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Laminin γ2-Chain Fragment in the Circulation: A Prognostic Indicator of Epithelial Tumor Invasion

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ABSTRACT

Laminin (LN) 5, the major component of epithelial-derived extracellular matrix (ECM), plays a major role in cell adhesion and motility. Previous reports stated that proteolytic processing of the NH_2-terminal region of the γ2 chain enhances cell motility on LN5, indicating that the degraded γ2 chain NH_2-terminal region would be shed from the ECM. However, soluble LN γ2 NH_2-terminal fragment (G2F) have not been detected in biological fluids. Here, we developed a double-monoclonal electrochemiluminescence immunoassay for human G2F and detected its presence in the normal human circulation (mean ± SD: 39.2 ± 10.3 ng/ml; $n = 10$). We also measured serum levels of G2F in nude mice orthotopically transplanted with three different human pancreatic carcinoma cell lines: MIApaca-II (secreting no LN5), HPAC (secreting the α3β3γ2 heterotrimer of LN5), or KP-1 (secreting the monomeric γ2 chain of LN5). Serum levels of G2F drastically increased in the nude mice transplanted with HPAC (mean ± SD: 351 ± 33 ng/ml, 5 weeks after transplantation), the most invasive tumor cells to generate extensive peritoneal dissemination *in vivo*. A moderate increase in serum levels of G2F was also observed in mice transplanted with KP-1 (87.9 ± 82 ng/ml, 5 weeks after transplantation), but no antigen was detected in the sera of MIApaca-II-transplanted mice. Therefore, circulating G2F was demonstrated to be a sensitive marker for LN5 production of primary pancreatic carcinomas, even if it was produced only as a monomeric γ2 chain. In 11 established human pancreatic tumor cell lines (6 of LN5-producing cells and 5 of nonproducing cells), LN5-secreting cells have significantly higher levels of cell surface expression of β4 integrin than nonsecreting cells. Thus, LN5 secretion is accompanied by cell surface expression of α6β4 integrin, participating in hemidesmosome reorganization to form invading edges of malignant epithelial carcinomas. These data reveal that the level of circulating G2F is a new, prognostic, tumor-characterizing marker for estimating the invasiveness and malignancy of epithelial carcinomas in cancer patients.

INTRODUCTION

Members of the LN[2] family of glycoproteins are major constituents of basement membranes (basal lamina), ECM components found in intimate contact with individual cells and cell layers (1). LNs have one heavy chain, α, and two light chains, designated β and γ, so 15 genetically distinct LN types are known at present (1, 2). LN5 is found in the basement membrane of epithelia, known as a major component of the anchoring filaments, and plays an important role in stabilizing the attachment of epithelial hemidesmosomes to the basement membrane via α6β4 integrin (2–4). Our present study is focused on the degradation and solubilization of the LN γ2 NH_2-terminal region. The 155 kDa mature form of the γ2 chain is cleaved into a 105 kDa COOH-terminal fragment, and some proteases mediate this extracellular processing to shed the globular domain IV and the epidermal growth factor-like rich domain V of the γ2 short arm from the 440 kDa high molecular weight form of LN5 (5). MMP2 (6), MT1-MMP (7), and bone morphogenic protein-1/type-I procollagen COOH-terminal peptidase (8) are considered to be candidates for LN γ2-processing proteases. The COOH-terminal fragments of LN γ2, with a molecular weight of 105 kDa, were shown previously to be additionally processed into the 80 kDa forms by MT1-MMP (7). The resultant degraded LN5 (400 kDa heterotrimer) is an active form that enhances epithelial cell motility and migration *in vitro* (6, 7). Therefore, the proteolytic release of the NH_2-terminal region of the γ2 chain can be an indicator for invasive LN5 deposition in the anchoring edges of epithelial cells. However, the contributions of the γ2 NH_2-terminal domain processing to hemidesmosome turnover or reorganization of LN5-rich anchoring contacts, probably acting in the epithelial cell invasion during tissue remodeling, are not well understood and require additional study (9).

