

No autoimmune phenomenon induced by immunisation with KIF20A-8 in HLA-A2 Tgm

Whether immunisation with KIF20A peptides induces autoimmune reactions is of great importance. The immunohistochemical analyses of several vital organs with anti-CD4 and anti-CD8 mAbs were performed in HLA-A2 Tgm after two-times vaccination with the KIF20A-8 peptide, as its amino-acid sequence was identical between human and mouse KIF20A. As shown in Figure 4B, no pathological change that suggests autoimmunity, such as lymphocyte infiltration or tissue destruction, was observed, indicating that lymphocytes stimulated with the KIF20A-8 peptide were safe at least in HLA-A2 Tgm.

Induction of KIF20A-reactive human CTLs from PBMCs of HLA-A2-positive healthy donors

To investigate whether the KIF20A-2, KIF20A-8, and KIF20A-28 peptides could generate KIF20A-specific human CTLs, CD8⁺ T cells sorted from PBMCs of HLA-A2-positive healthy donors were incubated with the autologous CD14⁺ cell-derived DCs pulsed with each peptide. After three-times stimulations, the cytotoxic activities of CD8⁺ T cells against peptide-pulsed T2 cells were examined by the ⁵¹Cr release assay (Figure 5A). Each CTL killed the T2 cells pulsed with KIF20A-2 (left), KIF20A-8 (middle), or KIF20A-28 (right) peptides, but not the T2 cells pulsed with irrelevant HLA-A2-binding HIV peptide or those without peptide loading. The data indicate that KIF20A peptides successfully induced human CTLs with peptide-specific cytotoxicity.

Next, the capacity of these CTLs to kill human cancer cell lines expressing both KIF20A and HLA-A2 was examined. As shown in Figure 5B, KIF20A-reactive CTLs stimulated with KIF20A-2 (left), KIF20A-8 (middle), or KIF20A-28 (right) peptides exhibited effective cytotoxicity to PANC1 (KIF20A⁺, HLA-A2⁺) and CaCo-2 (KIF20A⁺, HLA-A2⁺), but not to PK9 (KIF20A⁺, HLA-A2⁻). Furthermore, all CTLs exhibited cytotoxicity against SKHep1/KIF20A (KIF20A⁺, HLA-A2⁺), SKHep1 cells (KIF20A⁻, HLA-A2⁺) transfected with the *KIF20A* gene (Figure 2B, right), but not against SKHep1/Mock, SKHep1 cells transfected with an empty vector (Figure 5C). Those results suggested that CTL responses were specific to KIF20A expression and that the epitope peptides were naturally processed and expressed on the surface of cancer cells in the context of HLA-A2 molecules.

Blocking mAb specific to HLA-class I (W6/32) markedly reduced the number of INF- γ -producing CTLs generated by stimulation with KIF20A-2 (left), KIF20A-8 (middle), or KIF20A-28 (right) peptides by co-culture with PANC1 cells with statistical significance (Figure 5D, $P < 0.01$), whereas anti-HLA-DR mAb (H-DR-1) had no effect on CTL responses. The data verified that CTLs recognised KIF20A-expressing target cells in an HLA-class I-restricted manner.

DISCUSSION

In this study, whether KIF20A is applicable to a target of anticancer immunotherapy was carefully investigated. The *KIF20A* gene was overexpressed in pancreatic cancer cells but barely expressed in their normal counterparts and in many normal adult tissues as revealed by the cDNA microarray analysis, RT-PCR analysis, and immunohistochemical analyses. As a weak expression of *KIF20A* in the normal thymus in addition to the testis was observed in the cDNA microarray and RT-PCR analyses, KIF20A is not a perfect cancer/testis antigen but a TAA overexpressed in pancreatic cancer, as well as in bladder cancer, non-small cell lung cancer, and cholangiocellular carcinoma. We have reported that CDH3/P-cadherin is another TAA that is overexpressed in pancreatic cancer (Imai *et al*, 2008); however, it has been often

observed that using a single TAA as a target of anticancer immunotherapy does not yield satisfactory therapeutic outcomes in animal models. Using multiple TAAs in combination as targets drastically improved the outcome of anticancer immunotherapy (Fukushima *et al*, 2009), and therefore KIF20A could be not only a versatile tumour marker but also a TAA useful as a target of anticancer immunotherapy for pancreatic cancer by itself or in combination with other TAA(s). Furthermore, the involvement of KIF20A in pancreatic carcinogenesis suggests that KIF20A would be a promising immunotherapeutic target for pancreatic cancer as described below.

The KIF20A protein, also known as RAB6KIFL/MKlp2, was first identified to localise to Golgi apparatus and to have an important role in the dynamics in this organelle by an interaction with the GTP-bound form of Rab6 (Echard *et al*, 1998). KIF20A belongs to a large family of motor proteins that accumulate in mitotic cells, and microinjection of anti-KIF20A antibodies resulted in the failure of cytokinesis (Hill *et al*, 2000). The knockdown of *KIF20A* gene expression in pancreatic cancer cell lines by small-interfering RNA drastically inhibited the growth of those cells (Taniuchi *et al*, 2005).

The potential CTL target epitopes of KIF20A were listed among the HLA-A2 (*A*02:01*) binders predicted by the BIMAS software and surveyed using HLA-A2 Tgm. All three candidate epitope peptides identified could stimulate the generation of human CTLs that showed KIF20A-specific cytotoxicity in an HLA-A2-restricted manner in all three individuals examined. Importantly, immunisation of the KIF20A-8 peptide, of which the amino-acid sequence is conserved between human and mouse KIF20A, did not induce any signs of autoimmune phenomenon, suggesting the safety of this peptide for using anticancer immunotherapy targeting KIF20A. In this sense, the HLA-A2 is not only a powerful tool for rapid identification of HLA-A2-restricted CTL epitopes but also for evaluating the *in vivo* outcomes of immunotherapy using epitope peptides.

In conclusion, our data showed that KIF20A is a novel TAA and a potential target for anticancer immunotherapy for cancer cells expressing KIF20A at least in an HLA-A2-restricted situation. As KIF20A is highly expressed in a wider range of human malignancies, KIF20A is therefore a promising target for peptide-based immunotherapy for the treatment of malignancies, especially pancreatic cancer. Further investigation of the capability for induction of KIF20A-specific CTLs in pancreatic cancer patients thus remains an issue of great importance for clinical application.

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Hepatic Stellate Cells Accelerate the Malignant Behavior of Cholangiocarcinoma Cells

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ABSTRACT

Background. Although tumor–stromal interaction has been discussed, the role of hepatic stellate (HS) cells against cancer, especially cholangiocarcinoma (CC), has not been clarified. The aim of this study is to investigate the effect of HS cells on CC cell progression *in vitro* and *in vivo*.

Methods. The effects of CC conditioned medium (CC-CM) on activation and proliferation of HS cells (LI90 and LX-2), the influences of HS cell CM (HS-CM) on proliferation and invasion of CC cells (HuCC-T1 and RBE), and the effects of their interaction on HUVEC tube formation were assessed using each CM. The effect of HS cells on tumor growth was examined *in vivo* by subcutaneous co-injection. Cytokine array was performed to assess the secreted proteins induced by their coculture.

Results. CC-CM activated HS cells and increased their proliferation. HS-CM dose-dependently increased CC cell proliferation and invasion. Chemotherapy of CC cells was less effective when treated with HS-CM. HS-CM activated the mitogen-activated protein kinase and Akt pathways in tumor cells. The indirect interaction of CC and HS cells promotes tube formation of human umbilical venous endothelial cells. Subcutaneous co-injection of tumor cells with HS cells in nude mouse resulted in increased tumor size. Several proteins were found in the culture medium induced

by their coculture, thought to be key proteins which regulated tumor–stromal interaction.

Conclusions. This study indicates that HS cells play an important role in accelerating cholangiocarcinoma progression and may be a therapeutic target in cholangiocarcinoma.

Intrahepatic cholangiocarcinoma has poor prognosis due to its early lymphatic invasion and distant metastasis.^{1–3} Nonmalignant cells in the tumor microenvironment have been reported to play key roles in tumor progression. The tumor microenvironment comprises endothelial cells, pericytes, fibroblasts, and various bone marrow-derived cells (BMDCs) such as macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells, and mesenchymal stem cells.⁴ Among these, cancer-associated fibroblasts (CAFs), the so-called myofibroblasts, are reportedly associated with progression of breast, colon, prostate, and pancreas cancer.^{5–8}

Hepatic stellate (HS) cells are activated into myofibroblasts during hepatic fibrogenesis and react to hepatic injury by producing extracellular matrix factors or cytokines, such as transforming growth factor- β (TGF- β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF).⁹ Although these factors are important for the cancer microenvironment, the presence and role of HS cells in cancer have not been fully investigated. We have previously suggested that HS cells might be present in liver cancer stroma, and might promote progression of intrahepatic cholangiocarcinoma.¹⁰

Previous *in vitro* and *in vivo* studies on pancreatic cancer have suggested that the interaction of CAFs and cancer cells promotes cancer growth and invasion, and inhibits anti-cancer drug-induced apoptosis.^{5,11–13} These

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studies revealed that PDGF produced by pancreatic stellate cells enhances growth of pancreatic cancer cells. In addition, cancer cell-activated fibroblasts induced angiogenesis, resulting in production of vascular endothelial growth factor (VEGF).^{14,15}

The interaction between HS cells and cholangiocarcinoma cells has not been investigated; therefore, we examined whether HS cells could act as CAFs and accelerate the progression of cholangiocarcinoma cells *in vitro* and *in vivo*. Since we wanted to focus on the role of hepatic stellate cells, and cytokines and chemokines from stromal cells have been shown to be of great importance for tumor development, we used a cytokine array and assessed the secreted proteins associated with the tumor–stromal interaction.¹⁶

MATERIALS AND METHODS

Cell Culture

The HuCCT-1 cell line was obtained from the Cell Resource Center for Biochemical Research, Tohoku University (Sendai, Japan). The RBE cell line and the human HS cell line LI90 were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan).¹⁷ The human HS cell line LX-2 was a kind gift from Scott L. Friedman (Mount Sinai School of Medicine, NY, USA).¹⁸ Tumors were cultured in Roswell Park Memorial Institute (RPMI) medium, and HS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with penicillin/streptomycin. All cultures were maintained in 5% CO₂ air-humidified atmosphere at 37°C. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (VA, USA) and maintained using the EGM-2 bullet kit (Lonza, Basel, Switzerland).

