et al., 1997; Fujimoto et al., 2001]. In rats that undergo seizure induction by kainic acid, the levels of mRNA encoding SHARP1 or 2 are sharply increased within 1 h in the brain [Rossner et al., 1997]. In cultured human cells, both DEC1 and

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DEC2 are markedly induced in response to hypoxia [Miyazaki et al., 2002]. Co-transfection experiments with promoter reporters have identified functional hypoxia response elements in both DEC1 and DEC2 genes. These elements show high affinity toward hypoxia-inducible factor-1α (HIF-1α) and -β (HIF-1β), providing a molecular explanation on the co-regulatory phenomena of DEC1 and DEC2 during hypoxia response [Miyazaki et al., 2002]. We previously reported that DEC1 overexpression and Akt phosphorylation is mediated via PI-3K signaling [Bhawal et al., 2011] and DEC2 negatively regulates VEGF expression [Sato et al., 2008]. Rapid induction of these proteins in response to environmental stimuli suggests that DEC/STRA/SHARPs are protective against detrimental conditions.

Gingiva is covered by stratified squamous epithelium with architectural characteristics unique to dental areas. The vast majority of cells that compose the gingival oral epithelium are keratinocytes, characterized by their ability to produce cytoplasmic keratin. These epithelial keratinization processes appear to be prompted by the underlying connective tissue [Hassell, 1993]. Gingival fibroblasts also play a major role in normal connective tissue turnover, as well as in wound healing repair and regeneration [Pitaru et al., 1994]. The structural composition of the epithelial–connective tissue interface is influenced by interactions between the cells of two tissue types, epithelial cells and fibroblasts. Interleukin 1β (IL-1β) is a potent multifunctional pro-inflammatory polypeptide produced by monocytes and tissue macrophages [Dinarello, 1996]. IL-1β attracts and activates immune cells and controls the expression of most immunomodulatory genes [O'Neill, 2000]. An inflammatory microenvironment characterized by the presence of host leukocytes is observed in most, if not all, tumors, which coincide with IL-1β expression in many of these tumors [Balkwill and Mantovani, 2001].

As a consequence of increased cellularity and proliferation, as well as enhanced metabolism within a tumor, the oxygen concentration in solid neoplasms is generally lower than in the adjacent non-neoplastic tissue [Harris, 2002; Vaupel, 2004]. In fact, histopathological examination of carcinomas frequently reveals hypoxic areas within the tumor mass, mostly in the form of necrotic regions. In addition to hypoxia, more recent evidence suggests that non-hypoxic pro-inflammatory stimuli, including cytokines and growth factors, can also activate HIF-1α under normoxic conditions and modulate the transcription of hypoxia-associated genes [Frede et al., 2007]. HIF-1α regulated gene products play essential roles in tumor progression by promotion of cell proliferation, invasion, and metastasis [Semenza, 2000]. In addition to tumor promotion, HIF-1α also appears to play a role in inflammatory processes by regulating innate and acquired immunity [Lukashev et al., 2001; Kojima et al., 2002; Mecklenburgh et al., 2002; Cremer et al., 2003]. Levels of expression of HIF-1α and HIF-1 target genes (i.e., CA IX, ADM, VEGF, PGK1, DEC1, and DEC2) have been extensively studied. Vascular endothelial growth factor (VEGF) is the most commonly expressed cytokine of this group. Carbonic anhydrase IX (CAIX) has recently emerged as one of the most promising endogenous markers of cellular hypoxia. Adrenomedullin (ADM) is a multifunctional regulatory peptide, and its numerous biological actions support an integrator role in the cellular response to inflammation. HIF-1α exclusively induces the hypoxic transcription of glycolytic gene phosphoglycerate kinase 1 (PGK1). Recently, numerous reports have provided evidence that inflammation can facilitate the development of cancer [Semenza, 2000; Balkwill and Mantovani, 2001]. HIF-1α activity is up-regulated by pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and IL-1β. IL-1β and TNF-α are able to increase HIF-1α protein, and enhance the HIF-1α DNA binding [Hellwig-Burgel et al., 1999]. In this context, the very potent pro-inflammatory IL-1β may be of particular importance as a possible stimulator of HIF-1α in the gingiva under normoxic conditions. DEC1 and DEC2 genes are direct targets of HIF1α, and DEC1 and DEC2, induced by HIF1α, are crucial for the adaptation to hypoxia [Miyazaki et al., 2002]. Despite intensive studies, understanding of the non-hypoxic regulation of DEC and HIF-1α is still limited.