LN5 is known as one of the major scattering factors stimulating the invasive and metastatic ability of several types of tumor cells (10, 11). Some recent histochemical studies revealed that the γ2 chain of LN5 is strongly expressed in disseminating and infiltrating tumor cells at the invasive front of epithelial carcinomas, and its increased expression in tumor tissues is associated with poor prognosis (12–15). Another study reported that the monomeric γ2 chain (not associated with the α3 or β3 chain) is expressed as a single subunit specifically in some invading tumor cells (16). A recent interesting report demonstrated a correlated expression of the transcriptional regulator nuclear β-catenin and the LN γ2 chain in dedifferentiated tumor cells at the invasive site of carcinomas, suggesting that overexpression of LN γ2 in tissues is a potential marker of invasion of malignant and metastatic cancers (17).

Measurements of the concentration of soluble LN5 in cell culture supernatants were reported previously, but only in human keratinocytes (18). However, LN5 or its degraded fragments have not been detected yet in the biological circulation. Here, we developed an immunoassay system to measure the levels of soluble G2F and found that they are present in the normal human circulation. Additionally, we found that the concentration of fragments increased gradually in the circulation of experimental animals bearing human carcinoma cell lines, correlatively with the growth of the orthotopically transplanted tumors. We also showed that high levels of expression of cell surface α6β4 integrins can be observed with some of the human pancreatic carcinoma cell lines, synergistic with their ability to produce LN5 extracellularly. Here, we presumed that the cellular secretion of LN5 may be accompanied by the cell surface expression of its receptor, α6β4 integrin. α6β4 integrins on epithelial carcinoma cells are well known to be up-regulated correlatively with hemidesmosome reorganization in their invasive growth area, and localized in membrane protrusions associated with migration such as filopodia and lamellipodia (19). Thus, it was clearly shown that protease-mediated LN5 remodeling and expression of the counter receptor integrins on epithelial tumor cells contribute simultaneously to their invasion into the surrounding stroma, as suggested by the other reports (6, 7, 20). Therefore, the presence of G2F in the circulation is a new potential

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[2] The abbreviations used are: LN, laminin; ECM, extracellular matrix; MMP, matrix metalloprotease; MT1-MMP, membrane-type 1 matrix metalloprotease; G2F, laminin γ2 NH_2-terminal fragment; MoAb, monoclonal antibody; SEIA, sandwich enzyme immunoassay; ECL, electrochemiluminescence.

tumor marker for estimating invasiveness, by determining the accumulation of active LN5 and $\alpha6\beta4$ integrin expression in the invasive fronts.

MATERIALS AND METHODS

Cell Lines. KP-1, KP-2, KP-3, KP-4, SUIT-2, and BxPC-3 human pancreatic tumor cell lines were established as reported previously (21). Ten human tumor cell lines, including the breast carcinoma cell line T-47D, the lung carcinoma cell line A549, the gastric carcinoma cell line KATO-III, the gastric carcinoma cell line MKN45, the colorectal adenocarcinoma cell line COLO205, the osteosarcoma cell line MG-63, the fibrosarcoma cell line HT-1080, the cervical carcinoma cell line HeLa-S3, the glioblastoma cell line A-172, and the mouth epidermal carcinoma cell line KB were purchased from the Laboratory Products Department of Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). Human glioblastoma cell lines SK-N-SH and U-251 MG were provided from RIKEN Cell Bank (Wako, Japan). All of the other human tumor cell lines including 5 human pancreatic carcinoma cell lines (AsPC-1, HPAC, PSN-1, MIApaca-II, and PANC-1) used in this study were obtained from the American Type Culture Collection (Rockville, MD). All of the cell lines were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with streptomycin, penicillin, and 10% FCS (5×10^6 cells/75 cm^2 tissue culture flask in 25 ml culture medium) for 24 (KP-1, KP-2, KP-3, KP-4, AsPC-1, HPAC, SUIT-2, MIApaca-II, PSN-1, BxPC-3, PANC-1, MDA-MB435, T-47D, MDA-MB453, ZR-75-1, MCF-7, HT-29, SW620, NCI-H596, PC-14, A549, MKN45, DU145, PC-3, MG-63, U251, SK-N-SH, HeLa-S3, and KB) or 72 h (KATO-III, LX-1, WiDr, COLO205, LoVo, KP-1, and MIApaca-II) at 37°C in a humidified atmosphere containing 5% CO_2.

