Asialoglycoprotein Receptor Promotes Cancer Metastasis by Activating the EGFR–ERK Pathway

Suguru Ueno, Marija Mojic, Yoshimi Ohashi, Nobuaki Higashi, Yoshihiro Hayakawa, and Tatsuro Irimura

Abstract

Although the importance of glycans in malignant cell behavior is well documented, the potential involvement of endogenous lectins as modifiers of progression and metastasis in the tumor microenvironment has not been explored. In this study, we show that loss of the hepatic asialoglycoprotein receptor (ASGPR) in mice severely reduces the frequency of spontaneous lung metastasis after intrahepatic implantation of murine Lewis lung carcinoma (3LL) cells. Conversely, in vitro treatment with recombinant ASGPR increased the invasive and metastatic capacity of 3LL cells before intrahepatic implantation. ASGPR treatment in vitro increased the expression and production of matrix metalloproteinase-9 through activation of the epidermal growth factor receptor–extracellular signal-regulated kinase (EGFR–ERK) pathway. Our findings identify ASGPR as a novel important factor that responds to endogenous lectins in the tumor microenvironment to promote cancer metastasis by activating the EGFR–ERK pathway through interactions with counter-receptors on cancer cells.


Introduction

Tumor metastasis is a multistage process during which malignant cells alter their cellular behavior, spread from the primary tumor to distal organs, and establish distant metastatic lesions. All of these processes are greatly influenced by the tumor microenvironment (1). The process of cancer metastasis involves adhesion, invasion, migration, and proliferation, and these steps are also affected by the tumor microenvironment (2). According to the “seed and soil” hypothesis by Paget (3, 4), growth factors, chemokines, and adhesion molecules have been proposed as the molecular basis of the organ-selective natures of metastases, and the interactions between glycans on the surfaces of cancer cells and lectins on the host cells were considered to be essential (5, 6). Particularly, the interaction between the lectins expressed on endothelial cells (selectins) and their endogenous or exogenous counter-receptors on tumor cells plays an important role in the metastatic process (5–8). Indeed, the blockade of E-selectin function prevents tumor cell adhesion to endothelial cells in vitro and further attenuates the development of metastasis in vivo (9, 10). The typical ligands recognized by selectins are sialylated and fucosylated carbohydrate chains such as sialyl Lewis-a (sLea) and sialyl Lewis-x (sLex), and the expression of these molecules in a variety of carcinomas are inversely correlated to the prognosis of the patients (11–13). There are many type 2 transmembrane proteins with a C-type lectin fold involved in cellular interactions leading to changes in localization and gene expression (14). Interactions between some of these lectins, such as the asialoglycoprotein receptor (ASGPR), and their endogenous or exogenous counter-receptors are governed by binding to particular carbohydrate ligands in a calcium-dependent manner. ASGPR is expressed almost exclusively on hepatocytes and is involved in the capturing and subsequent endocytosis of glycoproteins that carry galactose or N-acetylgalactosamine (GalNAc) residues at the terminus of the glycans (15). ASGPR localizes to the vascular face of the hepatocyte to remove and to degrade potentially deleterious circulating glycoproteins (16, 17). Other reports show that platelets expressing asialoglycoproteins are removed from the circulation through recognition by ASGPR (18). Thus, the absence of ASGPR results in lethal coagulopathy and septic shock after bacterial infection that is not lethal in wild-type mice (18). Although the potential importance of ASGPR in other pathologies has been suggested, it remains unclear whether ASGPR is involved in the pathogenesis of cancer by signaling through its counter-receptors on cancer cells in the surrounding microenvironment.

In this study, we examined the role of ASGPR as a tumor microenvironmental factor in the formation of distant metastases, using in vivo models of spontaneous and experimental metastasis. Binding of ASGPR to tumor cells promoted lung
metastasis formation, and the mechanism, at least in part, involved transcriptional upregulation of matrix metalloproteinase (MMP)-9. Activation of the epidermal growth factor receptor–extracellular signal-regulated kinase (EGFR–ERK)-dependent pathway by ASPGR induced MMP-9. These results suggest that ASGPR plays a key role as a tumor microenvironmental factor to promote cancer metastasis.