On the basis of the above considerations, in the present study we set out to investigate the expression and role of HIF-1α and DEC1 in gingival inflammation. Here, we report that IL-1β can induce DEC1 and HIF-1α protein levels in gingival epithelial cells. We also demonstrate that the increase in DEC1 protein subsequently is followed by Akt phosphorylation.

## MATERIALS AND METHODS

### REAGENTS

Human recombinant-IL-1β was purchased from R&D Systems (Minneapolis, MN). LY294002 were purchased from Calbiochem (San Diego, CA). DEC1 antibody (CW27) was a kind gift from AL Harris (Cancer Research UK Molecular Oncology Laboratory, Oxford, UK). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

### PREPARATION OF CELLS

Human primary epithelial (HGE) and fibroblast (HGF) cells were prepared from healthy gingival tissues from donors (n = 3) with their informed consent. Briefly, the gingiva was treated with 0.025% trypsin and 0.02% EDTA overnight at 4°C and epithelial cells were isolated as described previously [Yuspa and Harris, 1974]. The cell suspension was centrifuged at 120*g* for 5 min, and the pellet was suspended in medium for epithelial cells containing 0.4% (v/v) bovine pituitary extract, 10 μg/ml insulin, 0.1 ng/ml hEGF, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B (HuMedia-KG2, KURABO, Tokyo, Japan). The cells were seeded in 60-mm plastic tissue culture plates and incubated in 5% $CO_2$/95% air at 37°C. When the cells reached sub-confluence, they were harvested and sub-cultured.

Human gingival fibroblast tissue were cut into small pieces and plated in 35-mm culture dishes (Corning Inc., Corning, NY) containing Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1 μg/ml of amphotericin B. When the human gingival fibroblasts formed a sub-confluent monolayer, they were harvested and seeded on a 100-mm culture dish (Corning). Human gingival epithelial and fibroblast cells at the fourth passage were used in the experiments.

IL-1β (10 ng/ml) was added to the cells for 24 h to evaluate the effect of treatment with an inflammatory cytokine. Cells were treated with LY294002 (10 μM) for 1 h prior to IL-1β treatment.

### QUANTITATIVE REAL TIME-PCR (QRT-PCR)

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). One-hundredth aliquot of the cDNA was subjected to real-time RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) for HIF-1α driven genes: CAIX, ADM, VEGF, PGK1, DEC1, and DEC2, and Pre-Developed TaqMan Assay Reagents (Applied Biosystems) for ACTB as an internal control. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to ACTB expression of each cell.

### SMALL INTERFERING RNA

The duplexes of each small interfering RNA (siRNA), targeting Akt and negative control (non-silencing siRNA or scrambled siRNA) were synthesized by Qiagen. The siRNAs were transfected into the cells using RNAiMAX (Invitrogen, Carlsbad, CA). The cells were incubated for 48 h and subjected to various analyses.

### WESTERN BLOTTING

Cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). SDS–PAGE were calibrated with molecular weight markers (Bio-Rad). DEC1 and DEC2 (1:1,000), Akt (1:1,000; Cell Signaling Technology, Inc., Danvers, MA), Phospho-Akt (1:2,000; Cell Signaling Technology), HIF-1α (1:500; BD Biosciences, San Jones, CA), and GAPDH (1:1,000; Cell Signaling Technology) were used as primary antibodies. Anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology) were each used at a dilution of 1:2,000. Bound antibodies were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham, Uppsala, Sweden), and images were analyzed by a Luminescent Image Analyzer (LAS-3000; Fuji Film Inc., Japan). The experiment was repeated three times. Quantitative analysis of relative protein expression of hDEC1, HIF-1α, Phospho-Akt, and GAPDH were calculated using Image J software.