Immunogens and MoAbs. Human LN5 was immunoisolated from MKN45 cell culture supernatants (11). The monomeric $\gamma2$ chain and the heterodimeric $\beta3\gamma2$ chains of human LN5 were isolated from A-172 and HT-1080 cell culture supernatants, respectively, essentially according to the previous procedure (11). BALB/c mice were immunized with LN5, and hybridomas secreting MoAbs reactive with LN5 were constructed according to the standard method (22). In the first trial, we established 4 hybridoma cell lines producing anti-LN5 MoAbs (19, 57, 2B10, and 8C2). The monomeric $\gamma2$ chain purified from A-172 cell culture supernatants, the LN5 heterodimer consisted with $\beta3\gamma2$ purified from HT-1080 cell culture supernatants, or the whole LN5 heterotrimers were absorbed onto microplates separately and used in an indirect ELISA for these MoAbs. Antihuman LN5 rabbit antiserum (11) was diluted appropriately and used as a positive control. Binding of antibodies to the various LN5 derivates immobilized on the plates was estimated by using peroxidase-labeled second antibodies and *o*-phenylenediamine substrates (Sigma Chemical Co.). In addition, 10 mg IgG of 8C2 MoAbs were immobilized onto cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions and used to immunoisolate the $\gamma2$ fragments of LN from the culture supernatants of human pancreatic carcinoma cell lines, KP-2 or BxPC-3. LN5 or its degraded fragments, eluted by 8 M urea from 8C2 MoAbs coupled to cyanogen bromide-activated Sepharose, was dialyzed against PBS. In the trial to construct the next murine hybridomas, we used this KP-2-derived LN5 as an immunogens to generate 2 new hybridomas secreting anti-LN5 MoAbs, which were individually named KP2-LN5-8C2E 12-1 and KP2-LN5-8C2E 18-4. All of the MoAbs obtained in this study were classified into IgG1 subclasses. A large amount of IgG was purified from the serum-free hybridoma cell culture supernatants by protein A-Sepharose column chromatography (Amersham Pharmacia). Purified MoAbs were labeled with horseradish peroxidase (Roche Diagnostics GmbH, Mannheim, Germany) according to the periodate method (22).

Western Blot Analysis. Human LN5 heterotrimers or $\gamma2$ chains were immunopurified from the culture supernatants of human pancreatic tumor cell line BxPC-3 using 8C2 MoAbs immobilized on a Sepharose 4B gel column. Isolated LN5 antigen was separated on a 4–20% gradient SDS-PAGE under nonreducing conditions and electrophoretically transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membrane was blocked in PBS containing 1% skim milk, and incubated in a solution containing each of the MoAbs established here. MoAbs bound on the membrane were visualized by antimouse IgG labeled with peroxidase and 4-chloro-1-naphtol substrates. The molecular masses on the membrane were determined by Kaleidoscope prestained markers (Bio-Rad Lab., Hercules, CA). Protein on the membrane was stained using coomassie brilliant blue (Wako Pure Chemical, Osaka, Japan).

Human Specimens. Fresh blood specimens were collected from 10 healthy male volunteers (average age 35.8 $\pm$ 4.2 years; range, 28–44 years) in our laboratories. These volunteers signed the informed consent from the Department of Research and Development, Eisai Co., Ltd., approved by the Institutional Commission of Ethics. Serum was separated from each sample by centrifugation after normal blood coagulation. Each serum sample was frozen at −40° until use.

Immunoassay. LN5, immunopurified from the culture supernatants of MKN45 cells, was basically used as the standard for the immunoassays performed in this study. We occasionally used the culture supernatants of BxPC-3 cells as the 100 units/ml control in the present experiments. The SEIA for the whole LN5 ($\alpha3\beta3\gamma2$ heterotrimer) was constructed using peroxidase-labeled 2B10 MoAbs and 57 MoAbs immobilized on 96-well microplates (Nunc A/S, Roskilde, Denmark). Another SEIA format using peroxidase-labeled KP2-LN5-8C2E 12-1 and immobilized KP2-LN5-8C2E 18-4 was also performed. This two-step SEIA was performed to measure the concentration of LN5 in the cell culture supernatant, essentially according to the published method (23). Briefly, purified IgG of 57 or KP2-LN5-8C2E 18-4 MoAb was naturally absorbed on the wells of the microplates and then blocked with PBS containing 1% skim milk. Standards and specimens were added into the wells and incubated for 1 h at room temperature. After incubation, the wells were washed three times with PBS, and the solution containing 2B10 or KP2-LN5-8C2E 12-1 coupled with peroxidases was added. One h later, the wells were washed with PBS, and tetramethyl benzidine liquid substrates were added (Sigma Chemical Co.). The enzyme reaction was terminated after 10 min of incubation by the addition of H_2SO_4, and the absorbance at 450 nm was measured by a T-max microplate reader (Molecular Devices Co., Sunnyvale, CA).