Preparation of Conditioned Media

The HS cell line, LI90, was grown to 70–80% confluency, medium was changed to serum-free DMEM for 48 h, and the supernatant was concentrated with Amicon Ultra filters for 3 kDa (Millipore, MA, USA). Protein content of the concentrated HS cell-conditioned media (HS-CM) was determined by Bradford assay, and aliquots were stored at –80°C until use. Conditioned media were similarly prepared from the two cholangiocarcinoma cell lines (CC-CM), HuCCT-1 and RBE.

Proliferation Assay

LI90 cells were seeded at 5,000 per well in 96-well plates and cultured overnight in DMEM/10% FBS. Medium was changed to serum-free DMEM, and concentrated

conditioned medium in which cholangiocarcinoma cells had been incubated (CC-CM) was added to cells at varying concentrations (0.05, 0.1, 0.5 µg/µl). Serum-free DMEM was added to control wells. Similarly, HuCCT-1 or RBE cells were seeded at 5,000 per well in 96-well plates and cultured overnight in DMEM/10% FBS. Medium was changed to serum-free DMEM, and concentrated HS-CM was added to cells at varying concentrations (0.05, 0.1, 0.5 µg/µl). Cell growth was analyzed after 48 h by adding the WST-8 reagent (Dojin Laboratories, Kumamoto, Japan) and taking the spectrophotometric reading 2 h later.

Invasion Assay

Biocoat Matrigel-coated invasion chambers (BD Biosciences, CA, USA) were used to examine cell invasiveness. In brief, 5×10^4 HuCCT-1 or RBE cells in 500 µl serum-free medium were added to the upper chamber. Medium containing 10% FBS or concentrated HS-CM (0.05, 0.1, 0.25, 0.5 µg/µl) was added to the lower chamber. Serum-free medium was added to the lower chamber of control wells. The cells were allowed to invade the Matrigel for 24 h at 37°C in 5% CO₂ atmosphere. The noninvading cells on the upper surface of the membrane were removed with a cotton swab. Cells were fixed in methanol, stained with 1% toluidine blue, and air-dried. Invading cells in three adjacent microscope fields for each membrane were counted at 20× magnification.

Immunocytochemistry

To demonstrate the effects of cholangiocarcinoma on LI90 cells, LI90 cells were seeded on glass coverslips in DMEM/10% FBS and incubated for 8 h. Medium was changed to concentrated CC-CM (0.25 µg/µl), and cells were incubated for 12 h. Serum-free DMEM was used as control. CC-CM was collected from HuCCT-1 or RBE cells. The cells were fixed and stained by α -SMA. For details, see the Supplementary Information.

Western Blot Analysis

To evaluate activation of HS cells in the presence of CC-CM, LI90 cells were seeded and grown in 6-cm dishes in DMEM/10% FBS for 8 h. Medium was changed to concentrated CC-CM (0.5 µg/µl) medium, or serum-free medium, and incubated for 12 h. To evaluate activation of the mitogen-activated protein kinase (MAPK) and Akt pathways in HS-CM, HuCCT-1 cells were grown in six-well plates in DMEM/10% FBS to 70% confluency. Cells were serum-starved overnight, and treated with HS-CM (0.5 µg/µl) for 0–120 min. The cells were lysed and

analyzed by Western blotting. For details, see the Supplementary Information.

HUVEC Tube Formation Assay

To investigate which cells played a greater role in angiogenic factor production, not only CMs from HS cells (HS-CM) and tumor cells (CC-CM) but also secondary CMs were prepared from tumor-stimulated HS cells (CC-HS-CM), and HS cell-stimulated tumor cells (HS-CC-CM); for details, see Supplementary Fig. 1. HUVECs (1×10^4 cells/well) were suspended in a mixture of CM (100 μ l) and EGM-2 medium (100 μ l) with 0.5% FBS, and seeded on 50 μ l Matrigel in 96-well culture plates. Serum-free DMEM (100 μ l) in place of conditioned media was used as control. After incubation for 12 h, photographs of each well were taken. The total capillary tube length and branching points in three random view-fields per well were examined, and the values were averaged.

Apoptosis Assay against Chemotherapy

HuCCT-1 cells were seeded in eight-well chamber slides (1×10^5 per well) (Nunc, NY, USA) in RPMI with 10% FBS. After incubation overnight, cells were washed, and medium was changed to RPMI with or without HS-CM (0.5 μ g/ μ l). Cells were treated with CDDP, 5-FU, or gemcitabine chemotherapeutic reagents, or vehicle. After 24 h, apoptotic cells were analyzed with terminal deoxynucleotidyl transferase-mediated dUTP nick end

labeling (TUNEL) assay (Promega, WI, USA), and nuclei were stained with Mayer's hematoxylin. The ratio of apoptotic cells was calculated as: (number of cells positive for TUNEL/number of nuclei stained with Mayer's hematoxylin) \times 100. Experiments were performed in triplicate, and 10 random fields were counted for each condition.

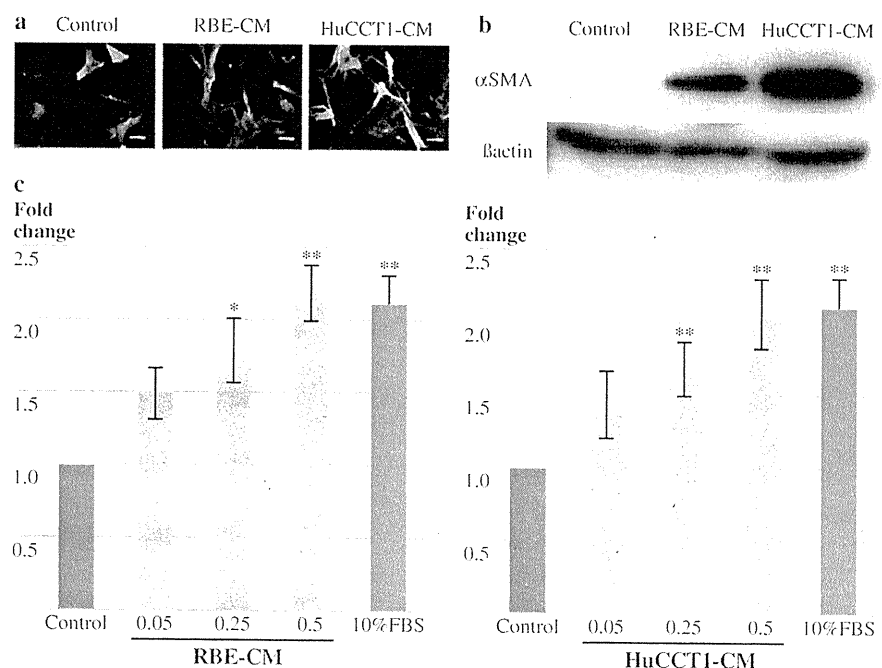
Subcutaneous Injection Model of Cholangiocarcinoma

All animal experiments were reviewed and approved by the University of Kumamoto Institutional Animal Care and Use Committee. Female athymic nude mice (BALB/c nu/nu; age, 6–8 weeks) were grouped into three sets of five mice. The five mice in each set were injected subcutaneously with one of the following: (a) 1.5×10^6 LI90 cells/100 μ l PBS, (b) 3×10^6 HuCCT-1 cells/100 μ l PBS, or (c) mixture of HuCCT-1 cells (3×10^6) + LI90 cells (1.5×10^6) in 100 μ l PBS. Mice were sacrificed 7 weeks after injection, the tumors were removed, and the tumor weights were recorded. Tumors were processed for histological examination.

Immunohistochemistry

To investigate the influence of HS cells on tumorigenesis in mice, we performed immunohistochemical study using α -SMA for evaluating myofibroblasts and CD31 for evaluating angiogenesis. For details, see the Supplementary Information.