Materials and Methods

Cells

The mouse Lewis lung carcinoma (3LL) cell line was kindly provided by Dr. Kazuyoshi Takeda (Juntendo University), and the luciferase-expressing 3LL (3LL-Luc2) cell line was prepared as previously described (19). 3LL and 3LL-Luc2 cells were maintained in RPMI-1640 medium (Nissui) supplemented with 10% FBS.

Mice

Asgr1+/− mice (129SV) were provided by Dr. Shun Ishibashi (Jichi Medical University; ref. 20) and were backcrossed (8 times) with C57BL/6 mice to establish Asgr1−/− mice (C57BL/6). Wild-type Asgr1+/+ and Asgr1−/− mice were bred and maintained in a specific pathogen-free facility. The absence of functional ASGPR in Asgr1−/− mice was confirmed by the absence of hepatocyte surface expression of ASGR1 by anti-ASGR1 antibody. All animal studies were approved by the Animal Care and Use Committee of the University of Tokyo.

Preparation of recombinant mouse ASGR1

The gene that encodes the carbohydrate recognition domain of mouse ASGR1 (rASGR1) was amplified from cDNA from the liver of a C57BL/6 mouse, using KOD Plus DNA polymerase (Toyobo) and the following primers: forward

5′-aaaggtacgctgctgctgattaagcgcagaatttc-3′

reverse primer

5′-aaagggccggctaaattagccttatccaactttgtctc-3′

The PCR products were separated in a 1% agarose gel, and extracted, purified with the QIAquick Gel Extraction Kit (Qiagen), and ligated with the pCR4 Blunt-TOPO Vector (Invitrogen) to determine the sequence. The cloned ASGR1 gene was inserted into pET-21a. Escherichia coli Rosetta (DE3) cells were transformed with the plasmid [pET-28a(+)+mASGR1], grown to mid-log phase at 37°C in 2×TY medium, and then treated with isopropyl-β-D-thiogalactoside (1 mmol/L). The cell lysates were prepared by freezing and thawing, and then DNase I (50 U/mL) and lysozyme (0.2 mg/mL) were added for a 1-hour incubation at 37°C. Next, the lysates were centrifuged at 15,000 × g for 10 minutes at 4°C. The pellets were washed with TBS containing 0.5% Triton X-100 and 10 mmol/L EDTA and then with H2O. The washed pellets were solubilized with 2 mol/L NH4OH and then added to 25 mmol/L MOPS buffer for dialysis. Soluble recombinant protein was purified by affinity chromatography on a column of galactose-Sepharose-4B, as previously described (21). The purity was confirmed by SDS-PAGE analysis. The binding activity of rASGR1 was tested by ELISA with saccharide-conjugated polyacrylamide (GlycoTech; ref. 21). The purified protein was biotinylated with N-hydroxysuccinimide biotin (Sigma-Aldrich), as described previously (22).

Reagents

To generate a rabbit anti-mouse rASGR1 polyclonal antibody, 2 female white rabbits were immunized with rASGR1 (1 μg) in Freund’s complete adjuvant (primary immunization) or in Freund’s incomplete adjuvant (first and second boost). The blood samples were collected from the carotid artery 1 week after the last immunization and were centrifuged at 3,500 rpm for 20 minutes at 4°C to collect serum. The immunoglobulin G (IgG) fraction of the rabbit serum was purified on a Protein G Sepharose column (GE Healthcare). For inhibiting ASGPR–carbohydrate interactions, anti-mouse rASGR1 polyclonal antibody or GalNAc (Sigma-Aldrich) was used. For control treatments, control rabbit IgG (Zymed; used at 1:1,000 dilution) or N-acetylgalcosamine (GlcNAc; Sigma-Aldrich) was used. The SCADS inhibitor kit was provided by the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area “Cancer” from The Ministry of Education, Culture, Sports, Science and Technology, Japan. For inhibiting EGFR or ERK phosphorylation, AG1478 (Sigma-Aldrich) or PD98059 (Sigma-Aldrich) was added to the culture.