The levels of G2Fs in human and experimental animal biological fluids were measured by the ECL immunoassay, using KP2-LN5-8C2E 12-1 MoAb labeled with Ruthenium (Igen International, Gaithersburg, MD) and KP2-LN5-8C2E 18-4 MoAb coupled to Dynabeads M-450 magnetic beads (Dynal A. S., Oslo, Norway), on a Picolumi 8220 automated clinical laboratory analysis system (Sanko Junyaku Co., Ltd., Tokyo, Japan). Human serum specimens were thawed, and 200 μl of each sample was transferred into each ECL reaction tube. The ECL immunoassay in this study was performed automatically by the programmed instruction for the two-step immunochemical reaction (the first step including mixture of the samples and the magnetic beads; the second step including mixture of the antigen-captured magnetic beads and the labeled antibodies), as described previously (24).

Orthotopical Transplantation Model. MIApaca-II, HPAC, and KP-1 human pancreatic tumor cell lines were cultured according to the method described above. Eight-week-old BALB/c *nu/nu* mice (Charles River Japan) were anesthetized as described previously (25). Trypsinized human pancreatic tumor cells were collected by mild centrifugation and washed three times with PBS. Seven million cells of each carcinoma line were injected into the head of the murine pancreas according to published methods (26). Mice were separated into three groups (bearing MIApaca-II, HPAC, or KP-1) and maintained under the same conditions. Three mice randomly selected from each group were sacrificed at 2, 3, 4, and 5 weeks, after intrapancreatic tumor injection. Their primary tumors were surgically removed, and blood was collected from each heart simultaneously. The removed wet primary tumors were weighed just after dissection. Mouse sera were separated by centrifugation and kept frozen at −40° until they were used for assay. Single regression analysis of the correlation between the serum G2F levels and the pancreatic tumor weights was performed by using Excel 2000 software (Microsoft Co.).

Flow Cytometry. Antihuman $\alpha1$ integrin (clone name: 5E8D9) and antihuman $\alpha2$ integrin (A2-IIE10) MoAbs were commercially available from Upstate Biotechnology (Charlottesville, VA). We purchased antihuman $\alpha5$ integrin (KH/33) from Seikagaku Co. (Tokyo, Japan) and antihuman $\beta4$ integrin (450–9D) from PharMingen/BD Bioscience (San Diego, CA). Antihuman $\alpha3$ integrin (P1B5) and antihuman αv integrin (CLB-706) MoAbs were purchased from Chemicon International (Temecula, CA). AMCI 7–4 (antihuman $\alpha6$ integrin MoAb), PLC37–1 (antihuman $\beta1$ integrin), and PLC13-6 (antihuman $\beta3$ integrin) are all murine IgG1 MoAbs, established in our