### IN VIVO EXPERIMENT

Twelve male Sprague–Dawley rats (3-week old) were obtained from Nihon SLC (Shizuoka, Japan) and housed in isolation cages throughout the experimental period. They were fed a standardized diet of hard briquettes and water, and maintained under a 12-h light/dark cycle at a temperature of 22°C and relative humidity of 50%. Rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in drinking water, ad libitum, for 4 days to reduce their native oral flora. This was followed by a 4-day antibiotic-free period. Rats were orally challenged with *Porphyromonas gingivalis* ATCC 33277 (laboratory stock), which was suspended in 5% carboxymethylcellulose. Each rat received 0.5 ml ($1 \times 10^9$ cells/ml) of the suspension by oral gavage (three times) at 48-h intervals. Control rats received carboxymethyl cellulose only. The animals were sacrificed after 3 days of gingivitis and the upper jaws were excised. Formalin-fixed specimens were decalcified with a 10% EDTA-2Na for 2 weeks and embedded in paraffin. The experimental procedures of this study were conducted under protocols approved by the Animal Care and Ethics Committee in accordance with Kanagawa Dental College guidelines.

### IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded human specimens (chronic gingivitis n = 11, chronic marginal periodontitis n = 11, healthy controls n = 5) and experimental rat gingivitis tissues. Mouse monoclonal anti-HIF-1α antibody (1:500; H1α67, Novus Biologicals, Littleton, CO), rabbit polyclonal anti-DEC1 CW27 (1:5000), anti-DEC1 (1:100), were used as primary antibodies. CSA system (Dako, Carpinteria, CA) was employed. Sections were initially immersed in Target Retrieval Solution (Dako) at 97°C for 40 min, and subsequent steps were performed according to manufacturer's instructions. Mouse monoclonal anti-RM-4 (1:50; RM-4, Transgenic Inc., Kobe, Japan), rabbit polyclonal anti-TNF-α (IHC world, LLC, Woodstock, MD), and rabbit polyclonal anti-IL-1β (1:200; Santa Cruz Biotechnology, Inc., Delaware, CA) antibodies were also used to detect the immunoreactivity in rat tissues. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

### STATISTICAL ANALYSES

Significant differences were analyzed by Fisher's exact test and a *P*-value of $<0.05$ was considered statistically significant.

## RESULTS

### INCREASED HIF-1α AND DEC1 IMMUNOSTAINING IN INFLAMMATORY GINGIVAL TISSUES

In all chronic inflammatory conditions, HIF-1α and DEC1 nuclear expression was observed in suprabasal layers of the epithelium. There was also positive staining present in some of the nuclei in the basal layer (Fig. 1A). We have confirmed the increased level of TNF-α, RM-4 (Fig. 1B), and IL-1β (Fig. 1C) protein expression in *P. gingivalis*-challenged rat gingival epithelium compared to the control. Immunohistochemical analysis revealed a higher expression in HIF-1α and DEC1 in the rat gingivitis tissues compared with that in the control tissues (Fig. 1C).

### EFFECTS OF IL-1β ON THE EXPRESSION OF HYPOXIA-RELATED GENES IN HUMAN PRIMARY GINGIVAL EPITHELIAL AND FIBROBLAST CELLS

Gingival cells were incubated for 24 h under IL-1β treatment to determine the time course for the induction of DEC1 gene expression in gingival cells. The concentration used in the experiment was not toxic for the cells as judged by MTS assay [Ito et al., 2012]. Steady-state levels of DEC1 mRNA derived from HGEs were up-regulated in the presence of IL-1β (10 ng/ml), as determined by real-time RT-PCR analysis (Fig. 2). On the contrary, DEC1 expression was down-regulated in HGF cells in response to IL-1β. Significant VEGF

