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Hypoxia upregulates adhesion ability to peritoneum through a transforming growth factor-β-dependent mechanism in diffuse-type gastric cancer cells

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ABSTRACT

Gastric cancer cells leaving the primary tumour are exposed to low oxygen levels in the peritoneal cavity; however, peritoneal metastatic phenotypes of hypoxic cancer cells remain unclear. We used 6 gastric cancer cell lines, including 3 diffuse-type gastric cancer (DGC) and 3 non-DGC cell lines. Using adhesion assay, we examined the effect of hypoxic conditions on their ability to adhere to peritoneal components. The expression level of *transforming growth factor*-β (TGF-β) and *integrins* mRNA of cancer cells was examined using reverse transcriptase-polymerase chain reaction. We further examined the effect of anti-integrin neutralising antibodies and a TGF-β receptor inhibitor on the adhesion ability of hypoxic cancer cells. The binding ability of DGC cells was higher than that of non-DGC cells; it was significantly increased by hypoxic (1% O_2) conditions compared to normoxic (21% O_2) conditions. In contrast, no remarkable change in adhesion ability was observed in the non-DGC cells under normoxic and hypoxic conditions. Integrins and TGF-β expression of hypoxic DGC cells was significantly higher than that of normoxic cells. TGF-β increased the adhesion ability and α2-, α3- and α5-*integrin* expression of hypoxic DGC cells, whereas the TGF-β receptor inhibitor decreased them. Neutralising antibodies against α2-, α3- and α5-integrin inhibited the adhesion ability of DGC cells. These findings suggested that hypoxic conditions promote the adhesion of DGC cells to the peritoneum. The upregulation of α2-, α3- and α5-*integrin* by TGF-β under hypoxic conditions may be one of the mechanisms responsible for the high metastatic potential of hypoxic DGC cells to the peritoneum.

1. Introduction

Hypoxic environment exists in most cancers because angiogenesis in solid carcinomas with a rapid growth is heterogeneous.[1] Although hypoxia is cytotoxic to both cancer and normal cells, some cancer cells acquire characteristics that allow them to survive and proliferate in a hypoxic environment.[2] These ischaemic conditions sometimes contribute to the metastatic phenotypes of cancer cells.[3,4] Clinical and experimental data also provide evidence for an association between hypoxic tumour microenvironment and poor prognosis.[5–7] However, the mechanisms responsible for the malignant progression of hypoxic cancer cells remain unclear.

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There are two types of gastric cancer: diffuse type and intestinal type. Prognosis of patients with diffuse-type gastric carcinoma (DGC), according to the Laurén classification[8], is poor because of the frequent incidence of peritoneal metastasis.[9–11] Peritoneal dissemination may arise from free cancer cells in the peritoneal cavity exfoliated from the serosal surface of the primary tumour.[12,13] Peritoneal lavage cytology at laparotomy has been a standard method for the detection of free tumour cells and a useful predictor of peritoneal recurrence in gastric cancer.[14] However, patients with positive cancer cells detected by cytology have not always developed peritoneal disease. Cancer cells leaving the primary tumour might be exposed to low oxygen levels in the abdominal cavity because no feeding vessel is found around these free cancer cells. In fact the abdominal cavity filled with ascites was severely hypoxic (<10 mm Hg) at the terminal stage after orthotopic implantation of human pancreatic cancer cell line.[15] Our preliminary clinical study showed that DGC cells detected by cytology had higher peritoneal metastatic potentials than non-DGC cells (data not shown). These findings suggest that hypoxia might affect the phenotype of free cancer cells in the peritoneal cavity.

Peritoneal dissemination processes are characterised by detachment of cancer cells from the primary gastric tumour, attachment to the peritoneum and growth at the site.[12] We hypothesised that only those cancer cells that adhere to the peritoneum under hypoxic conditions might cause peritoneal dissemination. Therefore, examination of the characteristics of hypoxic cancer cells is important.

We previously reported that α2-, α3- and α5-integrin, major receptors involved in the adhesion of cancer cells to the extracellular matrix (ECM), might play an important role in peritoneal metastasis of gastric cancer.[13,16] We also reported that transforming growth factor-β (TGF-β) upregulates α2-, α3- and α5-integrin expressions and increases the adhesion of cancer cells to the ECM.[17,18] It has been reported that TGF-β1 production is significantly increased under hypoxic conditions in a variety of cells,[19] including gastric cancer cells. Therefore, we hypothesised that TGF-β1 produced from hypoxic gastric cancer cells might affect their integrin expression, thus resulting in high metastatic potential to the peritoneum.

In the present study, we investigated the effect of hypoxia on adhesion ability of cancer cells and found that hypoxia upregulates the adhesion of DGC cells through a TGF-β-dependent mechanism. To our knowledge, this is the first report demonstrating the functional relationship between hypoxia and integrin expression in gastric cancer cells.

2. Materials and methods

2.1. Cell lines

Six gastric cancer cell lines were used. OCUM-2MD3[13], OCUM-8[20] and KATO-III[21] were derived from DGC, and MKN7[22], MKN45[22] and MKN74[22] were derived from non-DGC. Cells were cultured at 37 °C in 21% O_2 (normoxia) or 1% O_2 (hypoxia). Hypoxic conditions were maintained using a modular incubator chamber (Hirasawa Works, Tokyo, Japan) with 5% CO_2 and 1% O_2 balanced with N_2 gas. The culture medium consisted of Dulbecco's modified Eagle's medium (Nikken Bio., Osaka, Japan) with 10% foetal bovine serum (Life Technologies, Grand Island, NY), 100 IU/ml penicillin (ICN Biomedicals, Costa Mesa, CA), 100 μg/ml streptomycin (ICN Biomedicals) and 0.5 mM sodium pyruvate (Cambrex, Walkersville, MD).