FIG. 1 Effect of CC-CM on HS cell phenotype. **a** Immunohistochemical staining for α -SMA of HS cells. RBE-CM or HuCCT1-CM was added to HS cells, and the cell morphology was compared with that of HS cells in serum-free medium; bar, 100 μ m. **b** CC-CM-induced α -SMA expression was evaluated using Western blotting. **c** HS cell proliferation was measured in the presence of RBE-CM or HuCCT1-CM. Columns, mean of three experiments. * $P < 0.05$ and ** $P < 0.01$ versus serum-free media control

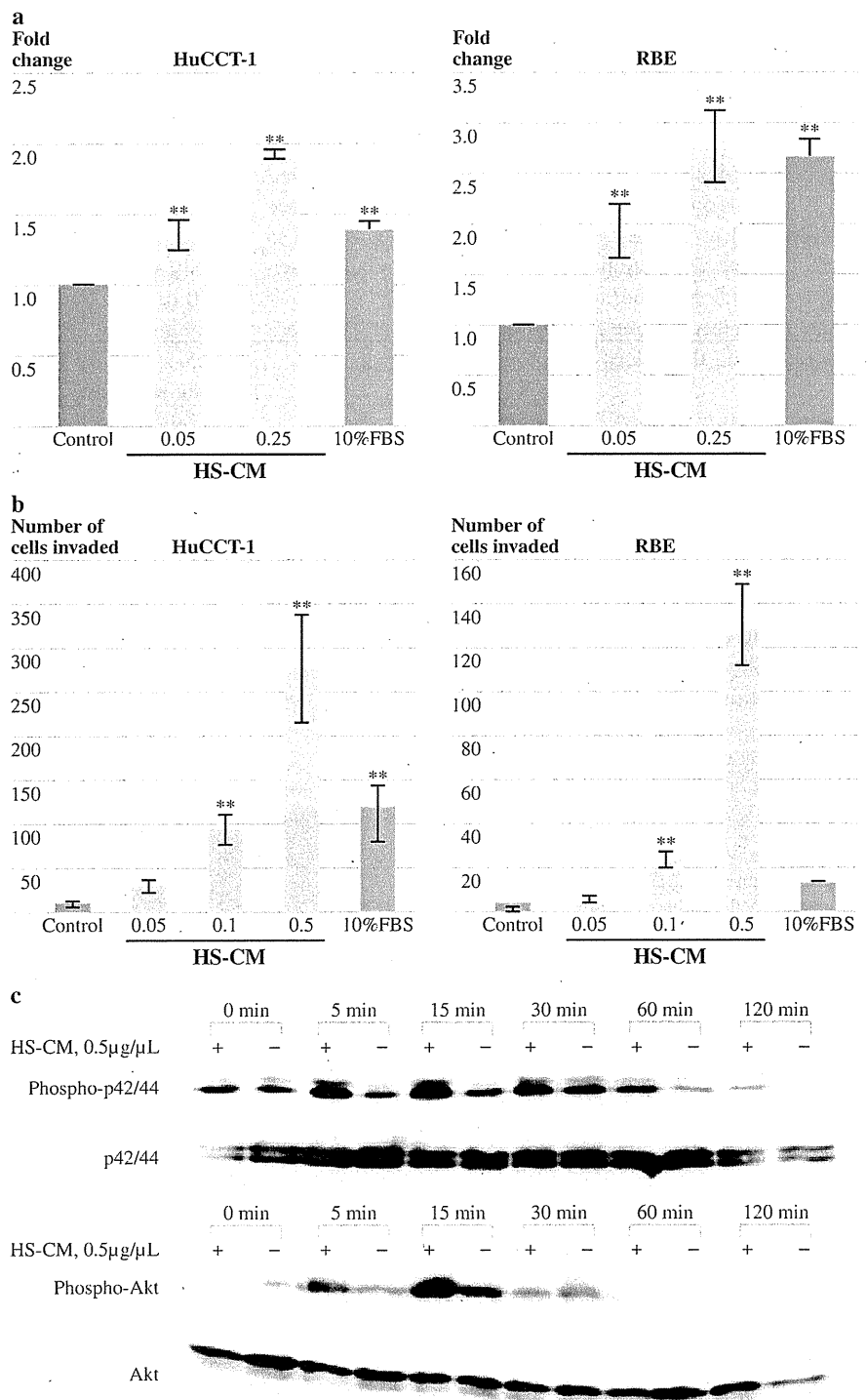


Cytokine Expression Analysis

To clarify secreted proteins induced by the interaction of cancer cells and stromal cells, we used a coculture model.

Cytokine concentrations in conditioned medium were quantified using a cytokine array (R&D, MN, USA) as per the manufacture's protocol. For details, see the Supplementary Information.

FIG. 2 Effect of HS-CM on cholangiocarcinoma cell phenotype. HS-CM ($\mu\text{g}/\mu\text{l}$) was added to RBE or HuCCT-1 cholangiocarcinoma cells. **a** Tumor cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 48 h. **b** Tumor cell invasion was evaluated at 24 h by using a Matrigel chamber assay. **c** Protein lysates from HuCCT-1 cells treated with HS-CM ($0.5 \mu\text{g}/\mu\text{l}$) were analyzed for Erk1/2 and Akt activation by Western blotting at various time points. Columns, mean of three experiments. ** $P < 0.01$ versus serum-free media control



Statistical Analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts Inc., Berkeley, CA, USA). Comparisons were carried out using Student's *t* test, and *P* values <0.05 were considered statistically significant. We analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test to compare multiple samples, with significance level of 0.05.

RESULTS

CC-CMs Induce Activation and Proliferation of HS Cells

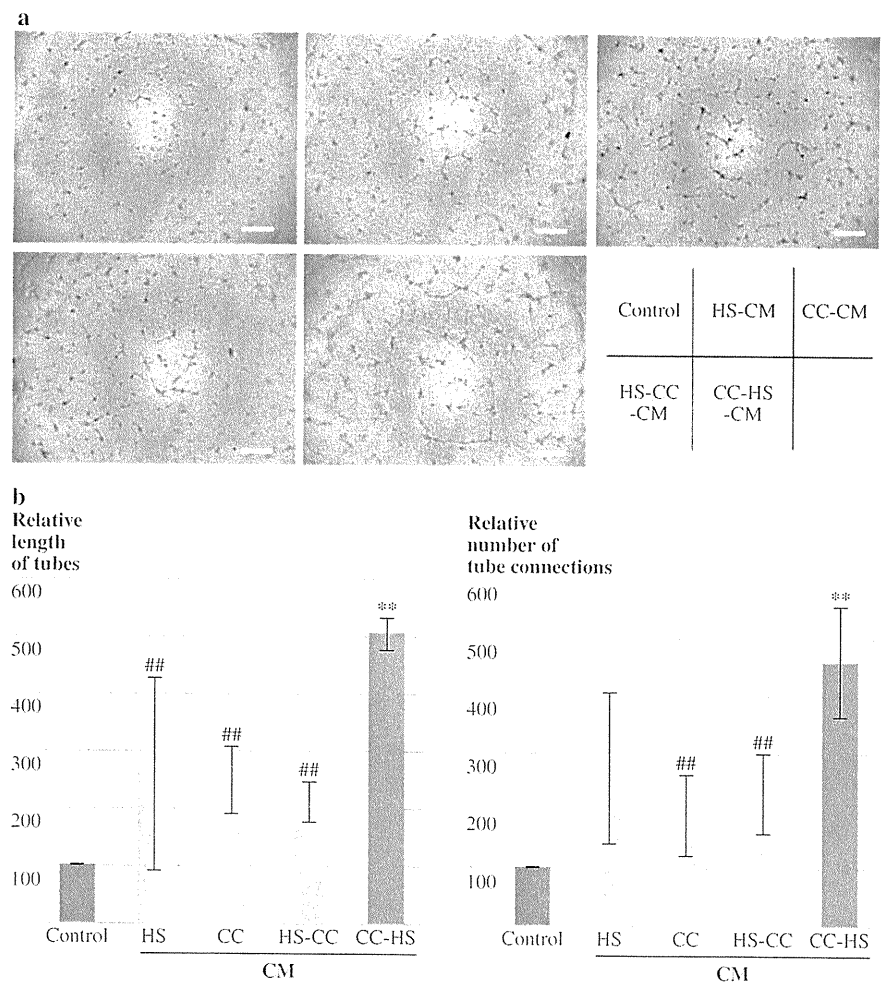
To confirm the activation of HS cells by cholangiocarcinoma, two CC-CMs were added to HS cells, and the resulting changes in morphology and proliferation were compared with the serum-free medium control. Compared with the serum-free control, addition of CC-CMs produced

by two cholangiocarcinoma cell lines altered the morphology of HS cells to that of myofibroblasts (Fig. 1a) and increased expression of α -SMA, a known marker of active HS cells (Fig. 1b). CC-CMs induced a dose-dependent increase in HS cell proliferation compared with serum-free medium controls (Fig. 1c).