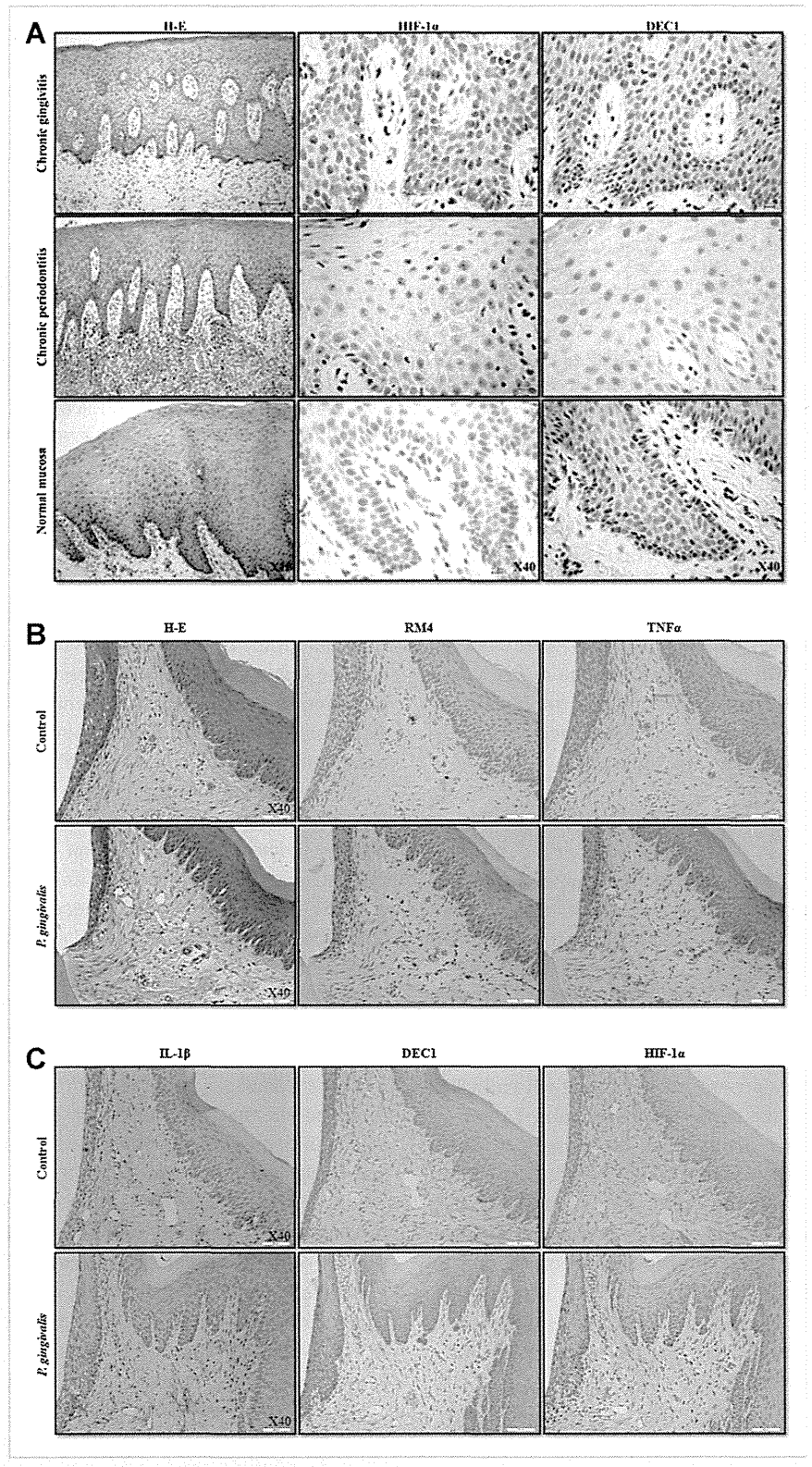


Fig. 1. Immunohistochemical analysis of DEC1 and HIF-1α in gingival inflammation. Four-micrometer-thick sections of formalin-fixed and paraffin-embedded specimens were deparaffinized and immunoreactivity was detected using the DAKO ENVISION Kit. The degree of staining was measured as the percentage of positively stained nuclei in inflammatory cells. A: DEC1 expression in normal human oral tissues was present predominantly in the granular and spinous layers of epithelial cells, while HIF-1α expression was almost absent. In human chronic gingivitis and chronic periodontitis samples, DEC1 expression was present predominantly in the suprabasal layer of epithelial cells. DEC1 and HIF-1α appeared highly expressed in patients with oral inflammation. B: Immunohistochemical analysis revealed a higher expression in RM-4 and TNFα expression in *P. gingivalis* challenged rat gingiva compared to the control. C: The increased expression of IL-1β was also observed in the experimental rat gingivitis model. DEC1 and HIF-1α were abundantly expressed in the *P. gingivalis* challenged rat gingiva samples. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