2.2. Adhesion assay

Using an adhesion assay, we examined the effects of hypoxic conditions on the adhesion ability of gastric cancer cells to peritoneal components. The binding of cancer cells to ECM components, matrigel (Collaborative Research Co., Bedford, MA), fibronectin (Mallinckrodt Specialty Chemicals Co., St. Louis, MO) and laminin (Mallinckrodt Specialty Chemicals Co.) was investigated. Ninety-six-well microtiter plates were coated with matrigel (8 μg/well), fibronectin (4 μg/well) or laminin (4 μg/well). These plates were left at 4 °C overnight and then incubated at 37 °C for 2 h. Gastric cancer cells (2×10^5 cells/well) were incubated under normoxic or hypoxic conditions for 48 h and then seeded onto the ECM components in the 96-well microtiter plates (Falcon, Lincoln Park, NJ). The cancer cells were allowed to adhere to each well for 30 min at 37 °C and then gently washed twice in phosphate-buffered saline (PBS). The adhering cancer cells were quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, Saint Louis, MO) colorimetric assay. The percentage of binding cells was calculated as follows: [OD of treated surface – OD of ECM component]/OD of total surface × 100. Total surface refers to the area of 2×10^5 cells seeded on the microtiter plates with matrigel, fibronectin and laminin.

To examine the effect of TGF-β on adhesion, cancer cells were incubated under normoxic conditions with the treatment of 10 ng/ml TGF-β1 (R&D Systems) for 24 h, following which the cells were used for the adhesion assay, as described above.

2.3. Effect of anti-integrin antibodies or TGF- receptor (TGF-R) inhibitor on adhesion activity of cancer cells

For integrin-blocking experiments, we used a neutralising antibody against integrins and a TGF-β receptor kinase inhibitor, SB431542 (Sigma). Further, 10 μg/ml each of neutralising monoclonal anti-integrin antibody [anti-α2β1-integrin (Covance, CA), anti-α3-integrin (Santa Cruz) and anti-α5β1-integrin (Chemicon, Temecula, CA)] was incubated with the cancer cells for 30 min before the adhesion experiment.

SB431542, a small synthetic molecule that interrupts the phosphorylation of Smad by TGF-βR, was used to study the inhibition of the TGF-β signalling pathway. Cancer cells were incubated under normoxic or hypoxic conditions in the presence of 10 μM SB431542 for 48 h, following which the cells were used in adhesion assays, as described above.

2.4. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time PCR was performed to examine *integrins* and *TGF-β1* mRNA expression. Cancer cells were incubated under

normoxic conditions with TGF-β and under hypoxic conditions with SB431542 for 48 h. After incubation, the total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After the removal of genomic DNA by DNAse, cDNA was prepared from 20 μg RNA with Maloney mouse leukaemia virus reverse transcriptase (Invitrogen) using random primers (Invitrogen). To determine fold changes in each gene, real-time RT-PCR was performed on the ABI Prism 7000 (Applied Biosystems, Foster City, CA), using commercially available gene expression assays for α1-, α2-, α3-, α5-, α6- and β1-*integrin* and *TGF*-β1 (Hs00235030, Hs00158148, Hs00233722, Hs00233732, Hs00173952, Hs01127543 and Hs00998130, respectively). PCR was performed at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalise mRNA levels for differences in sample concentration and loading. Fold changes in the expression of each target mRNA relative to GAPDH were calculated based on the threshold cycle (Ct) as $2^{-\Delta\ (\Delta Ct)}$, where ΔCt = Ct target – Ct GAPDH and Δ(ΔCt) = $\Delta Ct_{hypoxia} - \Delta Ct_{normoxia}$ Quantitative PCRs were performed in triplicate.

2.5. *Western blot analysis*

For examining the effect of hypoxia on Smad2 and Smad3 phosphorylation, cancer cells were incubated under normoxic or hypoxic conditions for 72 and 96 h, respectively. For examining the effect of TGF-β on Smad2 phosphorylation and inhibition by SB431542, the cancer cells were incubated with TGF-β(1 ng/ml) and/or with SB431542 (0.1 or 1 μM) under normoxic conditions. The cells were lysed in a lysis buffer, and aliquots containing 30 μg of total protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis; the protein bands were transferred to a polyvinylidene difluoride membrane (Amersham). The membrane was incubated in PBS-T (10 mM PBS and 0.05% Tween 20) supplemented with 5% non-fat milk or 5% bovine albumin (Sigma) at room temperature for 1 h. Next, the membrane was placed in a PBS-T solution containing the primary antibody p-Smad2 ($Ser^{465/467}$; 1:1000; Cell Signaling Technology, Danvers, CO), p-Smad3 ($Ser^{423/425}$, 1:1000, Cell Signaling Technology) or Smad2/3 (1:1000; Cell Signaling Technology) and allowed to react at 4 °C overnight. Next, each antibody was washed three times with PBS-T for 10 min, and a peroxidase-labelled