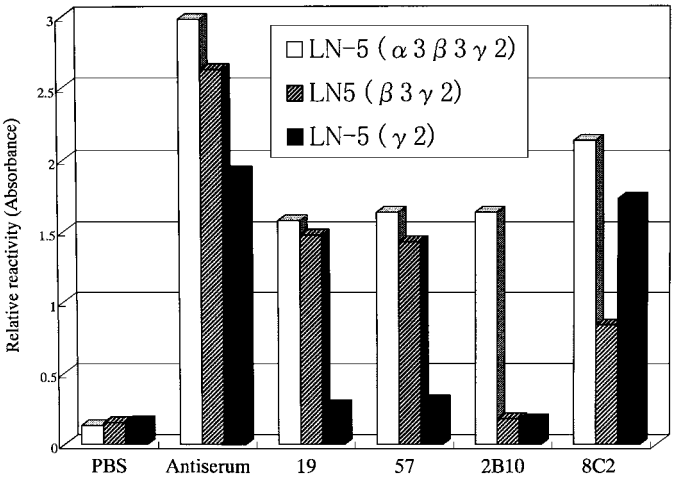

Fig. 1. Reactivity of the four established MoAbs to the heterotrimeric ($\alpha3\beta3\gamma2$), the heterodimeric ($\beta3\gamma2$), and the $\gamma2$ monomeric form of human LN5. Human LN5 heterotrimer ($\alpha3\beta3\gamma2$), heterodimer ($\beta3\gamma2$), or $\gamma2$ monomer was immunoisolated from the culture supernatants of MKN45, HT-1080, or A-172 cells, respectively. Relative binding of the MoAbs (or the antiserum as a positive control) to the LN5 antigen absorbed on the microplates was determined by measuring the absorbance obtained in the solid-phase immunoassay. PBS was used as negative control for binding. The 2B10 and 8C2 MoAbs reacted with the $\alpha3$ and $\gamma2$ chains, respectively. Both the 19 and 57 MoAbs recognized the $\beta3$ chain specifically, because they could not bind to the $\gamma2$ monomers.

laboratory. Trypsinized human pancreatic tumor cells were collected by mild centrifugation and washed three times with PBS. The cells were incubated in a solution containing antihuman integrin MoAbs at the IgG concentration of 1 μg/ml, and additionally treated with FITC-labeled antimouse IgG (Dako A/S, Glostrup, Denmark). Relative mean fluorescence intensity on the cell surfaces was measured by a FACScan system (Becton-Dickinson, San Jose, CA). Significance of differences in the integrin expression levels between the two groups was calculated by the test for equal variance using Excel 2000 software (Microsoft Co.).

RESULTS

Specificity of MoAbs. Four of the murine MoAbs (19, 57, 2B10, and 8C2) we established were selected to be reactive with human LN5 derived from the gastric carcinoma cell line MKN45, which has been shown to produce an $\alpha3\beta3\gamma2$ heterotrimer of LN5 (16, 27). The 8C2 MoAb can specifically react with LN5 derived from the human glioblastoma cell line A-172, which expresses only the $\gamma2$ subunit of LN5[3] and the other 3 MoAbs do not react (Fig. 1). Thus, the 8C2 MoAb was defined to recognize the $\gamma2$ chain of LN specifically. Both MoAbs 19 and 57 can bind to LN5 derived from the human fibrosarcoma cell line HT-1080, which expresses the $\beta3\gamma2$ heterodimer (27). Therefore, the 19 and 57 MoAbs were defined as binding specifically to the LN $\beta3$ chain. We observed that the 2B10 MoAb cannot react with the $\beta3$ or $\gamma2$ subunit of LN5, suggesting that this MoAb recognizes the $\alpha3$ chain of LN. The epitope specificity of these 4 MoAbs was reconfirmed in Western blotting experiments using these LN5 derivatives. The binding epitope of the 57 MoAb is quite similar to that of the 19 MoAb, and the 57 MoAb was used in the following study.

In the Western blot analysis, all of the MoAbs we established bound specifically to the heterotrimeric LN5, with a molecular weight of ~440 kDa (Fig. 2). The 8C2 MoAb, as well as the KP2-LN5-8C2E 12-1 and 18-4 MoAbs, also binds to the 85 kDa and the 50 kDa fragments of the $\gamma2$ chain isolated from the culture supernatants of human pancreatic carcinoma cell line BxPC-3. Considering the apparent molecular mass of these fragments, we conclude that they must be the proteolytic fragments derived from the NH_2-terminal region of the LN5 $\gamma2$ subunits, as reported previously (5–7). These data revealed that the 8C2, KP2-LN5-8C2E 12-1, and KP2-LN5-8C2E 18-4 MoAbs are reactive with the same region of the $\gamma2$ subunit NH_2 terminus. In addition, we observed that the KP2-LN5-8C2E 18-4 MoAb is partially competitive with 8C2, but not at all with the KP2-LN5-8C2E 12-1 MoAb, for binding to LN5 immobilized on the solid phase. Therefore, we selected the combination of peroxidase-labeled KP2-LN5-8C2E 12-1 and 18-4 MoAbs immobilized on microplates for the sandwich immunoassays described.