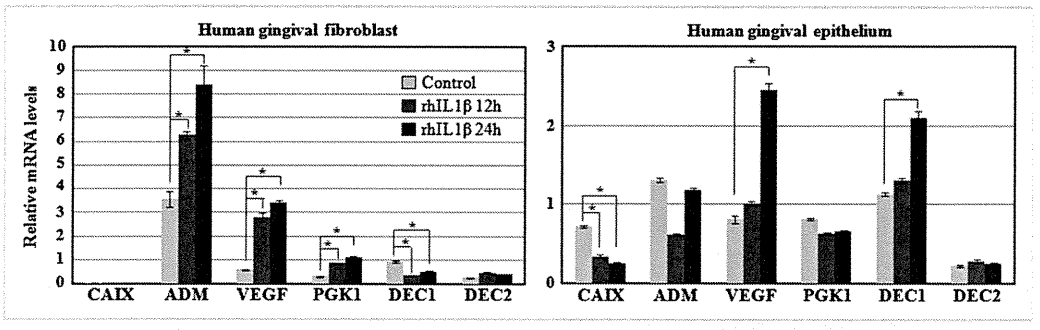


Fig. 2. Effects of IL-1β on the expression of hypoxia-related genes in human primary gingival epithelial and fibroblast cells. Human primary epithelial and fibroblast cells were prepared from healthy gingival tissues. Cells were incubated with 10 ng/ml of human recombinant IL-1β for 24 h. Total RNA was isolated and subjected to quantitative real-time PCR. DEC1 mRNA derived from HGEs was up-regulated in the presence of IL-1β. Significant VEGF expression was detected in both HGF and HGE cells after 24 h of IL-1β incubation. Relative mRNA levels were calculated as the ratio to that of house keeping gene (ACTB), and each bar represents the mean ± SD for at least three independent experiments (*$P < 0.05$).

expression was observed after 24 h of incubation with IL-1β in both HGF and HGE cells. There were no consistent differences in the mRNA levels of DEC2 after IL-1β incubation. ADM was markedly up-regulated in IL-1β-treated HGF cells, whereas there was no difference in IL-1β-treated HGE cells. The expression of CAIX was only observed in HGE cells and it was significantly downregulated in response to IL-1β. The mRNA levels of PGK1 were markedly increased in IL-1β-treated HGF cells only (Fig. 2).

### INVOLVEMENT OF PI3-K/AKT IN IL-1β-INDUCED DEC1 EXPRESSION

To determine whether PI-3K/Akt cascade plays an important role in IL-1β-induced DEC1 expression in gingival epithelial cells, pre-treatment of cells with a pharmacological inhibitor of PI-3K, LY294002 significantly attenuated the IL-1β-stimulated DEC1 expression, suggesting the involvement of PI-3K/Akt in DEC1 expression (Fig. 3A). Moreover, data in Figure 3B showed that IL-1β stimulated Akt phosphorylation in a time-dependent manner, which was significantly inhibited by pre-treatment of gingival epithelial cells with LY294002 during the period of observation. To ensure further that Akt was indeed involved in IL-1β-induced DEC1 expression, cells were transfected with Akt siRNA for 24 or 48 h. As shown in Figure 3C, transfection with Akt siRNA for 48 h significantly knocked down the Akt protein expression and had no effect on housekeeping GAPDH expression. Furthermore, data in Figure 3D, transfection with Akt siRNA for 48 h significantly inhibited IL-1β-induced DEC1 expression. These results suggest that PI-3K/Akt plays an important role for IL-1β-induced DEC1 expression in gingival epithelial cells.

## DISCUSSION

To help characterize the role of the newly discovered transcription factor DEC1 in inflammation, we studied the signaling pathway by which DEC1 expression was induced. We found that IL-1β-induced DEC1 expression is dependent on PI-3K/Akt signaling pathway. This conclusion is supported by the following results: (a) IL-1β induces 473 serine phosphorylation of Akt; (b) a PI-3K inhibitor (LY294002) inhibited the expression of DEC1 and the induction of DEC1 expression by IL-1β; (c) LY294002 inhibited the phosphorylation of Akt; and (d) the knock-down of Akt inhibited the DEC1 expression and the induction of DEC1 expression by IL-1β. These results are consistent with other reports showing an involvement of the PI-3K–Akt pathway and with data demonstrating that PI-3K inhibition antagonizes HIF-1α induction by IL-1β [Stiehl et al., 2002].