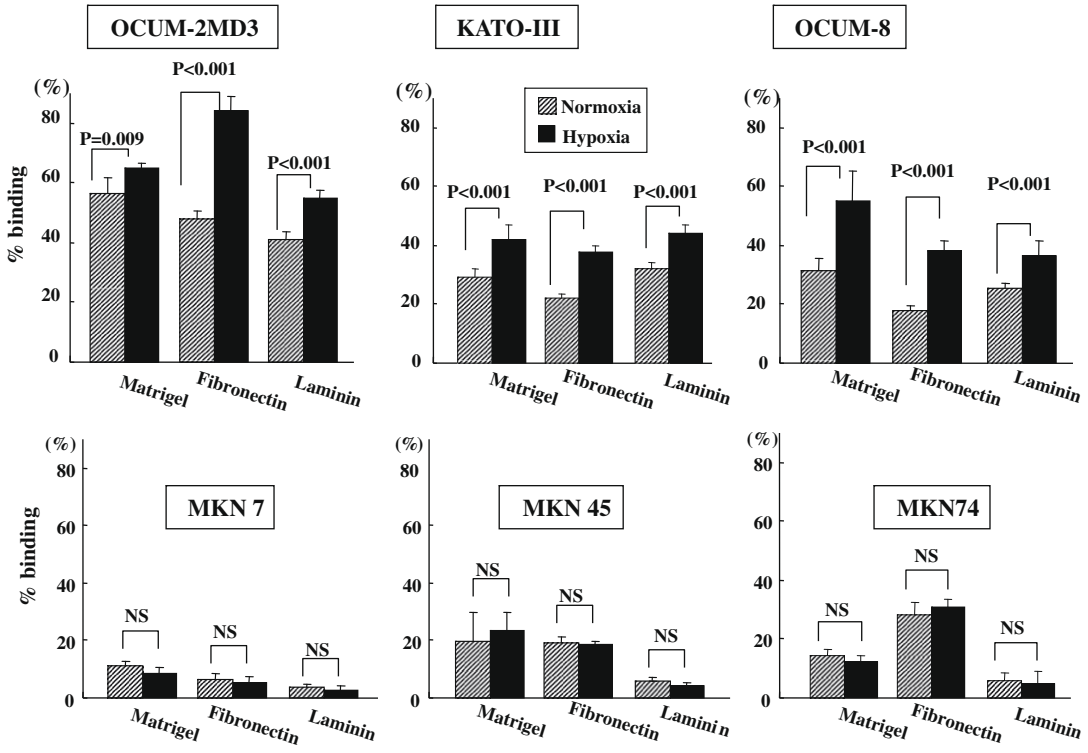


Fig. 1 – Adhesion ability of gastric cancer cells under normoxic or hypoxic conditions. The number of adherent diffuse-type gastric cancer (DGC) cells (OCUM-2MD3, OCUM-8 and KATO-III) was higher than that of the non-DGC cells (MKN7, MKN45 and MKN74). The number of OCUM-2MD3 cells adhering to the extracellular matrix (ECM) was significantly greater at 76.4% ($P < 0.001$) to fibronectin, 14.4% ($P = 0.009$) to matrigel, and 34.0% ($P < 0.001$) to laminin under hypoxic conditions, compared to that under normoxic conditions. The percentage of adherent KATO-III cells under hypoxic conditions was significantly greater at 69.1% ($P < 0.001$) to fibronectin, 42.3% ($P < 0.001$) to matrigel, and 36.6% ($P < 0.001$) to laminin, compared to that under normoxic conditions. The percentage of adherent OCUM-8 cells was also significantly greater under hypoxic conditions at 113.4% ($P < 0.001$) to fibronectin, 73.8% ($P < 0.001$) to matrigel, and 42.0% ($P < 0.001$) to laminin, compared to that under normoxic conditions. In contrast, there was no significant difference in the percentage of the adherent non-DGC cells MKN7, MKN45 and MKN74 under normoxic and hypoxic conditions.

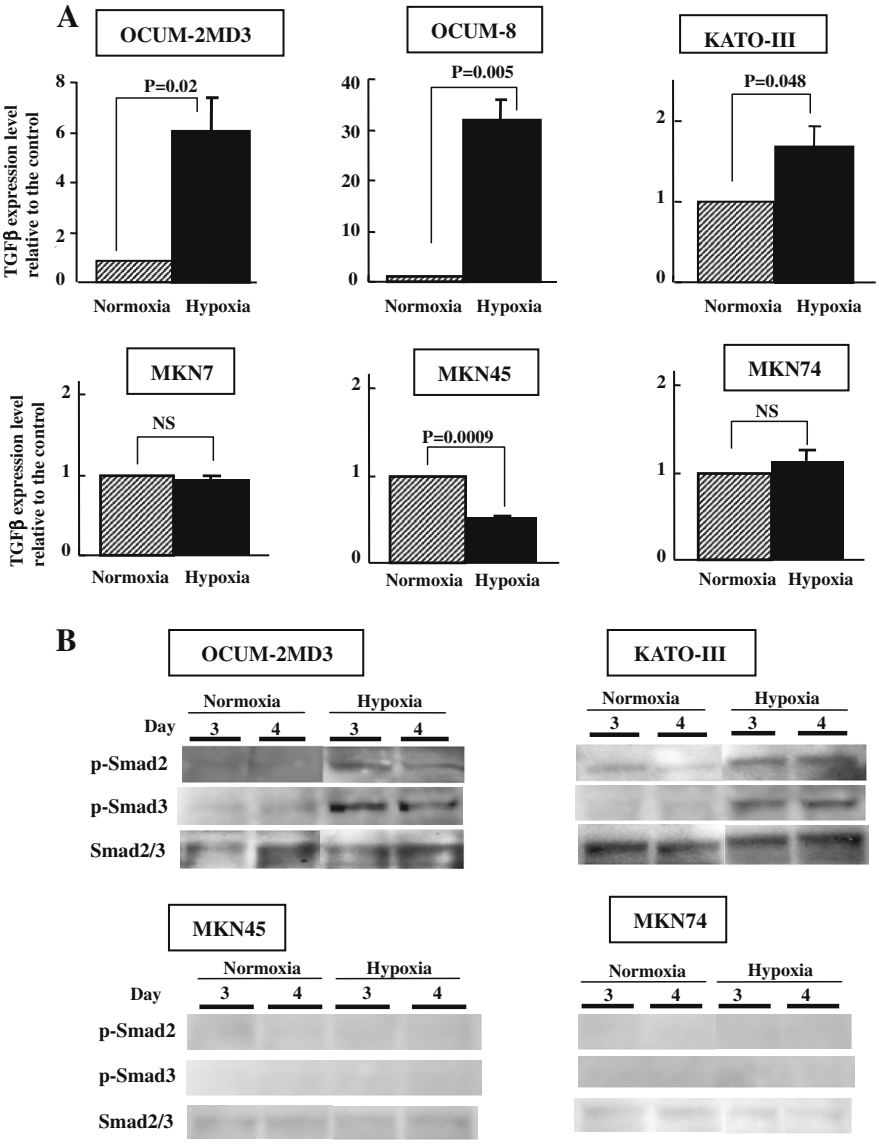


Fig. 2 – Effect of hypoxia on the *Transforming growth factor*-β1 and Smad2/3 expression. (A) *Transforming growth factor*-β1 (TGF-β1) mRNA expression under hypoxic conditions. *TGF*-β1 expression level of cancer cells under hypoxic conditions was significantly increased by 6.0-fold in OCUM-2MD3, 1.7-fold in KATO-III, 32.0-fold in OCUM-8, 0.95-fold in MKN7, 0.51-fold in MKN45 and 1.12-fold in MKN74, compared to that under normoxic conditions. (B) Smad2 and Smad3 phosphorylation level under hypoxic conditions. Hypoxia promoted Smad2 and Smad3 phosphorylation of OCUM-2MD3 and KATO-III cells, but not that of MKN45 and MKN74 cells.