HS Cells Stimulate Cholangiocarcinoma Cell Proliferation and Invasion

Addition of HS-CM to RBE cells increased tumor cell proliferation as compared with the proliferation in the case of serum-free medium controls (Fig. 2a). The effect was dose dependent, and a significant increase in proliferation compared with serum-free medium control wells was observed at 0.5 μ g/ μ l ($278 \pm 35\%$ of control; $P < 0.01$). The maximum effect of HS-CM on cell proliferation was greater on RBE cell proliferation than HuCCT1 cell proliferation, and was equal to the effect of 10% FBS (Fig. 2a). HS-CM also induced dose-dependent increases

FIG. 3 The interaction of tumor and stromal cells induces HUVEC tube formation. **a** Representative photographs of HUVEC culture in Matrigel (12 h); bar, 100 μ m. **b** HUVECs were treated with (a) HS-CM, (b) CC-CM, (c) secondary CM of RBE cells that were stimulated with the primary CM of HS cells (HS-CC-CM), and (d) secondary CM of HS cells that were stimulated with the primary CM of RBE cells (CC-HS-CM). Serum-free medium was used in place of CM as control; bar, μ m. The relative length and number of connections are shown. ** $P < 0.01$ versus serum-free media control, *** $P < 0.01$ versus CC-HS-CM



in HuCCT-1 and RBE cell invasion (Fig. 2b). HuCCT-1 invasion was significantly induced with as little as 0.05 $\mu\text{g}/\mu\text{l}$ HS-CM. The maximum dose (0.5 $\mu\text{g}/\mu\text{l}$ HS-CM) stimulated a 25.3-fold increase, and the minimum dose (0.05 $\mu\text{g}/\mu\text{l}$ HS-CM) stimulated a 2.8-fold increase in HuCCT-1 invasion compared with serum-free medium ($P < 0.0001$). The maximum dose of 0.5 $\mu\text{g}/\mu\text{l}$ HS-CM resulted in HuCCT-1 invasion at a higher level than that in the medium containing 10% FBS (278 ± 62 versus 119 ± 25 ; $P < 0.001$).

Factors Secreted from HS Cells Stimulate Activation of MAPK and Akt Pathways in Tumor Cells

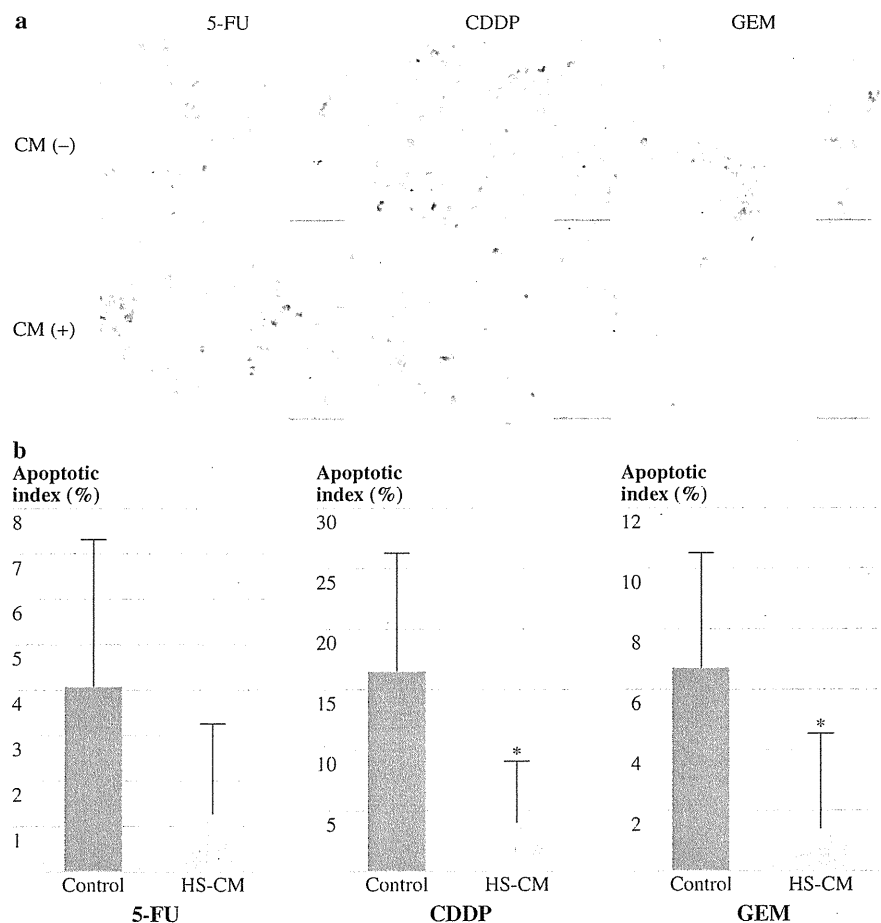
To determine signaling pathways that may be involved in the tumor-promoting effects of HS cells, HuCCT-1 tumor cells treated with HS-CM were examined for activation of MAPK and Akt by Western blotting. Increased phosphorylation of Erk1/2 was observed in cells treated with HS-CM compared with control cells from 5 to

120 min after treatment (Fig. 2c). Similarly, increased levels of phosphorylated Akt were observed from 5 to 60 min after stimulation with HS-CM.

Interaction between HS and Cholangiocarcinoma Cells Promotes HUVEC Tube Formation

We examined whether the interaction of HS cells and tumor cells induced angiogenesis in vitro using the HUVEC tube formation assay. HUVECs formed a network of capillary-like structures after 18 h of culture in Matrigel (Fig. 3a). CC-HS-CM significantly accelerated tube formation, in comparison with HS-CM or CC-CM, and the length and branching points of HUVEC tubes increased by 5.1 and 4.6 times, respectively (Fig. 3b). On the other hand, HUVEC tubes induced by treatment with HS-CC-CM were similar to those of either HS-CM or CC-CM (Fig. 3a, b). This indicated that tube formation was stimulated by the tumor cell-induced secretions of HS cells, but not by the HS cell-induced secretions of tumor cells. This is consistent with previous reports showing that

FIG. 4 Antiapoptotic effect of HS cells on cholangiocarcinoma cells against chemotherapy. HuCCT-1 cells were treated with 5-FU (500 $\mu\text{g}/\text{ml}$), CDDP (50 $\mu\text{g}/\text{ml}$), or gemcitabine (500 $\mu\text{g}/\text{ml}$) with or without HS-CM (0.25 $\mu\text{g}/\mu\text{l}$) for 24 h. **a** Apoptosis was detected using TUNEL assay, and cell nuclei were stained with Mayer hematoxylin. **b** Percentage of apoptotic cells was determined in five random fields for each condition. * $P < 0.05$



tumor cells stimulated fibroblasts to produce angiogenic factors in the indirect tumor–stromal cell interaction model.^{14,15}

HS Cells Inhibit the Effects of Chemotherapy against Cholangiocarcinoma Cells

Addition of HS-CM to HuCCT-1 cells treated with 5-FU (500 µg/ml), CDDP (50 µg/ml), or gemcitabine (500 µg/ml) resulted in an inhibition of apoptosis (Fig. 4a). The percentage of apoptotic cells treated by 5-FU, CDDP, or gemcitabine in serum-free medium were 4.1% ± 3.2%, 16.4% ± 4.1%, and 6.7% ± 1.4% versus 1.2% ± 2.1%, 4.1% ± 5.2%, and 1.4% ± 3.2% with addition of HS-CM (*P* = 0.134, 0.038, and 0.045, respectively; Fig. 4b). No significant differences were observed in total cell number in cells treated with chemotherapeutic reagents, such as 5-FU, CDDP, or gemcitabine, with or without the addition of HS-CM.

Co-injection of HS Cells and Tumor Cells Induces Tumor Progression in an In Vivo Subcutaneous Injection Model of Cholangiocarcinoma

Palpable tumors were not detected in the mice injected with HS cells alone, suggesting that HS cells do not have

tumorigenic potential. However, a significant increase in tumor volume was observed in the HuCCT-1 and LI90 mixed group versus the HuCCT-1 group when all data were analyzed (*n* = 5 per group) (Fig. 5a, b). The tumor weight at 6 weeks was also significantly greater in the mixed group than in the HuCCT-1 group. These findings suggest that the presence of HS cells accelerated the growth of cholangiocarcinoma. One mouse in the mixed group had a subcutaneous hemorrhage after cell injection, resulting in the disappearance of tumor cells at final evaluation. This outcome was ascribed to human error. To analyze whether *in vivo* tumor formation induced by HS cell co-injection is caused by increased myofibroblasts or increased angiogenesis, we performed immunohistochemical analysis. Mixed tumors of HuCCT1 and LI90 did not show more intense staining of α-SMA and CD31 than tumors formed by HuCCT1 cells alone (Fig. S2).

Co-culture of HS Cells and Cholangiocarcinoma Cells Induced Production of IL-1α, C5a, IL-1β, and G-CSF

We assessed the mediators secreted by cells in monoculture and coculture by performing cytokine arrays on conditioned media samples (Fig. 6a). Of the 36 proteins examined, 20 were detectable at the limits of sensitivity of this assay (Fig. 6b, c). Proteins were considered to be increased if they were present in the