Spontaneous and experimental metastasis assay

In the spontaneous metastasis assay, Asgr1+/+ or Asgr1−/− mice were anesthetized, and a small skin incision on the center of abdomen was made. 3LL-Luc2 cells (1 × 106; 10 μL) were directly injected into the left lateral lobe of the liver with a 29G needle and then the skin incision was closed with a surgical clip. The sizes of the primary tumor (length × width) and lung metastases were evaluated at 14 days after the tumor inoculation. For evaluating spontaneous lung metastasis, the lung was digested with prewarmed RPMI-1640 medium containing 1 mg/mL collagenase and 0.1% DNase I, and then with 0.25% trypsin (Sigma-Aldrich) or PD98059 (Sigma-Aldrich) or PD98059 (Sigma-Aldrich) was added to the culture.

Proliferation assay

Cells were seeded onto a 96-well plate (Sumilon; Sumitomo Bakelite) and incubated in complete RPMI-1640 medium [10% fetal calf serum (FCS)]. The Cell Counting Kit-8 (Dojindo Laboratories) was added at indicated times according to the manufacturer’s instructions, and the absorbance at 450/650 nm was measured on an Arvo X5 multilabel reader (Perkin Elmer).

Invasion assay

The invasive activity of 3LL cells was assayed in the Transwell cell culture insert (8-μm pore size; KURABO). The filters were preincubated with 20 μg/mL of rASGR1 or control bovine serum albumin (BSA; Calibiochem) in serum-free RPMI-1640 medium at 37°C for 48 hours. Cells (2.5 × 103; 100 μL) were injected into the tail vein of C57BL/6 mice, and the number of lung metastatic colonies was manually counted under the microscope 3 weeks after inoculation.
were precoated with fibronectin (Sigma-Aldrich; 1 µg/filter) on the lower surfaces, and the inside of the insert was coated with Matrigel (Becton Dickinson; 10 µg/filter). 3LL cells were preincubated with 5 µg/mL of soluble rASGR1 or control BSA at 37°C for 48 hours in serum-free RPMI-1640 medium. Cells were harvested, added into the upper compartment of the insert, and then incubated at 37°C for 13 hours. The invading cells on the filter were fixed and stained with Diff Quick Kit (Wako Pure Chemicals), and the remaining cells on the upper surface of the filter were removed. The number of the invading cells in 5 random fields was counted manually under the microscope.

**Gelatin zymography**

The cell-free culture supernatants were collected, and proteinase activity was analyzed by resolving on 10% SDS-polyacrylamide gels containing 1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature and then incubated with the regeneration buffer (50 mmol/L Tris-HCl, 0.5 mmol/L CaCl2, and 10⁻⁶ mol/L ZnCl2) for 13 hours at 37°C. The band intensity was evaluated by Image J software.

**Immunofluorescent staining**

Cells were fixed with ethanol and stained with a goat anti-mouse MMP-9 antibody (R&D Systems; 1:1,000) or isotype control IgG (Zymed; 1:1,000) at room temperature for 30 minutes, incubated with fluorescein isothiocyanate–conjugated rabbit anti-goat IgG (Zymed; 1:1,000) as a secondary antibody at room temperature for 30 minutes, and stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent imaging was obtained by confocal microscopy (TCS-SP5; Leica).