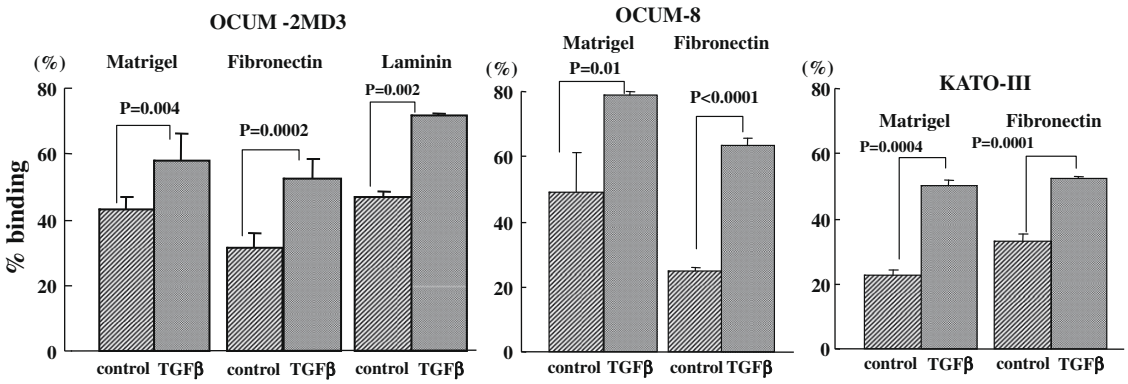


Fig. 3 – Adhesion ability of cancer cells treated with TGF-β under normoxic conditions. The adhesion abilities of cancer cells to fibronectin, matrigel and laminin were significantly increased by TGF-β compared to those of the control.