The association between chronic inflammation with a variety of epithelial malignancies has been recognized. Examples of inflammatory processes linked with an increased cancer risk include inflammatory bowel diseases and colorectal adenocarcinoma, atrophic gastritis and gastric cancer, cholangiocarcinoma related to chronic cholecystitis, and esophageal carcinoma following reflux esophagitis [Weitzman and Gordon, 1990; Balkwill and Mantovani, 2001; O'Byrne and Dalgleish, 2001]. As a consequence, there is now enough evidence that the increased risk for malignant transformation is related to inflammation-associated damage to DNA (such as oxidative damage) and disruption of tissue architecture and function via the "activation" of stromal cells and components able to influence cell survival, growth, proliferation, differentiation, and movement [Mignogna et al., 2004].

It is well established that IL-1β is a major cytokine involved in the inflammatory process in periodontitis [Tatakis, 1993]. IL-1β acts directly on local fibroblasts in inflammatory condition, inducing a variety of genes and helping to create an activated phenotype characterized by hyperplasia and invasiveness [Walsh et al., 1998]. Recent evidence has identified a link between inflammation, and the activation of HIF-1 expression. Inflammatory cytokines induce HIF-1α accumulation and HIF-1 DNA binding in different cell types [El Awad et al., 2000; Dame et al., 2004]. IL-1β can increase HIF-1 DNA binding and VEGF production, which suggests a link between this cytokine and HIF-1α regulation [Thornton et al., 2000; Koyama et al., 2002]. HIF-1α protein was abundantly expressed by macrophages in inflamed rheumatoid synovia while being absent in healthy synovial [Hollander et al., 2001]. Conditional knockout of HIF-1α in macrophages and other myeloid lineage cells leads to decreased myeloid cell infiltration and activation, to impaired