**Reverse transcriptase PCR**

Cells were transferred to Ultraspec reagent (Biotecx Laboratories), and total RNA was extracted according to the manufacturer’s instructions. After treatment with DNase I (Qiagen), RNA was subjected to reverse transcription with Superscript II (Invitrogen) with oligo(dT). PCR was carried out with 1 µL of cDNA with AmpliTaq Cold (Applied Biosystems). The PCR conditions were as follows: 95°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 7 minutes. The PCR products were separated on 1.0% agarose gels and stained with ethidium bromide and then the band intensity was evaluated by Image J software. The following primers were used for detecting MMP-9 and β-actin:

- **MMP-9**
  - Forward: 5’-CCTTGGGTAGCACAACAGC-3’
  - Reverse: 5’-ATACTCGATGCGCTATGTCG-3’
- **β-Actin**
  - Forward: 5’-CTTCTACAATGAGCTCGTGG-3’
  - Reverse: 5’-TGATGACCTGGCCGTACGGC-3’

**Hepatocyte–tumor cell coculture**

Isolation of hepatocytes from the mice was adapted from the method of hepatocyte isolation in the rat (23, 24). Mice were anesthetized, and 24G Surflew needles were inserted into the portal vein. The liver was perfused with 30 mL of prewarmed solution A (137 mmol/L NaCl, 5.37 mmol/L KCl, 0.5 mmol/L NaH₂PO₄, 0.42 mmol/L Na₂HPO₄, 10 mmol/L HEPES, 0.017 mmol/L phenol red, 5 mmol/L EGTA, 4.17 mmol/L NaHCO₃, and 5 mmol/L glucose) and then 30 mL of prewarmed solution B (137 mmol/L NaCl, 5.37 mmol/L KCl, 5 mmol/L CaCl₂, 0.5 mmol/L NaH₂PO₄, 0.42 mmol/L Na₂HPO₄, 10 mmol/L HEPES, 0.017 mmol/L phenol red, 4.17 mmol/L NaHCO₃, 0.05% collagenase, and 0.005% trypsin inhibitor) at the same flow level. The hepatic lobes were collected after perfusion and excised in RPMI-1640 medium containing 0.005% DNase I (Roche) on ice. The excised liver lobes were resuspended in 20 mL of RPMI-1640 medium containing 0.005% DNase I, and the cells were gently separated by pipetting and filtered by a cell strainer (70 µm nylon mesh; Falcon). The cells were resuspended with 12.5 mL of RPMI-1640 medium with 0.005% DNase I and then 1 mL of 1.5 mol/L NaCl and 9 mL of Percoll (GE Healthcare) were added. The cells were centrifuged and gently washed with RPMI-1640 medium containing 0.005% DNase I. Hepatocytes were seeded to a gelatin precoated 24-well plate (Sumilon) in Williams E medium (10% FCS, 10⁻⁹ mol/L insulin, 10⁻⁹ mol/L dexamethasone, 100 µg/mL penicillin, and 100 µg/mL streptomycin) and incubated at 37°C overnight. Before subjecting them to a coculture with 3LL cells, hepatocytes were fixed with 1% gluteraldehyde at 4°C for 10 minutes.

**Western blotting**

Cells were treated with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate. The antibodies against EGFR, anti-phospho-EGFR (Tyr1068), anti-ERK 1/2, and anti-phospho-ERK 1/2 (Thr202/204; Cell Signaling Technology) were used. The band intensity was quantified by Image J software.

**Statistical analysis**

The Mann–Whitney U test was conducted to determine statistical significance in the metastasis assays. Other assays were evaluated by Student’s t test. The values of P < 0.05 were considered statistically significant.

**Results**

**ASGPR promotes lung metastasis of 3LL cells**

ASGPR is composed of the heterodimeric, transmembrane glycoproteins ASGR1 and ASGR2 (25). ASGR1-deficient mice also do not express ASGR2 or ASGR1 (20), thus lacking functional ASGPR. Despite the deficiency in functional ASGPR expression, ASGR1-deficient mice appear overtly normal and do not accumulate asialoglycoproteins in their blood (20). To determine whether the absence of ASGPR affects growth and lung metastasis, 3LL-Luc2 cells were injected intrahepatically. Although we did not observe any differences in the size of the primary tumors in the liver between Asgr1⁻⁻ and Asgr1⁻¹/² mice (Fig. 1A), the spontaneous lung metastasis of 3LL-Luc2...
cells that was determined by the relative luciferase activity (RLU) in the lung was significantly lower in Asgr1−/− mice than in Asgr1+/+ mice (Fig. 1B). To examine whether ASGPR directly promoted the metastatic colonization of 3LL cells, we incubated these cells in vitro with rASGR1 and injected them intravenously into syngenic B6 mice. When these cells were pretreated with rASGR1, the number of metastatic colonies in the lung significantly increased (Fig. 1C), suggesting that ASGPR induced changes in 3LL cells that enhanced the metastatic potential of the lung.