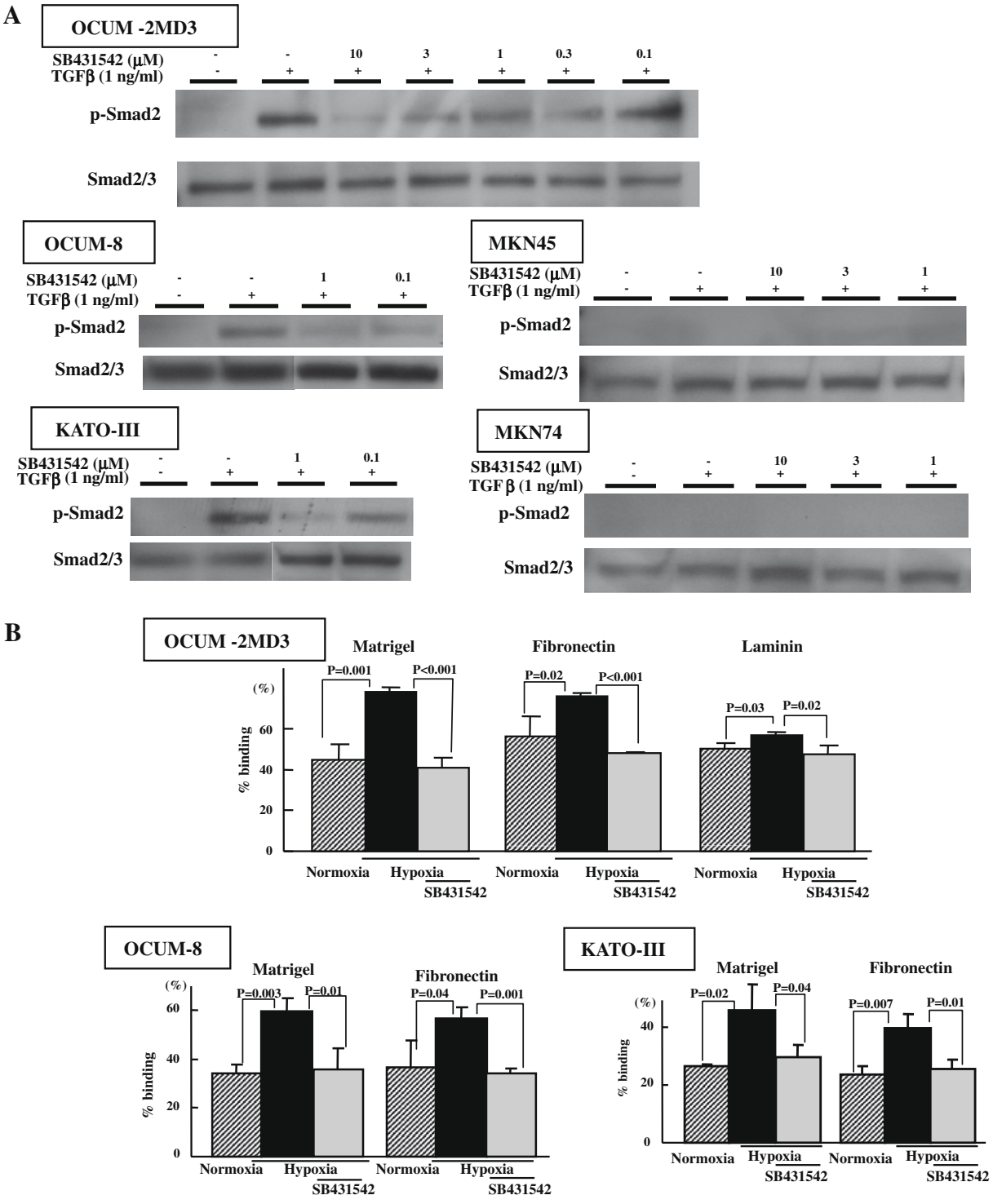


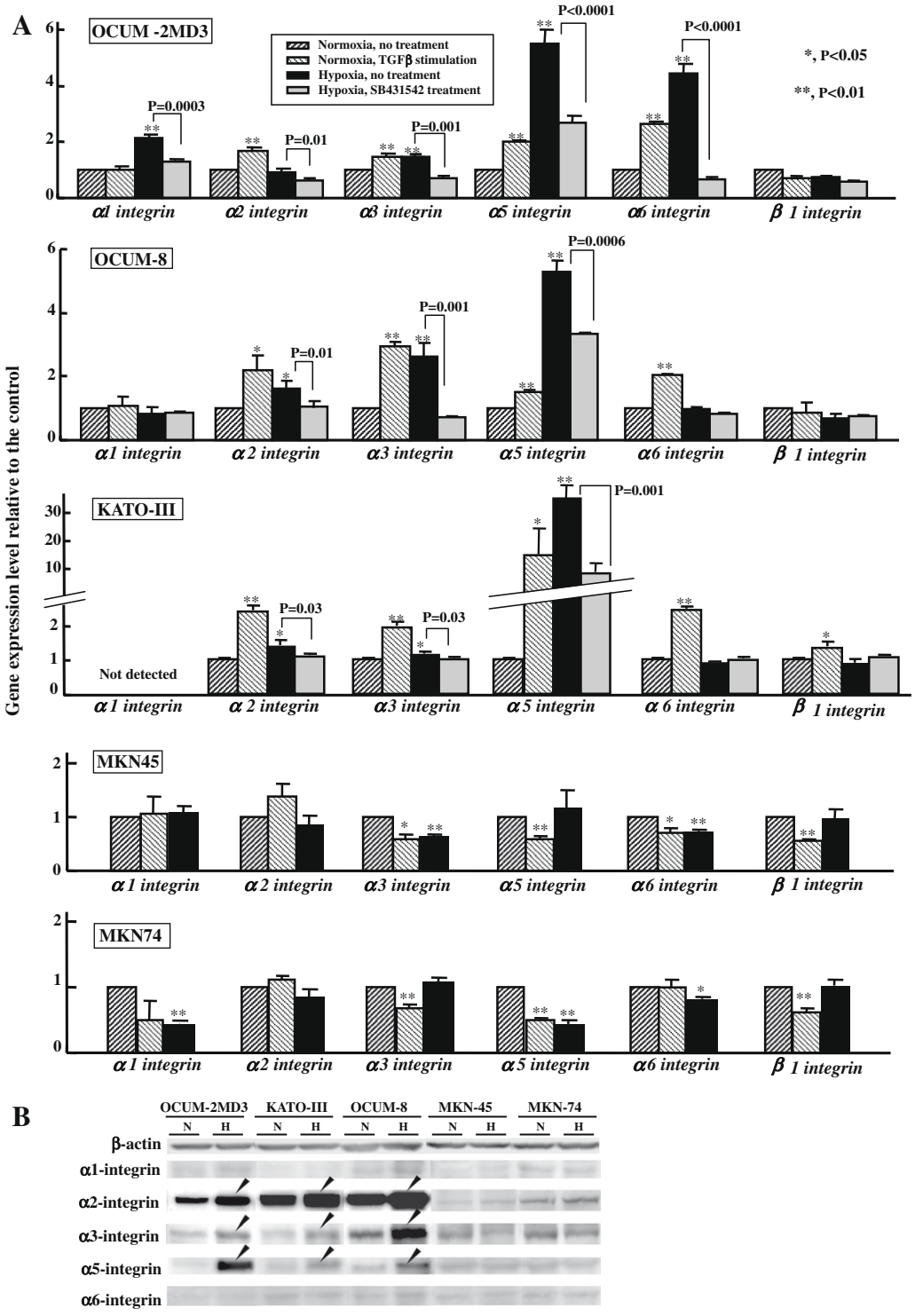
Fig. 4 – Effect of TGF-βR kinase inhibitor. (A) Inhibition of Smad2 phosphorylation by TGF-βR kinase inhibitor, SB431542. TGF-β treatment promoted Smad2 phosphorylation in the 3 DGC cell lines. Smad2 phosphorylation was not shown in MKN45 and MN74 cells. SB431542, a TGF-βR kinase inhibitor, inhibited Smad2 phosphorylation by TGF-β. (B) Inhibition of adhesion ability of hypoxic cancer cells by SB431542. Hypoxia significantly increased the adhesion ability of OCUM-2MD3, OCUM-8 and KATO-III cells, compared to normoxic conditions. SB431542 at 1 μM inhibited the hypoxia-mediated increase in adhesion ability of OCUM-2MD3 cells to fibronectin, matrigel and laminin by 36.7% ($P < 0.001$), 47.5% ($P < 0.001$) and 15.5% ($P = 0.02$), respectively. In KATO-III cells, SB431542 inhibited the adhesion ability to fibronectin and matrigel by 35.5% ($P = 0.01$) and 35.2% ($P = 0.04$), respectively. In OCUM-8 cells, SB431542 inhibited the adhesion ability to fibronectin and matrigel by 40.2% ($P = 0.001$) and 40.0% ($P = 0.01$), respectively.

secondary antibody (Amersham) reactive with the primary antibody was added. The bands were detected using an enhanced chemiluminescence system (Amersham).

For examining the effect of hypoxia on protein level of integrins, cancer cells were incubated under normoxic or hypoxic conditions for 96 h. The protocol is as above. The primary antibody was follows; α1-integrin (1:1000, Chemicon), α2-integrin (1:1000, Chemicon), α3-integrin (Chemicon), α5-integrin (1:1000, Chemicon), α6-integrin (1:1000, Abcam, Cambridge, MA) and β-Actin as an internal control (1:1000, Cell Signaling Technology).

2.6. Statistical analysis

Comparisons among the data sets were made with an analysis of variance, followed by Student's *t*-test. Differences were considered to be statistically significant when the *P* value was $\leqslant 0.05$.



3. Results

3.1. Effect of hypoxia on adhesion ability of gastric cancer cells

Fig. 1 shows the adhesion ability of gastric cancer cells to the ECM of matrigel, fibronectin and laminin, under normoxic or hypoxic conditions. The adhesion ability of the DGC cells OCUM-2MD3, OCUM-8 and KATO-III was significantly increased under hypoxic conditions compared to that under normoxic conditions. In contrast, no remarkable change was observed in the adhesion ability of the non-DGC cells MKN7, MKN45 and MKN74 under similar conditions. Hypoxia significantly decreased the ability of MKN7 and MKN45 cells to adhere to matrigel and laminin, respectively. Therefore, the following examinations were performed using the 3 DGC cell lines.

3.2. Effect of hypoxia on TGF-1 mRNA expression, and Smad2 and Smad3 phosphorylation of gastric cancer cell lines

Expression level of *TGF*-β1 mRNA significantly increased in the 3 DGC cell lines under hypoxic conditions compared to that under normoxic conditions. In contrast, the expression of *TGF*-β1 mRNA did not change under hypoxic conditions in MKN7 and MKN74 cells. In MKN45 cells, *TGF*-β1 mRNA expression was significantly decreased (Fig. 2A). Smad2 and Smad3 phosphorylation level was also increased in OCUM-2MD3 and KATO-III cells under hypoxic conditions compared to that under normoxic conditions, while that in MKN45 and MKN74 cells was decreased (Fig. 2B).