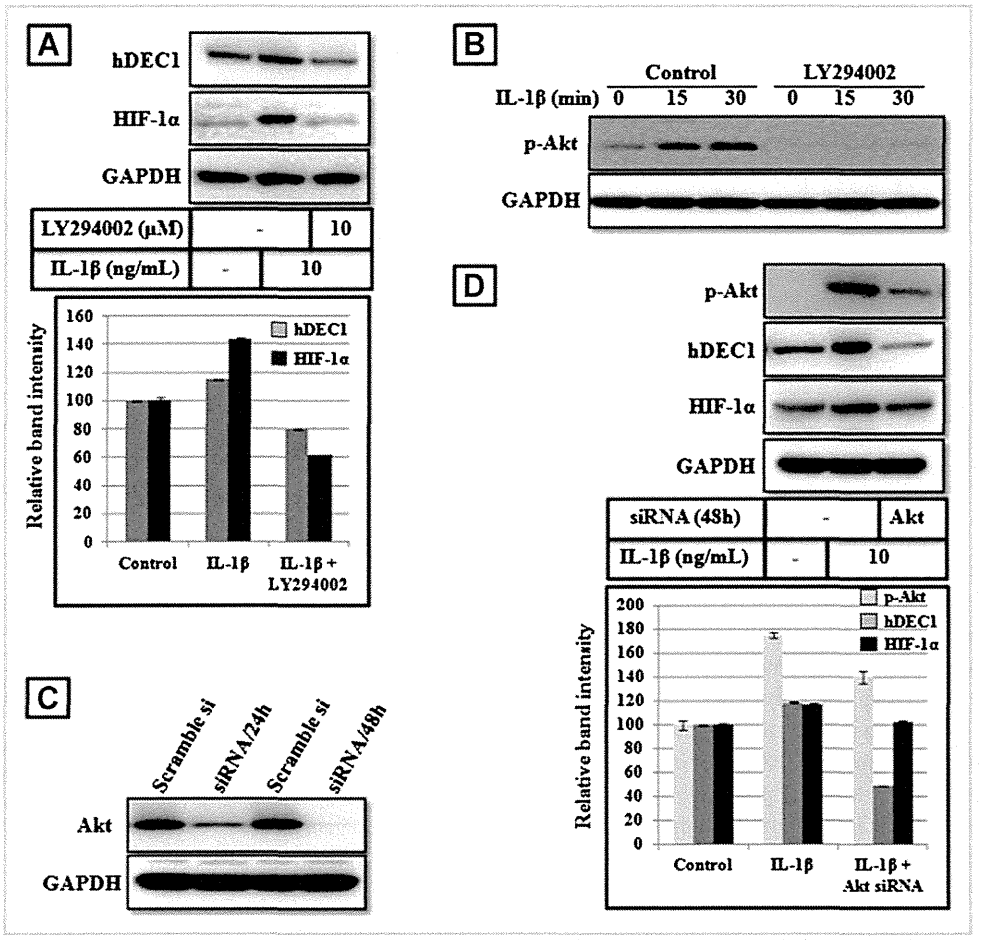


Fig. 3. PI-3K/Akt plays a critical role in IL-1β-stimulated DEC1 expression. A: Human primary gingival epithelial cells were pre-treated with LY294002 for 1 h and then incubated with IL-1β (10 ng/ml) for 24 h. The cell lysates were assayed to determine the expression of DEC1 and HIF-1α as described in Materials and Methods Section. Densitometric measurements normalized to those of the house keeping gene (GAPDH) were analyzed and shown as mean values ± SD of three independent experiments. B: Human primary gingival epithelial cells were treated with 10 ng/ml IL-1β for the indicated times in the absence or presence of 10 μM LY294002. The cell lysates were assayed to determine the phosphorylation of Akt. C: Human primary gingival epithelial cells were transfected with Akt siRNA for 24 or 48 h. The cell lysates were assayed by Western blot analysis using an anti-Akt polyclonal antibody. Membranes were stripped and re-probed with an anti-GAPDH antibody as a control. D: Human primary gingival epithelial cells were transfected with Akt siRNA. After transfection for 48 h, the cells were stimulated with IL-1β (10 ng/ml) for 24 h, and then cell lysates were analyzed for DEC1 and HIF-1α protein by Western blot analysis. Densitometric measurements normalized to those of the house keeping gene (GAPDH) were analyzed and shown as mean values ± SD of three independent experiments.

chronic cutaneous inflammation, and to decreased joint inflammation in a rheumatoid arthritis [Cremer et al., 2003]. Taken together, these studies implicate HIF-1α as an important mediator of inflammatory responses in macrophages.

Studies to uncover the signaling pathways involved in non-hypoxic HIF-1α activation revealed the involvement of MAPKs and PI-3K/Akt in HIF-1α accumulation and transactivation [Minet et al., 2000; Michiels et al., 2001; Zhou et al., 2003]. We demonstrate that a major inflammatory cytokine, IL-1β, up-regulates HIF-1α protein via an inflammatory signaling pathway involving Akt. Inhibition of Akt significantly reduced IL-1β-induced DECs expression, which shows an important role for Akt in cytokine induced DEC1 expression. We thus conclude that Akt and NF-κB independently contribute to the expression of DEC1.

Binding of DEC1 to cytokine-regulated elements prompted us to examine the effect of cytokines on the DEC expression. DEC1$^{-/-}$, in comparison with wild-type CD$^{+}$ T cells, produced less interferon-gamma (IFN-γ) and IL-4. Reintroduction of DEC1 in these cells fully rescued IFN-γ and IL-4 expression in DEC1$^{-/-}$ cells upon their differentiation into Th1 cells, indicating that expression activation of these cytokines depends on the DEC1 function. Endogenous DEC1 expression was significantly increased upon treatment with IL-2, IL-6, IL-12, IL-15, TNF-α, and IFN-β, while IL-1β, IL-4, IL-7, IL-18, and IL-21 effects were less pronounced, after 3 h of stimulation in human NK-92 natural killer cell line [Ivanova et al., 2004]. We also performed a preliminary screening of IL-1β-treated human epithelial cells for 24 h and found IL-1β, IL-6, IL-8, IL-12 (P40), TNF-α, and GM-CSF protein in the analyzed supernatants (Supplementary data). Such a broad spectrum of cytokines affecting DEC1 transcription implies an important role for DEC in functionally pleiotropic processes of cell growth, differentiation, apoptosis, immune response, etc. Our data suggest that various cytokines

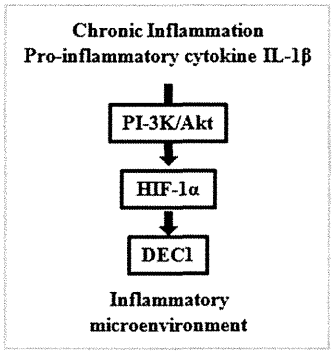


Fig. 4. Schematic representation in a model of DEC1 participation in inflammation. DEC1 is constitutively expressed in gingival epithelium. Expression of DEC1 was up-regulated in gingival inflammation in a PI-3K/Akt-dependent pathway.

converge upon Akt to participate in DEC1 regulation and that DEC1 activation may occur in response to diverse cellular stimuli.