**ASGPR promotes cell invasion and proteinase activity of MMP-9 in 3LL cells**

Tumor cells that are highly metastatic to distant sites are known to be capable of adhering to vascular walls, invading basal lamina, and surviving under harsh conditions (2). To elucidate how the ASGPR–cancer cell interaction enhanced metastatic capacity in vivo, changes in the behavior of 3LL cells upon ASGPR treatment were examined. Although in vitro treatment of 3LL cells with rASGR1 did not significantly affect proliferation rates (Fig. 2A) and adhesion to fibronectin (data not shown), their invasion was strongly enhanced (Fig. 2B). Elevation of the proteinase activity of MMP-9 (Fig. 2C) but not MMP-2 (data not shown), enzymes involved in the invasion of malignant cells, was observed by gelatin zymography after the in vitro treatment of 3LL cells with rASGR1.

**ASGPR-specific induction of MMP-9 expression in 3LL cells**

To determine whether the effect of ASGPR on invasion and increased proteinase activity of MMP-9 was due to specific interactions between rASGR1 and its counter-receptors on 3LL cell surfaces, an anti-ASGR1 polyclonal antibody was used to disrupt lectin–carbohydrate recognition. As shown in Figure 3A, the anti-ASGR1 antibody suppressed MMP-9 production by 3LL cells induced by rASGR1 treatment, which strongly supports the hypothesis that the upregulation of MMP-9 production is caused by ASGPR binding. The ASGPR interaction elevated MMP-9 protein and mRNA as indicated by immunofluorescent staining (Fig. 3B) and reverse transcriptase PCR (RT-PCR; Fig. 3C), respectively. These changes were blocked by preincubation of the samples with anti-ASGR1 polyclonal antibodies or GalNAc, both of which interrupt the carbohydrate binding of ASGPR.

**Endogenous hepatic ASGPR induces MMP-9 production in 3LL cells**

We further confirmed that ASGPR present on hepatocytes induced the production of MMP-9 by 3LL cells. As shown in...
Figure 4, MMP-9 production by 3LL cells increased when these cells were cocultured with hepatocytes from Asgr1+/+ mice, whereas hepatocytes from Asgr1−/− mice or the addition of the anti-ASGR1 polyclonal antibody had no effect (Fig. 4). Collectively, these results clearly support the hypothesis that endogenous hepatic ASGPR induces MMP-9 production by interacting with its counter-receptors during the process of metastasis to the lung.

**ASGPR activates the EGFR–ERK pathway in 3LL cells**

To identify the downstream signals activated in 3LL cells following the binding of ASGPR, a chemical screening was carried out with a library of small molecular weight compounds (SCADS inhibitor kit), each of which inhibits a particular cellular signaling pathway. Among a variety of compounds, AG1478 and PD98059, specific inhibitors of the phosphorylation of EGFR and ERK, respectively, significantly suppressed the MMP-9 production in 3LL cells induced by rASGR1 binding (Table 1). To further confirm that the EGFR and ERK pathways were activated in 3LL cells upon their interaction with ASGPR, the phosphorylation status of EGFR and ERK in 3LL cells was determined after the incubation with rASGR1. As shown in Figure 5A, Western blot analysis revealed that EGFR and ERK were phosphorylated as early as 15 minutes after stimulation with rASGR1. Severe impairment in the phosphorylation of ERK was observed in the presence of the EGFR inhibitor AG1478 (Fig. 5B). Importantly, we confirmed that both AG1478 and PD98059 treatment inhibited the upregulation of MMP-9 mRNA by rASGR1 stimulation (Fig. 6). Collectively, these results show that the EGFR–ERK pathway was activated in 3LL cells upon their interaction with ASGPR and that the process leads to the induction of MMP-9 transcription.