3.3. TGF-β stimulated the adhesion ability of DGC cells

The percentage of OCUM-2MD3 cells adhering to ECM components increased significantly after TGF-β treatment compared to that of the control. 66.0% to fibronectin ($P = 0.0002$), 35.3% to matrigel ($P = 0.004$), and 51.7% to laminin ($P = 0.0002$). In OCUM-8 cells, the increase in adherence was 157.9% to fibronectin ($P < 0.0001$) and 59.6% to matrigel ($P = 0.01$); in KATO-III cells, the increase in adherence was 58.6% to fibronectin ($P = 0.0001$) and 123.8% to matrigel ($P < 0.0001$; Fig. 3).

3.4. Effect of the TGF-R kinase inhibitor SB431542 on Smad2 phosphorylation and adhesion ability of hypoxic cancer cells

Under normoxic conditions, Smad2 phosphorylation was increased by 1 ng/ml TGF-β1 and decreased by 1 μM SB431542 in OCUM-2MD3, OCUM-8 and KATO-III cells. In contrast, Smad2 phosphorylation was not found in MKN45 and MKN74 cells (Fig. 4A). The adhesion ability of these DGC cells was significantly increased under hypoxic conditions compared to that under normoxic conditions. SB431542 at 1 μM significantly inhibited the adhesion abilities of hypoxic OCUM-2MD3 cells to the ECM, and SB431542 inhibited those of KATO-III and OCUM-8 to fibronectin and matrigel (Fig. 4B).

3.5. Effect of TGF-β and SB431542 on the expression of integrin mRNA of cancer cells under normoxic conditions

Expression levels of α2-, α3-, α5- and α6-integrin mRNA of OCUM-2MD3, OCUM-8 and KATO-III cells after incubation with TGF-β were significantly increased compared to the control. In contrast, expression levels of α3-, α5- and β1-integrin mRNA were significantly decreased in both MKN45 cells and MKN74 cells. Hypoxia significantly stimulates cell surface expression of selective integrin subunits, α2-, α3-, α5-, α6- and β1-integrin mRNA in DGC cells, but inhibited the expression of α1-, α3-, α5- and α6-integrin mRNA in MKN45 and MKN74 cells. SB431542 treatment significantly inhibited

Fig. 5 – Effect of hypoxia on the integrin expression of gastric cancer cells. (A) expression level of *integrin* mRNA. TGF-β treatment significantly increased α2-*integrin* expression by 1.6-fold, α3-*integrin* by 1.4-fold, α5-*integrin* by 1.9-fold and α6-*integrin* by 2.6-fold in OCUM-2MD3 cells, compared to untreated cells. In OCUM-8 cells treated with TGF-β under normoxic conditions, α2-*integrin* expression significantly increased by 2.1-fold, α3-*integrin* by 2.9-fold, α5-*integrin* by 1.4-fold and α6-*integrin* by 2.0-fold, compared to untreated cells. In KATO-III cells treated with TGF-β, α2-*integrin* expression significantly increased by 2.3-fold, α3-*integrin* by 1.9-fold, α5-*integrin* by 14.1-fold, α6-*integrin* by 2.4-fold and β1-*integrin* by 1.3-fold, compared to untreated cells. In MKN45 cells treated with TGF-β, α3-*integrin* by 0.6-fold, α5-*integrin* by 0.6-fold, α6-*integrin* by 0.7-fold and β1-*integrin* by 0.5-fold, compared to untreated cells. In MKN74 cells treated with TGF-β, α3-*integrin* by 0.7-fold, α5-*integrin* by 0.5-fold and β1-*integrin* by 0.6-fold, compared to untreated cells. In OCUM-2MD3 cells, hypoxic conditions significantly increased the expression level of α1-, α3-, α5- and α6-integrin by 2.1-, 1.4-, 5.4- and 4.4-fold, respectively, compared to that under normoxic conditions. In OCUM-8 cells, hypoxia significantly increased α2-*integrin* expression by 1.6-fold, α3-*integrin* by 2.6-fold and α5-*integrin* by 5.2-fold, compared to that under normoxic conditions. In KATO-III cells, hypoxia increased α2-*integrin* expression by 1.3-fold, α3-*integrin* by 1.12-fold and α5-*integrin* by 34.3-fold, compared to that under normoxic conditions. In MKN45 cells, hypoxia significantly decreased α3-*integrin* expression by 0.6-fold and α6-*integrin* by 0.7-fold, compared to that under normoxic conditions. In MKN74 cells, hypoxia significantly decreased α1-*integrin* expression by 0.4-fold, α5-*integrin* expression by 0.4-fold and α6-*integrin* by 0.8-fold, compared to that under normoxic conditions. Under hypoxic conditions, SB431542, a TGF-βR kinase inhibitor, significantly inhibited α1-*integrin* expression by 38.2%, α2-*integrin* by 32.1%, α3-*integrin* by 51.8%, α5-*integrin* by 51.0% and α6-*integrin* by 84.8% in OCUM-2MD3 cells. In OCUM-8 cells, SB431542 inhibited α2-*integrin* expression by 29.2%, α3-*integrin* expression by 72.3% and α5-*integrin* expression by 36.9%; in KATO-III cells, SB431542 significantly inhibited α2-*integrin* expression by 21.6%, α3-*integrin* by 11.3% and α5-*integrin* by 77.0%. (B), Protein level of integrin. In diffuse-type gastric cancer cells, hypoxic condition elevated the expression levels of α2-, α3- and α5-integrin, in comparison with normoxic condition. In contrast, no remarkable change was observed under hypoxic condition in non-diffuse-type gastric cancer cells.