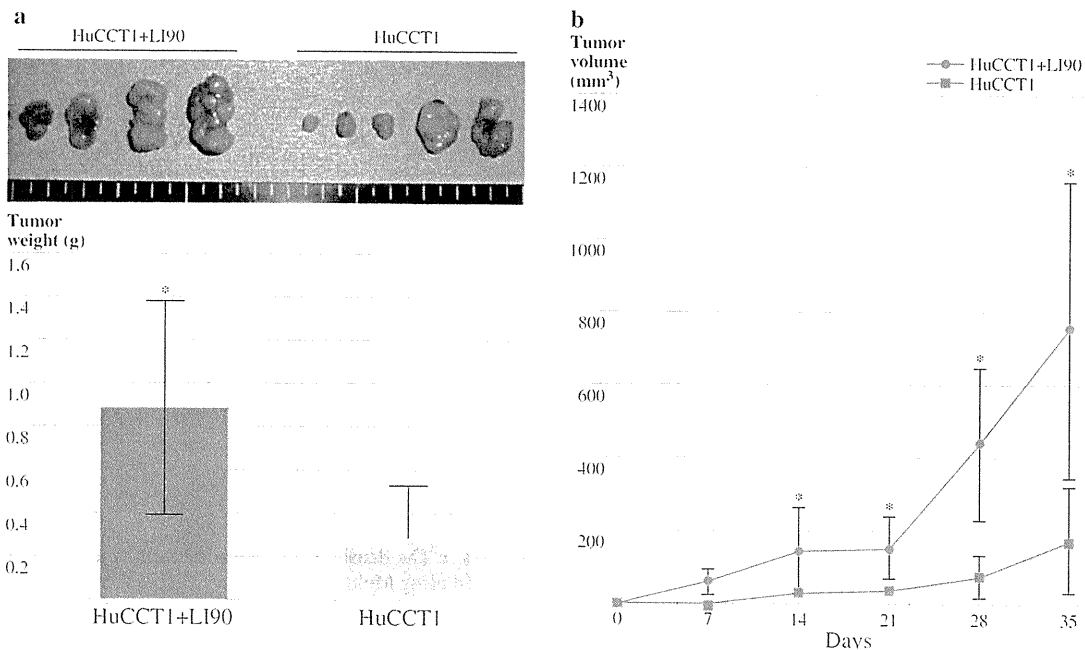


FIG. 5 Subcutaneous co-injection of HS cells with HuCCT-1 cells in nude mice resulted in increased tumor size and weight. **a** Mice were sacrificed after 35 days, and the resulting tumors were removed, photographed, and weighed (mean ± SD). * *P* < 0.05. **b** Tumor size

was measured weekly, determined using a caliper rule. Tumor volume was calculated by using the formula $(a \times b \times c)/2$, where *a* and *b* are the short and long diameters of the tumors, respectively, and *c* is the thickness

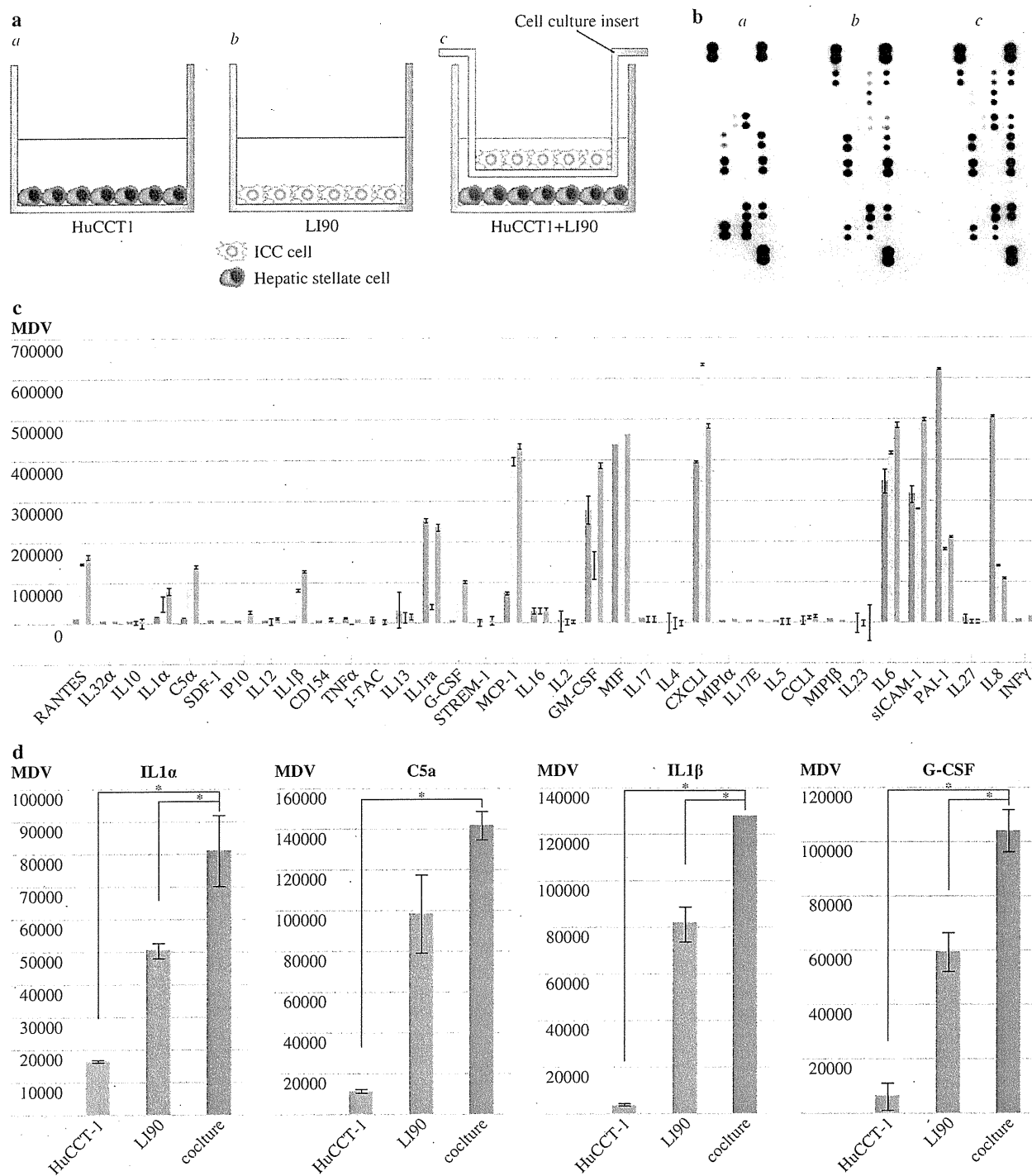


FIG. 6 Quantitative evaluation of cytokines and chemokines in conditioned media samples from monoculture and coculture based on cytokine arrays. **a** CM samples were prepared from (a) monocultured HuCCT-1, (b) monocultured LI90, and (c) cocultured HuCCT-1 and LI90. **b** Representative membrane arrays from three samples after development. A total of three membranes that included 36 molecules measured for duplicates were processed to generate data used in our

analyses. **c** The densitometric value of each locus on the array was measured using Multi Gauge software (FUJIFILM, Japan). The value will be referred to as the mean densitometric value (MDV) of each cytokine. **d** The molecules IL-1 α , C5a, IL-1 β , and G-CSF were selected such that the value of coculture (c) was more than the total value of the two monocultures (a + b). * $P < 0.05$

coculture at a value that was greater than the total value of the two monocultures, and four proteins met this criterion: interleukin (IL)-1 α , C5a, IL-1 β , and granulocyte colony-stimulating factor (G-CSF). These proteins were detected at much lower level in the cancer cell monoculture than in the HS cell monoculture. Among these four cytokines, the level of IL-1 α , IL-1 β , and G-CSF was significantly different between the HS cell monoculture and coculture (Fig. 6c).

DISCUSSION

Tumor–stromal interaction is an important aspect of cancer progression that warrants further clarification. Many investigators have shown that CAFs, so-called myofibroblasts, play a pivotal role in tumor progression, and have studied CAFs for the purpose of stroma-targeted therapy.^{19,20} We previously suggested that HS cells might promote cholangiocarcinoma progression, and the present study demonstrated the interaction of HS cells and cholangiocarcinoma cells in vitro and in vivo.¹⁰ Consistent with our expectations, HS cells were activated by tumor cells and promoted the growth of them. The interaction of these cells in vitro and in vivo has never been investigated, and the current study supports the notion that HS cells may be CAFs in cholangiocarcinoma.

Previous studies focused on the role of stellate cells in pancreatic cancer, and various mechanisms were found.^{5,11,13,21–23} Pancreatic stellate cells induced tumor growth and invasion, and inhibited chemotherapy-induced apoptosis in pancreatic cancer. Previous studies suggested that PDGF was the most important factor for stellate cell-induced tumor growth using a neutralizing antibody assay.^{11,13} Similar to these reports, HS cells induced a dose-dependent increase in the proliferation and invasion of tumor cells in our study.

Several studies suggested that stellate cells influenced tumor response to chemotherapy in pancreatic cancer.^{24,25} We hypothesized that HS cells behave like pancreatic stellate cells, and confirmed the antiapoptotic effect of HS cells on tumor cells using TUNEL assay. We used common clinical chemotherapeutic agents against cholangiocarcinoma, and determined that HS cells have an antiapoptotic effect on CDDP and gemcitabine treatments.

Interestingly, stromal cells are indispensable in tumor angiogenesis, and therefore antiangiogenic therapies must be aimed to target stromal cells as well as cancer cells.^{14,15} We observed that tumor cells induced secretions in HS cells that stimulated tube formation, but this effect was not seen when we replaced tumor cells with stromal cells. This result emphasized the importance of stromal cells in angiogenesis.

The presence of HS cells significantly increased the tumor growth in vivo, and this supports the idea that HS cells provide an advantageous microenvironment for cholangiocarcinoma cells. We examined the amount of stromal component and the microvessel density in excised tumors, but we did not observe any differences between the tumor cell-only injection group and the co-injection group. LI90 cells are not immortalized, and their active lives are very limited. LX2 may be a better cell line for use in the tumor formation assay, or in other detailed in vivo analyses.