Figure 3. ASGPR-specific induction of MMP-9 production and requirement of carbohydrate binding in ASGPR-induced MMP-9 expression. A, 3LL cells were incubated with soluble rASGR1 or control BSA at 37°C for 48 hours. To inhibit ASGPR interactions, an anti-ASGR1 polyclonal antibody was added. MMP-9 production in cell-free culture supernatants was measured by gelatin zymography. Data represent the means ± SEM (n = 3). **, P < 0.01. B, 3LL cells were incubated with soluble rASGR1 or control BSA at 37°C for 24 hours. To inhibit ASGPR interaction, rASGR1 was pretreated with an anti-ASGR1 polyclonal antibody. Cells were subjected to immunofluorescent staining with antibodies against MMP-9. Scale bar, 100 μm. C, 3LL cells were incubated with soluble rASGR1 or control BSA at 37°C for 4 hours and then they were harvested to determine the expression levels of MMP-9 or β-actin mRNA by RT-PCR. To inhibit ASGPR interactions, an anti-ASGR1 polyclonal antibody or GalNAc was added to the culture, and as a control treatment, rabbit IgG or GlcNAc was added, respectively (n = 3).

Figure 4. MMP-9 production by 3LL cells increased when these cells were cocultured with hepatocytes from Asgr1+/+ mice, whereas hepatocytes from Asgr1−/− mice or the addition of the anti-ASGR1 polyclonal antibody had no effect (Fig. 4). Collectively, these results clearly support the
Discussion

It has been widely documented that surface glycans of highly metastatic tumor cells are different from those present on poorly metastatic tumor cells (26). These disparities in glycans may represent an accumulation of genetic and epigenetic changes during the progression of tumor cells. The unique glycans may also be directly involved in the interactions between tumor cells and cells in the host, which potentially promote growth at the primary sites and/or initiate formation of metastases. Among various endogenous lectins on the host cells that were previously considered to be involved in these processes, lectins specific for glycans with terminal galactose or GalNAc residues, such as galectins and C-type lectins, should be considered as candidates to promote malignant cell growth and/or metastasis because peanut lectin stimulates proliferation in colonic explants from patients in vitro (27) or in human colon cancer cell lines (28). However, it is not clear whether endogenous galactose-type lectins play a role in regulating malignant behavior of tumor cells. In this study, we showed that a galactose-type, C-type lectin, ASGPR, functioned as a tumor microenvironmental factor to promote lung metastasis and activate the EGFR–ERK signaling pathway to stimulate MMP-9 production in murine lung carcinoma 3LL cells.

Considering that the induction of MMP-9 by rAGSR1 was suppressed by pretreatment with an anti-ASGR1 antibody or

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Target</th>
<th>Inhibition rate (%)</th>
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<tbody>
<tr>
<td>AG1478</td>
<td>EGFR</td>
<td>59</td>
</tr>
<tr>
<td>PD98059</td>
<td>MEK</td>
<td>45.1</td>
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<tr>
<td>AG825</td>
<td>HER2, EGFR</td>
<td>28.1</td>
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<tr>
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<td>p38 (MAPK)</td>
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<td>AG490</td>
<td>Jak-2</td>
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<tr>
<td>PD169316</td>
<td>p38 (MAPK)</td>
<td>16.5</td>
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<tr>
<td>LY294002</td>
<td>PI3K</td>
<td>7.5</td>
</tr>
<tr>
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<td>AG957</td>
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<tr>
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<tr>
<td>Wortmannin</td>
<td>PI3K</td>
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<tr>
<td>H-89, HCl</td>
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<td>Bisindolylmaleimide</td>
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<tr>
<td>H-7</td>
<td>PKC, PKA</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Androgen receptor</td>
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NOTE: 3LL cells were preincubated with each compound at 37°C for 1 hour and then rASGR1 was added. After incubation for 48 hours, the MMP-9 in the cell-free culture supernatant was measured by gelatin zymography (n = 3). The inhibition rate was calculated as follows: [(ASGR-dependent MMP-9 production−ASGR-dependent MMP-9 production with inhibitor)/ASGR-dependent MMP-9 production] × 100.