LN5 Levels in Human Tumor Cell Culture Supernatants. The concentration of LN5 in culture supernatants of several human tumor cell lines was measured by the SEIA using the 57 MoAbs (anti-LN $\beta3$) immobilized on the microplates and the 2B10 MoAbs (anti-LN $\alpha3$) labeled with peroxidase, with the BxPC-3 culture supernatant as the 100 units/ml control (Fig. 3). None of the human breast carcinoma cell lines (MDA-MB-435, T-47D, MDA-MB-453, ZR-75-1, and MCF-7) secretes LN5 extracellularly. Nonepithelial tumor cell lines MG-63, U251, and SK-N-SH do not produce LN5. Both the HeLa-S3 and KB cell lines can be classified as the epidermal tumors, but they do not secrete LN5. Human prostate adenocarcinoma cell lines DU-145 and PC-3 showed moderate expression of LN5 (Fig. 3). Although LN5 was undetectable in the culture supernatant of lung carcinoma cell line A549, it was apparently observed in the supernatant of lung carcinoma cell line PC-14 and slightly detected in the supernatant of lung adenosquamous carcinoma cell line NCI-H596. Human colorectal adenocarcinoma cell lines HT-29 and SW620 secreted a slight amount of LN5. The 11 human pancreatic carcinoma cell lines were clearly classified into LN5-producing types (KP-2, KP-3, HPAC,

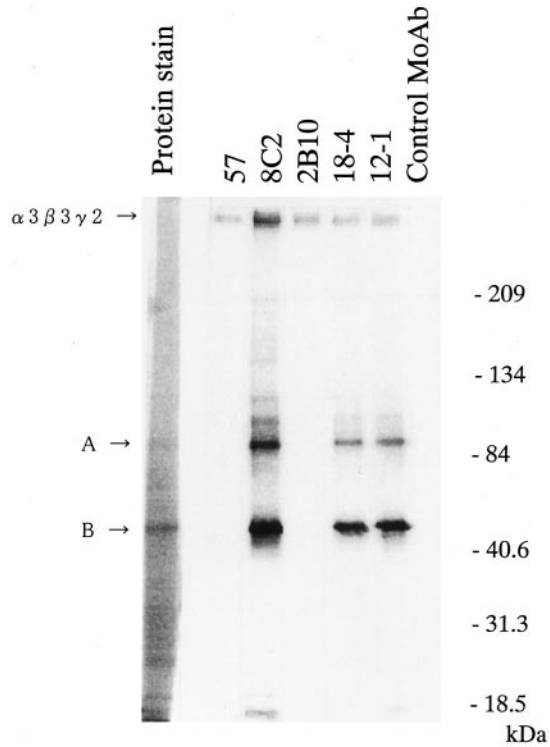

Fig. 2. Western blot analysis for LN $\gamma2$ chain-derived fragments isolated from human pancreatic carcinoma cell culture supernatants. Detailed procedures for SDS-PAGE and immunoblotting are described in "Materials and Methods." The 8C2, KP2-LN5–8C2E 18-4, and KP2-LN5-8C2E 12-1 MoAbs were all reactive with the same fragments derived from the NH_2-terminal region of the LN $\gamma2$ chain, mainly consisting of fragments of ~85 kDa (*A*) and 50 kDa (*B*). All of the MoAbs recognized small amounts of intact LN5 (400 kDa) at the upper limit of the gel.

[3] Y. Fukushima, personal communication.