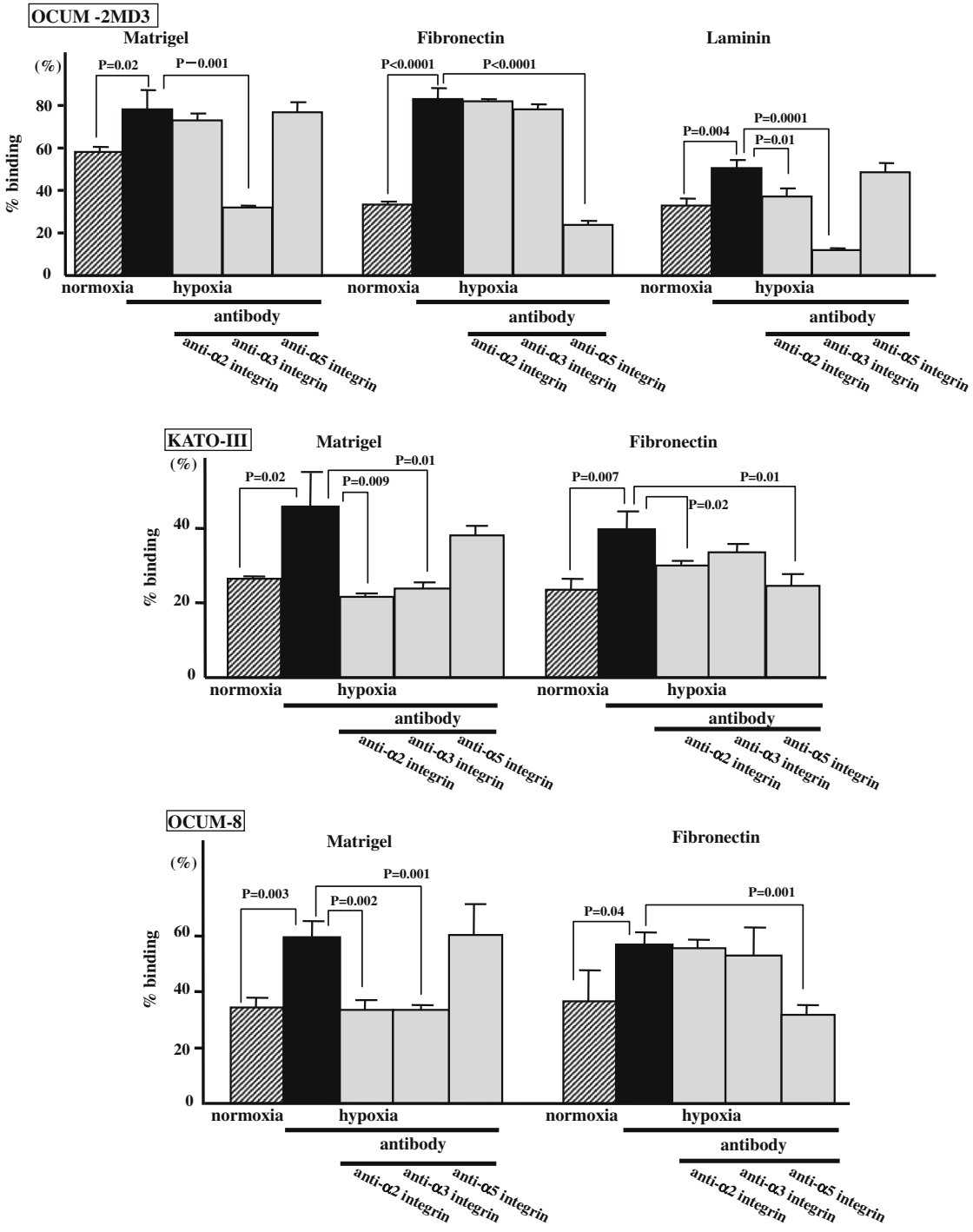


Fig. 6 – Blocking adhesion by anti-integrin neutralising antibody. In OCUM-2MD3 cells, treatment with anti-α2β1-integrin antibody significantly (*P* = 0.01) inhibited the adhesion ability under hypoxic conditions by 26.4% to laminin; anti-α3-integrin antibody inhibited the increase in adhesion to laminin by 75.9% (*P* < 0.0001) and to matrigel by 59.1% (*P* = 0.001). Anti-α5β1-integrin neutralising antibody inhibited the increase in adhesion to fibronectin by 71.1% (*P* < 0.0001). In KATO-III cells, treatment with anti-α2β1-integrin antibody inhibited the increase in adhesion abilities under hypoxic conditions to fibronectin by 24.3% (*P* = 0.002) and to matrigel by 52.9% (*P* = 0.009); anti-α3-integrin antibody inhibited the increase in adhesion abilities under hypoxic conditions to matrigel by 48.1% (*P* = 0.01), and anti-α5β1-integrin antibody inhibited the increase in adhesion to fibronectin by 38.4% (*P* = 0.01). In OCUM-8 cells, treatment with anti-α2β1-integrin antibody inhibited the increase in adhesion abilities under hypoxic conditions to matrigel by 43.4% (*P* = 0.002), anti-α3-integrin antibody inhibited the increase in adhesion to matrigel by 43.6% (*P* = 0.001). Anti-α5β1-integrin antibody inhibited the increase in adhesion to fibronectin by 44.0% (*P* = 0.001).

expression levels of α1-, α2-, α3-, α5- and α6-integrin in OCUM-2MD3 cells and α2-, α3- and α5-integrin expression in OCUM-8 and KATO-III cells (Fig. 5A).

3.6. *Effect of hypoxia on protein levels of integrins of cancer cells*

In DGC cells, α2-, α3- and α5-integrin expression levels under hypoxic condition were elevated in comparison with normoxic condition. In MKN-45 and MKN74 cells, those had no remarkable change (Fig. 5B). These protein levels of integrins corresponded to mRNA level.

3.7. *Effect of anti-integrin antibodies on adhesion ability of cancer cells in hypoxia*

To examine the relationship between adhesion activity of cancer cells and integrins, we tested whether neutralising antibodies against integrin could neutralise the adhesion activity of DGC cells. In OCUM-2MD3 cells, anti-α2β1-integrin antibody significantly inhibited adhesion to laminin compared to the control. Anti-α3-integrin antibody decreased adhesion to laminin and matrigel, and anti-α5β1-integrin antibody decreased adhesion to fibronectin. In KATO-III, anti-α2β1-integrin antibody significantly decreased the adhesion to fibronectin and matrigel of cancer cells under hypoxic conditions compared to control. In OCUM-8 cells, anti-α2β1- and anti-α3-integrin antibodies significantly decreased the adhesion abilities to matrigel of hypoxic cancer cells compared to the control. An anti-α5β1-integrin antibody decreased adhesion abilities to fibronectin (Fig. 6).