Abbreviations: MEK, mitogen-activated protein/extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C.

Figure 5. ASGPR activates the EGFR–ERK pathway. A, 3LL cells were incubated with soluble rASGR1 at 37°C for 15, 30, 45, and 60 minutes, and the phosphorylation of EGFR or ERK was determined by Western blot analysis. B, 3LL cells were treated with AG1478 (10 μmol/L) or PD98059 (10 μmol/L) at 37°C for 1 hour, and the phosphorylation of ERK was determined by Western blot analysis at 45 minutes after the incubation with soluble rASGR1. Data represent the means ± SEM (n = 3); **, P < 0.01.
by GalNAc (Fig. 3C), the recognition of glycan chains by ASGPR should be responsible for the MMP-9 induction. Interestingly, some plant lectins, such as concanavalin A and phytohemagglutinin-L4, were reported to induce MMP-9 production by macrophages (29), hepatic stellate cells (30), microglia, and astrocytes (31). The MMP-9 production by 3LL cells was also induced by a coculture with hepatocytes from wild-type mice but not with those from Asgr1−/− mice (Fig. 4), indicating that the endogenous ASGPR on hepatocytes functioned as a tumor microenvironmental factor to regulate tumor cell behavior.

Many types of tumor cells express elevated levels of EGFR (32, 33), and the glycosylation status of EGFR potentially regulates its function (34–39). For example, N-glycan on Asn-120 of EGFR suppressed ligand-independent spontaneous oligomerization (38). In the present study, we showed that the EGFR–ERK pathway in 3LL cells was activated by the interaction between ASGPR and EGFR (Fig. 5). Phosphorylation of c-jun-NH2-kinase (JNK), but not proteins in the p38 pathway, was observed during ASGPR stimulation (data not shown). The transcription factor AP-1 is a heterodimer of the 2 protooncogene families Jun and Fos, and they are downstream targets of activated ERK and JNK, respectively. Our result that ASGPR stimulation induced the production of MMP-9 but not MMP-2 in 3LL cells was consistent with the finding that the differential regulatory mechanism of MMP-2 or MMP-9 (40) and further the binding sequences of AP-1 are present in the 5′-region of MMP-9 and several other MMP genes but not in the MMP-2 gene (41).

The activation of the EGFR–ERK pathway mediates cell proliferation in a biphasic, dose-dependent manner (42, 43). However, O-charoenrat and colleagues showed that MMP-9 induction in head and neck squamous carcinoma cells increased in an EGF concentration–dependent manner (44). In the present study, we determined that rASGR1 did not affect the proliferation of 3LL cells in vitro (Fig. 2A) and that the growth rate of tumors in the liver in Asgr1−/− mice was not significantly different from that of wild-type mice (Fig. 1A). Thus, the responses induced in 3LL cells by ASGPR binding are apparently different from the response to EGF, although a detailed comparison should be conducted.

The importance of ASGPR in the pathogenesis in human cancers remains to be determined. Preliminarily, we found that MMP-9 production by other cancer cell lines in both mouse (B16F10 and MC38) and human (HT-29) origins were stimulated by incubation with rASGR1 (data not shown); therefore, the phenomenon observed with 3LL cells may be applicable as a mechanism of lung metastasis of colon carcinoma via the liver in a physiologic context. Thus, the interaction between cancer cells and ASGPR may play a general role in the formation of a metastatic niche in the cancer microenvironment. To understand the relevance of ASGPR and related lectins in cancer progression, identification of ASGPR counter-receptors, which may be EGFR or a glycoprotein linked to EGFR, on cancer cells would be of great interest. Further elucidation of the molecular mechanism by which ASGPR or other C-type lectins can cross-talk with cancer cells may be important in establishing new therapeutic strategies for controlling the metastatic spread of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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