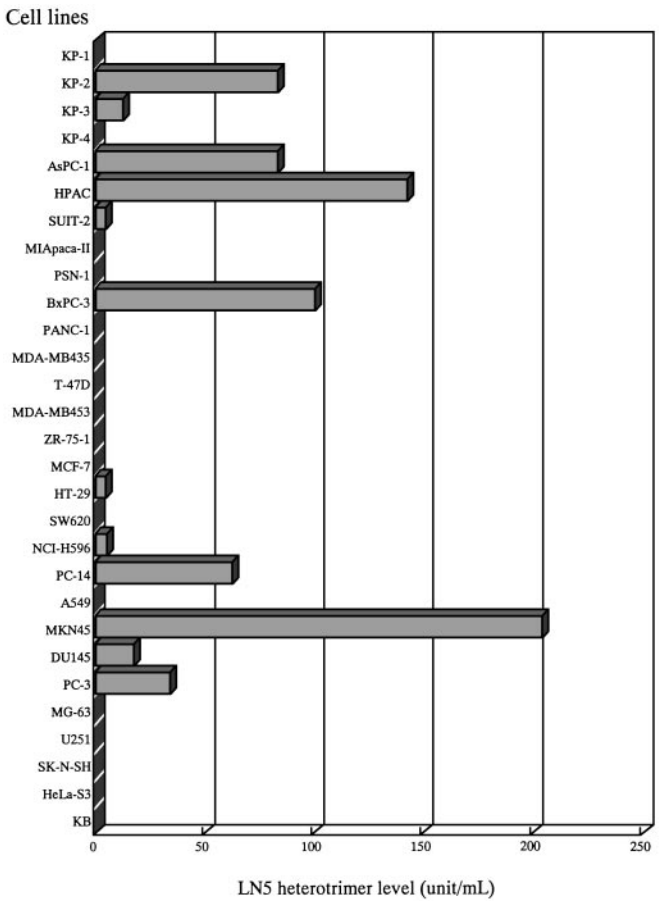

Fig. 3. Relative levels of heterotrimeric LN5 ($\alpha3\beta3\gamma2$) in the culture supernatants of several human tumor cell lines. All of the tumor cell lines were established from human cancers. Pancreatic carcinoma cell lines (KP-1, KP-2, KP-3, KP-4, AsPC-1, HPAC, SUIT-2, MIApaca-II, PSN-1, BxPC-3, and PANC-1), breast carcinoma cell lines (MDA-MB435, T-47D, MDA-MB-453, ZR-75-1, and MCF-7), colorectal carcinoma cell lines (HT-29 and SW620), lung carcinoma cell lines (NCI-H596, PC-14, and A549), a gastric carcinoma cell line (MKN45), prostatic carcinoma cell lines (DU145 and PC-3), an osteosarcoma cell line (MG-63), glioblastoma cell lines (U251 and SK-N-SH), a cervical carcinoma cell line (HeLa-S3), and a mouth epidermal carcinoma cell line KB were used. The relative concentrations of LN5 were measured by the SEIA using the 57 (anti-LN $\beta3$ chain) and 2B10 (anti-LN $\alpha3$ chain) MoAbs. LN5 levels in the culture supernatants were estimated by regarding those in the culture supernatants of BxPC-3 cells as 100 arbitrary units per milliliter.

AsPC-1, BxPC-3, and SUIT-2) or nonproducing types (KP-1, KP-4, PSN-1, PANC-1, and MIApaca-II), as shown in Fig. 3.

Detection of the Monomeric LN $\gamma2$ Chain by SEIA in Human Tumor Cell Culture Supernatants. Human carcinoma cell lines including KATO-III, LX-1, WiDr, COLO205, LoVo, KP-1, and MIApaca-II were cultured. The LN $\gamma2$ chain or its degraded products (mainly consisting of the 85 and 50 kDa fragments) released into the culture supernatant can be detected by the SEIA using the peroxidase-labeled KP2-LN5-8C2E 12-1 and 18-4 MoAbs immobilized on the microplates, and MKN45-derived LN5 as the assay standards (Fig. 4). As expected, intact LN5 heterotrimers were also detectable with the SEIA system using the KP2-LN5-8C2E 12-1 and 18-4 MoAbs, because most of the tumor cell lines presented in Fig. 4 secrete the LN5 heterotrimeric form ($\alpha3\beta3\gamma2$), which is detected efficiently by this SEIA (Fig. 4). KATO-III cells were shown to produce the LN5 heterotrimeric form, but the secreted levels of the $\gamma2$ chain were relatively elevated. The human lung carcinoma cell line LX-1 expresses all of the subunits, as well as the 3 colorectal adenocarcinoma cell lines WiDr, COLO205, and LoVo. It is surprising that the pancreatic carcinoma cell line KP-1 secretes only the monomeric $\gamma2$ chain and expresses neither the $\alpha3$ nor $\beta3$ chains (Fig. 4). In contrast, another pancreatic carcinoma cell line, HPAC, efficiently expresses high amounts of the LN $\gamma2$ chain into the culture supernatants at a concentration of $\geq$10,000 ng/ml (not shown in Fig. 4). These data also showed that there was no expression of the $\alpha3$, $\beta3$, or $\gamma2$ subunits of LN5 by cells of the human pancreatic carcinoma cell line MIApaca-II.