The current study identified three candidate molecules that may participate in the tumor–stromal interaction: IL-1 α , IL-1 β , and G-CSF. The significance of IL-1 β in the tumor–stromal interaction has been reported.^{16,26,27} Inflammation is one of the important aspects of cancer development, and CAFs are known to behave like myofibroblasts in the wound. Similarly, we believe to have found the same characteristics between hepatic stellate cells in carcinoma and those in damaged liver. Identification of the key characteristics of hepatic stellate cell for tumor development remains a problem for future study, and we would like to elucidate this issue using several candidate secreted proteins identified by the current study. We believe that these proteins may provide clues to therapeutic targets focusing on CAFs.

In conclusion, our data provide evidence that HS cells interact with tumor cells to promote tumor progression in cholangiocarcinoma in vitro and in vivo. Thus, HS cells may be a therapeutic target for inhibition of tumor–stromal interaction in cholangiocarcinoma.

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Perioperative Intra-Arterial and Systemic Chemotherapy for Pancreatic Cancer

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ABSTRACT

Background. Even after curative resection of pancreatic cancer, there is a high probability of systemic recurrence. This indicates that subclinical metastases are already present at the time of operation. The purpose of this study was to assess the feasibility and outcomes of patients who received a novel multimodality therapy combining pancreatic resection and intraoperative radiation therapy (IORT) with pre- and postoperative chemotherapy for pancreatic cancer.

Methods. For eligible patients with pancreatic cancer, 5-FU was administered at a dose of 125 mg/m²/day on days 1–5 every week as a continuous pancreatic and hepatic arterial infusion, and gemcitabine was infused intravenously at a dose of 800 mg/m² per day once per week for 2 weeks for preoperative chemotherapy. Pancreatic resection combined with IORT was performed 1 week after preoperative chemotherapy. Postoperative chemotherapy was performed in the same way as preoperative chemotherapy. We performed an intention-to-treat analysis for all enrolled patients.

Results. This study enrolled 44 patients. The most common toxicities were hematological and gastrointestinal events. Grade 3/4 hematological toxicities were observed during preoperative chemotherapy, although there were no grade 3/4 nonhematological events. Postoperative chemotherapy-related toxicities were more critical and frequent than preoperative ones. There were no pre- or postoperative

chemotherapy-associated deaths. Median overall survival was 36.5 months with 30.5% overall 5-year survival.

Conclusions. This multimodality therapy is feasible and promises to contribute to survival. It should be evaluated in a phase III setting.

Pancreatic adenocarcinoma remains a lethal disease, with an overall 5-year survival rate ranging from 0.4 to 5%.^{1,2} Even after curative resection of pancreatic cancer, there is a high probability of systemic and/or local recurrence.^{3–5} This indicates that subclinical metastases are already present in most patients at the time of operation, even if preoperative radiological imaging or intraoperative examination revealed no metastatic lesions. Therefore, a multimodality strategy, including not only local control but also treatment of micrometastases, is required for patients with pancreatic cancer. For local control, beginning in 1984 we introduced extended radical pancreatectomy combined with intraoperative radiation therapy (IORT).⁶ This approach provided the best control of local recurrence, but there was no survival benefit because of blood-borne metastases.⁵ To treat unresectable pancreatic cancer, we introduced a combination of chemotherapy using 5-fluorouracil (5-FU) pancreatic and hepatic arterial continuous infusion and systemic gemcitabine administration; this combined therapy was well tolerated, with a 1-year survival rate of 50.9%.⁷

We studied a novel multimodality therapy combining pancreatic resection and IORT with pre- and postoperative chemotherapy using 5-FU intra-arterial continuous infusion and systemic gemcitabine administration in patients with potentially resectable pancreatic cancer. The purpose of this study was to evaluate the feasibility and outcomes of this multimodality therapy.

PATIENTS AND METHODS

Patients

All patients were advised of the investigational nature of the study and gave their written, informed consent to participate before the beginning of the study. All patients underwent a standard pretreatment evaluation that included a physical examination, a thin-section, contrast-enhanced, multiphase spiral computed tomography (CT) of the abdomen, and ultrasonography. The absence of liver metastasis was confirmed by CT during arterial portography combined with CT-assisted hepatic arteriography (CTAP + CTHA), as described previously.⁸ The absence of lung metastasis was confirmed by chest CT. The protocol required patients with potentially resectable disease as assessed by a physical examination and the following objective radiographic criteria: (1) no evidence of remote metastases; (2) no evidence of tumor extension to the celiac axis or the superior mesenteric artery. We included only patients in whom it was technically possible to resect and reconstruct the superior mesenteric vein (SMV) or the portal vein (PV), if the tumor involved SMV or PV. We excluded cases in which the tumor was 1 cm or smaller in diameter, because of the very low possibility of systemic spreading of the disease. Patients were required to have an Eastern Cooperative Oncology Group performance status of ≤ 2 .

Perioperative Chemotherapy

The treatment schema is shown in Fig. 1. The pre- and postoperative chemotherapy consisted of the combination

of 5-FU arterial continuous infusion and systemic gemcitabine administration. In all cases, the catheter for arterial infusion was introduced from the femoral artery under local anesthesia. After the closure of the distal tip of the catheter, a side hole was made at an appropriate site in the celiac axis to allow the distribution of 5-FU to both the pancreatic tumor and the liver preoperatively, and in the hepatic artery to distribute the drug to the whole liver postoperatively. An arterial port was implanted in the subcutaneous tissue. 5-FU was administered at a dose of 125 mg/m² per day on days 1–5 each week as continuous infusion through the arterial port for 2 weeks during preoperative chemotherapy and for 8 weeks during postoperative chemotherapy. Gemcitabine was infused intravenously for 30 min at a dose of 800 mg/m² once weekly for a total of 2 doses preoperatively and for a total of 18 doses postoperatively. The doses of these drugs were based on our preliminary results for the combination chemotherapy using 5-FU intra-arterial infusion and systemic gemcitabine for unresectable pancreatic cancer.⁷

In cases of grade 3 or higher toxicity according to the National Cancer Institute–Common Toxicity Criteria (NCI-CTC) version 3.0, drug infusion was interrupted until recovery. History, physical examination, and complete blood counts (CBCs) were repeated weekly before infusion of the drugs. Chemistry profiles were performed every 2 weeks. The catheter and port for arterial infusion were removed after the completion of intra-arterial infusion of 5-FU.

Surgery

Patients with cancer of the head of the pancreas underwent a substomach-preserving pancreaticoduodenectomy (SSPPD), a pylorus-preserving pancreaticoduodenectomy (PPPD), or a Kausch-Whipple resection: the last of these was performed if a tumor directly invaded the duodenum or antrum of the stomach, or if a distal gastrectomy had been performed before. Patients with cancer of the body or tail of the pancreas underwent a distal pancreatectomy. Patients underwent resection with reconstruction of SMV or PV if a tumor was thought during surgery to involve these vessels. For IORT, a dose of 30 Gy with a 12 MeV of electron beam was delivered to the operative field using a special pentagon applicator following dissection, as described previously.⁶

Hospital death was defined as death during hospitalization. Major surgical complications included any occurrence of anastomotic leak, postoperative intra-abdominal or gastrointestinal hemorrhage or fistula, intra-abdominal abscess, pneumonia, catheter-related sepsis, thromboembolic events, and reoperation. Pancreatic fistula was assessed according to an international study group (ISGPF) definition.⁹

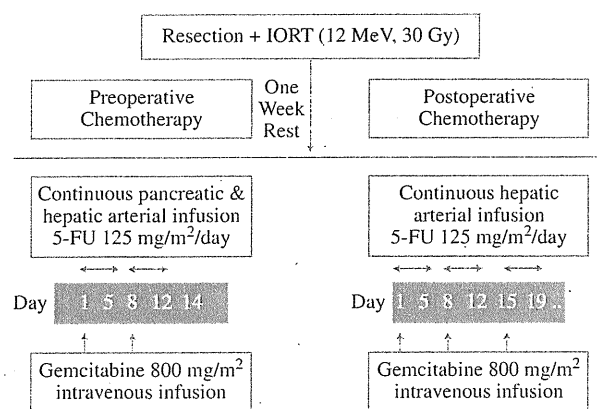


FIG. 1 Treatment schema. 5-FU was administered on days 1–5 every week as a continuous arterial infusion combined with gemcitabine infused once weekly for 2 weeks followed by pancreatic resection combined with IORT. Postoperative chemotherapy was performed in the same way as preoperative chemotherapy

Toxicity and Outcome Evaluation

Toxicities were graded according to NCI-CTC version 3.0. Survival was calculated from the day of surgery and estimated by the Kaplan–Meier method. The first site of disease recurrence was documented for outcome analysis.

All patients were evaluated every 3–4 months by physical examination as well as by chest and abdominal CT after surgery. For those without any recurrence after 2 years, follow-up was at 6-month intervals. Cytologic or histologic confirmation of disease recurrence was not required.

RESULTS

Patient Characteristics

From May 2001 through September 2008, 44 patients were enrolled in this study. The patients' characteristics are outlined in Table 1. The primary pancreatic lesion was located in the head in 33 patients, in the body in 9, and in the tail in 2. All patients underwent pancreatic resection. Pancreatic ductal adenocarcinoma was confirmed in all patients histologically. R0 resection was performed in 37 patients, R1 resection in 5 (11.4%), and R2 resection in 2 (4.5%). The median tumor size was 3 (range, 1.3–8.7) cm. Lymph node metastases were identified in 30 patients (68.2%), including para-aortic lymph node metastases in 3 patients. Resection and reconstruction of SMV or PV were necessary in 22 patients (50%), although 13 (29.5%) were proven to have histological portal invasion. Thirty-four patients received IORT after resection. All of the patients began postoperative chemotherapy after recovery from surgery, although 20 patients (45.5%) were completely treated according to the postoperative schedule. The mean pre- and postoperative doses of total 5-FU administered per patient were 2.8 and 5.2 g. The mean pre- and postoperative doses of total gemcitabine were 5.2 and 14.3 g.