The results in our study present evidence that IL-1β-induced DEC1 protein, with a subsequent increase in gene transcription of several HIF-1α driven genes. Our group has previously demonstrated the differences in the gene expression profiles of HGE and HGF cells using DNA microarray technique [Abiko et al., 2004]. We believe that these differentially expressed genes in this study play important roles in the processes of epithelial–fibroblast interaction through the secretion of their gene products into the microenvironment at disease sites. Our findings suggest that IL-1β-mediated DEC1 up-regulation is a physiological response to inflammatory conditions (Fig. 4). We have thus hypothesized that IL-1β may be restricted with regard to the cell types it can efficiently activate. Therefore, although gingival epithelial cells may be potently activated by IL-1β, other cell types, such as gingival fibroblasts (monocytes/macrophages), produce pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, may be activated relatively weakly by IL-1β.

This study provides a basic mechanism for inflammation and reveals multiple molecular targets for the development of therapeutic agents. Our current findings are the first to show that IL-1β participates in DEC1 expression downstream of the PI-3K/Akt pathway. Our studies are underway to determine the roles and mechanisms of the differentially expressed genes in the pathogenesis of gingivitis and to elucidate the molecular basis for the signaling pathways involved in epithelial–fibroblast communications under both physiological and pathophysiological conditions.

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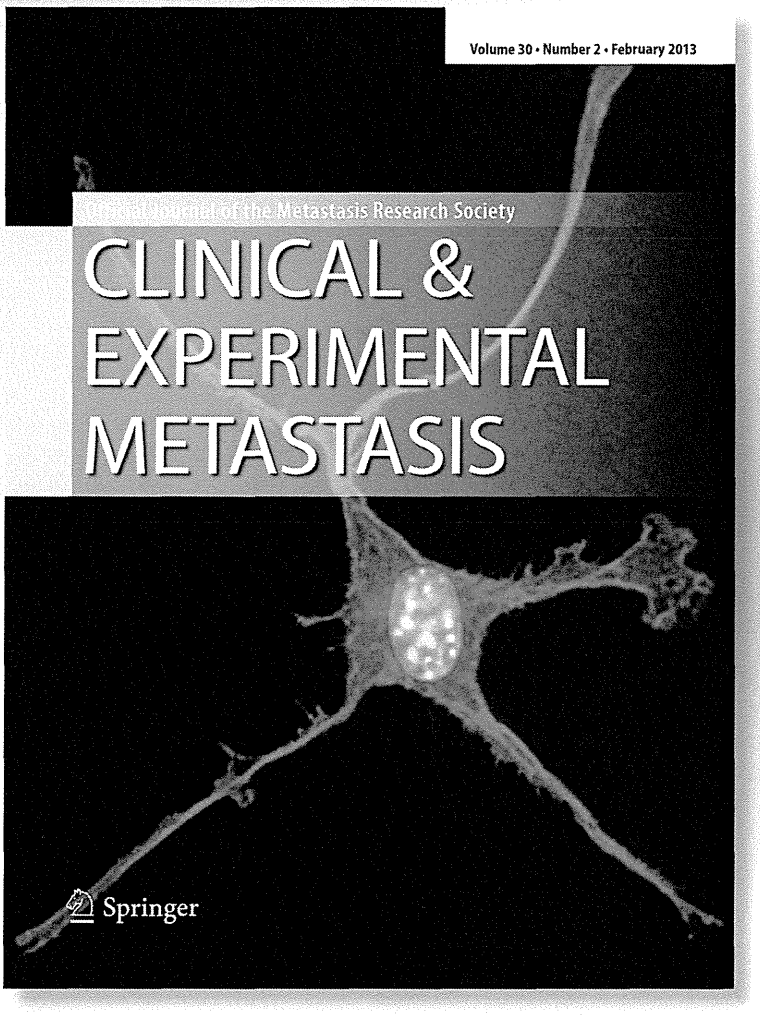


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