4. Discussion

DGC frequently metastasizes to the peritoneum.[9–11] Since the gastric cancer cells leaving the primary tumour are exposed to low oxygen levels in the abdominal cavity, examination of the cancer phenotype under hypoxic conditions is important for understanding the mechanism responsible for peritoneal metastasis. In this study, hypoxic conditions significantly increased the adhesion ability of the DGC cells OCUM-2MD3, OCUM-8 and KATO-III to the peritoneum components of fibronectin, laminin and matrigel. These findings suggest that the upregulation of adhesion properties of hypoxic cancer cells might be one of the mechanisms responsible for high metastatic potential of DGC cells to the peritoneum.

We know from clinical experiences that patients with free cancer cells in the abdominal cavity have not always developed peritoneal implantation, and our preliminary clinical study showed that DGC cells detected by cytology had higher peritoneal metastatic potentials than the non-DGC cells (data not shown). In this study, hypoxia either decreased or did not change the adhesion ability of the non-DGC cells MKN7, MKN74 and MKN45. The binding ability of DGC cells was higher than that of the non-DGC cells. These findings might explain one of the reasons for poorer prognosis of cytology-positive patients of DGC compared to the non-DGC patients.

We investigated the effect of hypoxia on different integrin expression levels because it has been reported that integrin expression might play an important role in adhesion to the peritoneum.[23] Furthermore, the effect of hypoxia on integrin expression is still controversial. Although Rohwer et al. reported that hypoxia-inducible factor 1 alpha (HIF-1α) mediates anoikis resistance through suppression of α5β1-integrin[24], Spangenberg et al. identified the regulatory mechanism of cellular adhesion to fibronectin through HIF-dependent α5 β1-integrin induction. ERBB2-mediated transcriptional upregulation of α5β1-integrin fibronectin receptor promotes tumour cell survival under adverse conditions.[25] In our study, hypoxia upregulated mRNA expression levels and protein levels of α2-, α3- and α5-integrin in DGC cell lines. We previously reported that the upregulation of integrin expression is closely associated with peritoneal metastasis of gastric cancer.[13,16] Upregulation of integrin expression might provide an explanation for the increase in adhesion ability of hypoxic gastric cancer cells. To determine whether integrins are associated with adhesion ability of cancer cells under hypoxic conditions, we tested whether neutralising antibodies against integrins affected adhesion-stimulating activity under hypoxic conditions in DGC. Adhesion activity was inhibited by neutralising antibodies against α2-, α3- or α5-integrin. These *in vitro* findings suggested that α2-, α3- or α5-integrin, which were upregulated by hypoxia, might play an important role in the adhesion of free DGC cells in the abdominal cavity. The adhesion ability of the 3 cell lines to fibronectin under hypoxic conditions was most strongly inhibited by treatment with α5 β1-integrin antibody. α5β1-integrin constitutes the major cellular receptor for fibronectin.[26] The adhesion ability to laminin was most strongly inhibited by α3-integrin antibody treatment. These findings suggested that α5- and α3-integrin might play an important role in the adhesion of the peritoneum in hypoxic DGC cells to fibronectin and laminin.

We previously reported that TGF-β increased the α2-, α3- and α5-integrin mRNA expression and promoted the adhesion ability of DGC cells to the ECM.[18] In the present study, TGF-β1 expression was increased under hypoxic conditions in DGC cell lines, and Smad2 and Smad3 phosphorylation was promoted by hypoxia. TGF-β1 significantly increased the adhesion ability of the 3 cell lines and upregulated integrin expression. The TGF-βR kinase inhibitor SB431542 inhibited phosphorylation of Smad2 by TGF-β1 stimulation. The upregulated integrin expressions under hypoxic conditions were significantly decreased by SB431542. α5-integrin expression was severely inhibited by SB431542, followed by α3-integrin expression. These findings suggest that hypoxia-induced upregulation of integrin expression through TGF-β1 and subsequent activation of Smad 2/3 signalling, thus resulting in the increase in the adhesion ability of DGC cells.

MKN45 and MKN74 cells have been reported to be TGF-β signal-deficient while no mutation was found in TGF-β receptor-II.[27] Moreover, it has been reported that TGF-β receptor-I was not expressed in MKN45 cells.[28] In this study, Hypoxic condition stimulated neither TGF-β1 expression nor Smad2/Smad3 phosphorylation in non-DGC, MKN45 and MKN74 cells. In addition, TGF-β1 did not stimulate Smad2/Smad3 phosphorylation in both cell lines. These findings also suggested that TGF-β signalling pathway of the non-DGC cells,

MKN45 and MKN74 cells, is functionally inactivated. Although a mutation in the TGF-β signalling pathway was not evident in these cell lines, unidentified molecule(s) that transmit TGF-β signals might be involved in the non-DGC cells, such as epigenetic alteration of TGF-β receptor or transcriptional corepressors. Since there might be a novel mechanism underlying TGF-β pathway inactivation in the non-DGC cells, epigenetic study is therefore necessary in the future.

TGF-β-dependent induction of integrin expression in hypoxic tumour cells offers a new insight to antitumour strategies by inhibiting TGF-βR for the treatment of peritoneal metastasis of DGC. TGF-βR kinase inhibitor might therefore be considered useful for the inhibition of peritoneal metastasis initiation of DGC.

HIF-1 binds to hypoxia-response elements of various target genes and activates transcription of these genes. HIF-1 is upregulated by hypoxia and is an essential component in changing the transcriptional response of tumours under hypoxic conditions.[29] HIF-1 has been reported to be associated with the metastatic potential of various types of cancer cells.[1] Our preliminary study showed that TGF-β and integrin expression of cancer cells were not decreased by HIF-1 siRNA, although the expression of HIF-1 was increased by hypoxia in DGC cell lines (data not shown). These findings suggested that HIF-1 is not associated with the adhesion process of DGC cells under hypoxia.

In conclusion, hypoxia plays an important role in the adhesion ability of DGC cells, which may be involved in the initiation of peritoneal metastatic progression. Active TGF-β signalling induced by hypoxia might be associated with the upregulation of adhesion ability of DGC cells by increasing the expression of α2-, α3- and α5-integrin.

Conflict of interest statement

None declared.

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