Toxicities of Pre- and Postchemotherapy and Surgery

All 44 patients were included in the toxicity analysis. The overall toxicity profiles related to pre- and postoperative chemotherapy are outlined in Table 2. The most common toxicities were hematological and gastrointestinal events.

Nineteen patients (43.2%) experienced grade 3/4 neutropenia during preoperative chemotherapy. All preoperative chemotherapy-related toxicities abated after discontinuation of drug infusion. Forty-three patients underwent surgery 1 week after the completion of preoperative chemotherapy. Only one patient experienced a delay in surgery because of grade 4 neutropenia. Five major complications occurred in five patients after surgery,

TABLE 1 Patient characteristics

Characteristics	No. of patients	%
Total no. of patients	44	
Median age (yr)	65 (37–79)	
Male/female	26/18	
Site of primary lesion		
Head	33	75
Body	9	20.5
Tail	2	4.5
Pancreatectomy		
PPPD	16	36.4
SSPPD	13	29.5
PD	4	9.1
DP	11	25
Stage		
Ia	3	6.8
Ib	1	2.3
IIa	10	22.7
IIb	26	59.1
III	1	2.3
IV	3	6.8
Histologic differentiation		
Well	16	36.4
Moderately	22	50
Poorly	5	11.4
Adenosquamous	1	2.3
Tumor size (cm)		
1.0–2.0	6	13.6
2.1–4.0	33	75
>4.1	5	11.4
Nodal involvement		
Present	30	68.2
Absent	14	31.8
Portal vein invasion		
Present	13	29.5
Absent	31	70.5
Residual tumor		
R0	37	84.1
R1	5	11.4
R2	2	4.5

PPPD pylorus-preserving pancreaticoduodenectomy; SSPPD substomach-preserving pancreaticoduodenectomy; PD pancreaticoduodenectomy; DP distal pancreatectomy

including grade C pancreatic fistula in two patients, intra-abdominal abscess in one, and cerebral infarction in one. Three patients recovered from complications by means of conservative therapies. One patient underwent reoperation for grade C pancreatic fistula. Hospital death was observed in one patient because of liver failure after intra-abdominal bleeding caused by pancreatic fistula.

TABLE 2 Pre- and postoperative chemotherapy-related grade 3/4 toxicities

	Preoperative	Postoperative
Hematological		
Anemia	0	4 (9.1)
Leukopenia	8 (18.2)	14 (31.8)
Neutropenia	19 (43.2)	24 (54.5)
Thrombocytopenia	2 (4.5)	3 (6.8)
Others		
Perforation of small intestine	0	1 (2.3)
Liver abscess	0	3 (6.8)
Cardiac ischemia/infarction	0	2 (4.5)
Renal dysfunction	0	1 (2.3)
Cholangitis	0	3 (6.8)
Appetite loss	0	1 (2.3)

Percentages are shown in parentheses

Toxicities are defined by NCI-CTC for Adverse Events v3.0

Postoperative chemotherapy was initiated between 3 and 12 weeks after surgery. Twenty-four patients (54.5%) experienced grade 3/4 neutropenia during postoperative chemotherapy, although these toxicities abated after drug infusion was interrupted. Perforation of the small intestine in one patient occurred 1 year after pancreatic resection. This patient underwent emergency surgery and recovered. Grade 3/4 cardiac ischemia occurred in two patients and liver abscess in three patients (6.8%) during postoperative chemotherapy. No intra-arterial catheter-related toxicity occurred in any of the patients. Neither pre- nor postoperative chemotherapy-associated death was observed.

Survival and Outcome

The median follow-up period was 28.2 (range, 5.5–93.3) months. The 1, 3, and 5-year actuarial overall survival rates in all the patients were 78.8, 50.3, and 30.5%, respectively (Fig. 2). The median survival time was 36.5 months.

At last follow-up, 22 of the 44 patients (50%) had died. Seventeen (38.6%) had died as a result of recurrence. There were five (11.4%) non-cancer-related deaths, including one hospital death. Twenty-two patients (50%) remained alive. The median time of tumor recurrence was 24.0 months from the day of surgery. Liver metastases were observed in four patients (9.1%), peritoneal dissemination in six (13.6%), lung metastases in one (2.3%), pleural dissemination in one, bone metastases in one, and local recurrence in four (Table 3). Eight patients survived more than 32.3 months. The two patients with R2 resection died of peritoneal dissemination within 12 months.

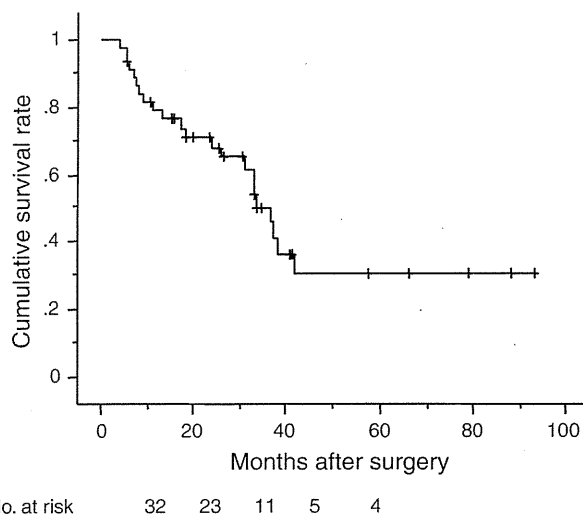


FIG. 2 Overall survival curve for all patients

TABLE 3 Outcomes after this multimodality therapy for patients with pancreatic cancer

	No.
Cancer deaths	17
Liver metastases	4
Lung metastases	1
Pleural dissemination	1
Peritoneal dissemination	6
Local recurrence	4
Bone metastases	1
Non-cancer-related deaths	5
Alive	22
Total	44

DISCUSSION

To our knowledge, this is the first report of perioperative intra-arterial and systemic chemotherapy for pancreatic cancer. This treatment was clearly operator-dependent. Grade 3/4 neutropenia was relatively frequent during perioperative chemotherapy, although the toxicities abated after interruption of drug infusion. Grade 3/4 nonhematological toxicities were observed during postoperative chemotherapy. Liver abscess occurred in three patients. This was thought to be influenced by regurgitated cholangitis, because all of the patients underwent hepaticojejunostomy after PD. Perforation of the small intestine occurred in one patient 3 months after completion of postoperative chemotherapy. Cardiac ischemia required hospitalization for two patients. However, the relationship between these events and chemotherapy was unclear. Toxicities were more critical and frequent during postoperative chemotherapy than during preoperative. Intra-arterial infusion was acceptable

for perioperative chemotherapy, because no catheter-related toxicity was observed.

Practical and theoretical advantages of preoperative treatment of pancreatic cancer were proposed as an early treatment for micrometastases and optimized patient selection for surgery.^{10–12} Circulating tumor cells in the blood proved to be present in 28% of patients with pancreatic cancer, and the prevalence increased with tumor stages.¹³ Moreover, complications, which occurred after 30–45% of major pancreatic resections, delayed the initiation of post-operative chemotherapy.^{14,15} These are supported to introduce preoperative chemotherapy for pancreatic cancer.

The rationale for intra-arterial infusion of chemotherapeutic agents appears to be promising from the point of view of the drug-concentration response, because most liver metastases (>3 mm) have an arterial blood supply.^{16,17} Locoregional adjuvant chemotherapy has been reported to have 3-year survival rates ranging from 48 to 54%, and lower recurrence rates of liver metastases ranging from 8 to 17% for pancreatic cancer compared with no-adjuvant studies.^{3,4,18,19} This study also showed that liver metastases diminished to 9.1%, indicating that intra-arterial chemotherapy might be effective to prevent liver metastases.

We adopted pancreatic resection combined with IORT for local control in this series. Local recurrence was observed in only four patients (9.1%). Single-institution experiences suggest that local failure rates were lower in radiation groups (10–26%) than in no-radiation groups (50–80%).^{20–24} This indicated that resection combined with IORT could provide good control of local recurrence.

Recently, a phase III randomized trial (CONCO 001 study) demonstrated that adjuvant gemcitabine significantly delayed the development of recurrence after resection of pancreatic cancer, with a median survival time of 22.1 months.²⁵ Evans et al. reported on a phase II trial of neoadjuvant gemcitabine-based chemoradiation for stage I/II pancreatic cancer.²⁶ The median survival time of 36.5 months in our study is similar to the 34 months in the Evans group trial despite a greater proportion of patients with node-positive (68.2%) and R2 resection (4.5%) in our study than in the Evans group trial. Because our perioperative chemotherapy is complicated, it will be necessary to clarify which adjuvant treatment is most effective for pancreatic cancer to simplify treatment.

In conclusion, this perioperative chemotherapy for pancreatic cancer is feasible and promises to contribute to survival. It should be evaluated in a phase III setting.

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NO donor and MEK inhibitor synergistically inhibit proliferation and invasion of cancer cells

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Abstract. Nitric oxide (NO) shows tumoricidal activity. We had previously reported that NO downregulates the phosphatidylinositol-3-kinase/Akt pathway, but upregulates the MEK/ERK pathway downstream of growth factor signaling. We hypothesized that NO donor and MEK inhibitor in combination synergistically inhibit the viability of cancer cells compared to either NO donor or MEK inhibitor alone. We determined the effects of S-nitrosoglutathione (GSNO, NO-donor) and U0126 (MEK inhibitor) on insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) signaling, proliferation and invasion in cancer cell lines. GSNO inhibits phosphorylation of IGF-I receptor (IGF-IR), EGF receptor (EGFR) and Akt, but upregulates ERK1/2 phosphorylation in MIA PaCa-2 and HCT-116 cells after stimulation by IGF-I and EGF. On the other hand, U0126 inhibits phosphorylation of ERK1/2, but upregulates phosphorylation of IGF-IR and EGFR in MIA PaCa-2 and HCT-116 cells. The combination of GSNO and U0126 downregulates phosphorylation of IGF-IR, EGFR, Akt and ERK1/2 after stimulation by IGF-I and EGF. GSNO as well as U0126, inhibits the proliferation of MIA PaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells in a dose-dependent manner. GSNO and U0126 in combination synergistically inhibit proliferation and invasion of cancer

cells. These results indicate that the combined treatment of NO donor and MEK inhibitor may be promising in cancer therapy.

Introduction

Insulin-like growth factor (IGF) and epidermal growth factor (EGF) signaling play a key role in cancer proliferation and invasion (1-5). IGF-I and EGF bind to IGF-I receptor and EGF receptor and then phosphorylate the tyrosine of the cognate receptors. Insulin receptor substrate (IRS)-1, an adaptor protein, exists mainly in the cytosol, and binds to phosphorylated IGF-IR, resulting in phosphorylation and activation of IRS-1. IRS-1 and EGF receptor (EGFR) transduce phosphatidylinositol-3-kinase (PI3K), which in turn activates further downstream components, including Akt. Alternatively, phosphorylated and activated EGFR and IRS-1 can also bind to another adaptor protein, Grb-2, which activates the MEK/ERK pathway, another major IGF and EGF signaling cascade parallel to the PI3K/Akt pathway (6,7).

Recent studies have shown that nitric-oxide (NO) plays a role in the posttranslational modification of proteins (8-11). Controversial results have been reported regarding the roles of NO in cancer. Recent papers reported that endogenous NO promotes oncogenesis and angiogenesis in various cancers (12,13). In contrast, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, *in vitro* and *in vivo* (14-21). These studies suggest that NO can act either as a tumor suppressor or as a tumor enhancer depending on cell type and the level of NO in the cells. However, the molecular mechanism underlying the inhibitory effects of NO on cancer viability, remains unclear. We have reported that NO inhibits cancer cell proliferation and invasion through downregulation of IRS-1 protein, resulting in inhibition of the PI3K/Akt pathway (14). In contrast, NO enhances nitrosylation and activates N-Ras and H-Ras proteins (22). Members of the Ras family of small GTPase proteins including K-Ras, N-Ras, and H-Ras, play central roles in the transduction of growth factor signals (23). Thus, NO downregulates the PI3K/Akt pathway, but upregulates the MEK/ERK pathway. Therefore, we hypothesized that the combination of NO donor and MEK inhibitor synergistically inhibits the proliferation and invasion of cancer cells

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Abbreviations: IGF-IR, IGF-I receptor; EGF, epidermal growth factor; EGFR, EGF receptor; NO, nitric-oxide; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol-3-kinase; GSNO, S-nitrosoglutathione; ERK, extracellular signal-regulated kinase; PTC, papillary thyroid carcinoma

Key words: nitric oxide, MEK, insulin-like growth factor receptor, epidermal growth factor receptor, Akt, extracellular signal-regulated kinase, cancer proliferation, cancer invasion, pancreatic cancer, colon cancer

compared to either NO donor or MEK inhibitor alone. In the present study, we demonstrated that combination treatment of NO donor and MEK inhibitor shows greater inhibitory effects on the proliferation and invasion of cancer cells compared to either MEK inhibitor or NO donor alone, which are associated with the inhibition of both the PI3K/Akt and MEK/ERK pathways. These data provide new insight into the molecular basis underlying the regulation of cancer viability.

Materials and methods

Materials. S-nitrosoglutathione (GSNO) and U0126 were purchased from Calbiochem (San Diego, CA). Recombinant IGF-I and EGF were purchased from Peprotech (London, UK). Antibodies against phospho-Tyr 1135/1136 IGF-I R β , phospho-Tyr1068EGFR, phospho-Ser 473 Akt, phospho-ERK1/2, IGF-IR, EGFR, Akt, and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. MIAPaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂.

Cell lysis. Cells were lysed with cell lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM DTT, and 1% NP-40]. Following incubation on ice for 30 min, lysate samples were centrifuged at 13,000 g for 30 min. Aliquots of the supernatant containing equal amounts of protein, determined using Lowry assay, were subjected to immunoblotting followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting. A total of 20 μ g of each protein sample was mixed with 5X sample buffer including 10% β -mercaptoethanol and the mixture was boiled for 5 min. The total cellular protein extracts were separated on 10% SDS polyacrylamide gels. The extracts were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), the membranes were blocked with 5% nonfat dried milk for 2 h at room temperature, and incubated with primary antibody for either 2 h at room temperature or overnight at 4°C. This was followed by incubation with secondary antibody conjugated with anti-rabbit or mouse IgG antibody peroxidase for 2 h at room temperature. Detection was done with an enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, NJ). Bands of interest were scanned by using Colorio GT-X970 (Epson, Tokyo, Japan) and were quantified with NIH Image 1.62 software (NTIS, Springfield, VA).

Cell proliferation assay. The mixture of GSNO (NO donor), U0126 (MEK inhibitor) and 100 μ l of medium containing 5x10³ cancer cells was added to each well (of a 96-well plate). Cell proliferation assay was performed using Cell Counting kit-8 containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) (Dojin Laboratories, Kumamoto, Japan) after the incubation for 72 h.

At the end of each experiment, the cell proliferation reagent WST-8 was added to each well and the plates were incubated at 37°C for 2 h. Optical density (OD) (A450 nm) was measured using an automatic microplate reader (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate.

Invasion assay. The invasive activities of cultured MIAPaCa-2 and HCT-116 cells were assayed using BD BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA). MIAPaCa-2 cells were initially seeded on a 24-well plate at a density of 5x10⁴ cells/well and cultured in DMEM/10% FBS, in the presence of 1% glutamine and antibiotics (1% penicillin and streptomycin sulfate) at 37°C in a humidified atmosphere of 5% CO₂. Medium containing NIH3T3 fibroblasts in the lower chamber served as a chemoattractant. The cells were then exposed to NO donor (GSNO 200 μ M) and MEK inhibitor (U0126 10 μ M). The chambers were incubated for 24 h, to determine invasion efficiency. HCT-116 cells were initially seeded on a 24-well plate at a density of 1x10⁵ cells/well and cultured in RPMI-1640/10% FBS, in the presence of 1% glutamine and antibiotics (1% penicillin and streptomycin sulfate) at 37°C in a humidified atmosphere of 5% CO₂. Medium containing NIH3T3 fibroblasts in the lower chamber served as a chemoattractant. The cells were then exposed to NO donor (GSNO 200 μ M) and MEK inhibitor (U0126 10 μ M). The chambers were incubated for 48 h, to determine invasion efficiency. MIAPaCa-2 and HCT-116 cells on the upper surface of the filter were removed using a cotton-wool swab, and the cells that had invaded the lower surface were stained with 1% toluidine blue after fixation in 100% methanol. The number of invading cells was counted in 5 randomly selected fields under a light microscope. Each experiment was performed in triplicate.

Statistical analysis. Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts Inc., Berkeley, CA). Comparisons were carried out using Student's t-test, and P<0.05 were considered statistically significant. ANOVA followed by Tukey-Kramer post hoc test was used to compare multiple samples, with a significance level of 0.05.

Results

NO donor and MEK inhibitor influence IGF-I signaling in MIAPaCa-2 cells. Stimulation of IGF-I resulted in marked phosphorylation of IGF-IR, Akt and ERK1/2 in MIAPaCa-2 cells. GSNO, a NO donor, inhibited IGF-I-stimulated phosphorylation of IGF-IR and Akt. On the other hand, GSNO induced phosphorylation of ERK1/2 without stimulation of IGF-I, and enhanced IGF-I-stimulated phosphorylation of ERK1/2. However, GSNO did not influence IGF-IR, Akt, and ERK1/2 protein expression in MIAPaCa-2 cells. U0126, a MEK inhibitor, inhibited IGF-I-stimulated phosphorylation of ERK1/2. On the other hand, surprisingly, U0126 enhanced IGF-I-stimulated tyrosine phosphorylation of IGF-IR, without the influence of IGF-IR protein expression. In addition, U0126 upregulates IGF-I-stimulated phosphorylation of Akt. The combination of GSNO and MEK inhibitor inhibited IGF-I-stimulated phosphorylation of IGF-IR, Akt, and ERK1/2 without the influence of IGF-IR, Akt, and ERK1/2 protein expression (Fig. 1).