Integrins Expressed on Cells of the Pancreatic Carcinoma Cell Lines. The relative levels of $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$, αv, $\beta1$, $\beta3$, and $\beta4$ integrins expressed on cells of the pancreatic tumor cell lines were quantified by flow cytometric analysis. We additionally averaged the relative expression levels of these integrins on cells of the 6 cell lines secreting LN5 or the 5 nonsecreting cell lines, separately (Table 1). The average levels of $\alpha2$ or $\beta1$ integrin are quite similar (relative ratio of 0.99 or 1.09, respectively) between these two groups, but the levels of $\beta4$ integrin on cells of LN5-producing cell lines are drastically elevated compared with those on cells of nonproducing cell lines (relative ratio of 8.19). The expression levels of $\alpha1$ or $\alpha3$ integrins seemed to increase moderately in the 6 cell lines secreting LN5 (1.4 or 1.44, respectively) as compared with those in the cells of the nonsecreting cell lines, but no statistical significance was observed. Up-regulation of $\alpha6$ integrins (ratio, 2.22) and down-regulation of $\alpha v\beta3$ integrins (ratios of 0.86 and 0.53, respectively) on cells of the LN5-producing cell lines was observed, but differences in these changes were not significant. On the contrary, the cell lines without LN5 secretion tended to express significantly higher levels of cell surface $\alpha5$ integrin than cells of the LN5-producing cell lines (Table 1). Statistically significant differences were observed between LN5-secreting cells and LN-5 nonsecreting cells in the expression levels of $\beta4$ integrins ($P < 0.002$) and $\alpha5$ integrins ($P < 0.002$).

Measurement of LN5 Levels in Human Sera. We could immunologically detect LN5 in human sera from 10 healthy male subjects using the ECL sandwich immunoassay with two different MoAbs reacting to the distant sites of the NH_2-terminal domain of the $\gamma2$ chain (KP2-LN5–8C2E 12-1 and 18-4). On the contrary, any of the SEIAs described here were not suitable for the detection of the circulating form of LN5 or its fragments, suggesting that high assay sensitivity is necessary for the quantification of the low amount of LN5 antigen in the human circulation. In the construction of the ECL

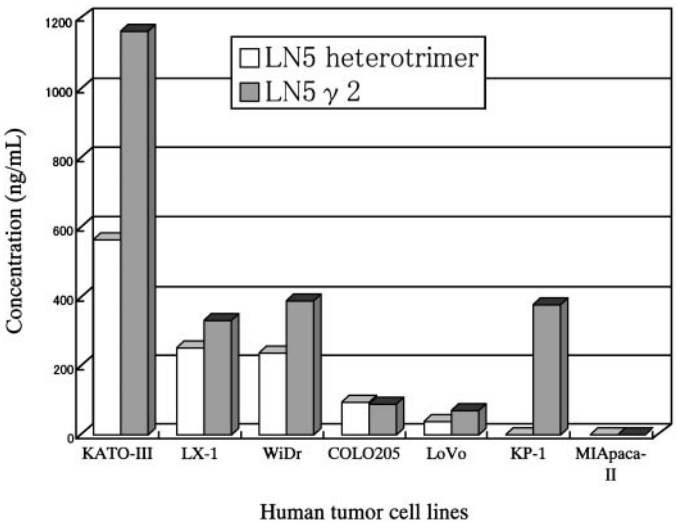

Fig. 4. Measurement of the monomeric $\gamma2$ chain in the culture supernatants of human carcinoma cell lines. The concentrations of the LN5 heterotrimer ($\alpha3\beta3\gamma2$) were measured by the SEIA using the 57 (anti-LN $\beta3$ chain) and 2B10 (anti-LN $\alpha3$ chain) MoAbs. The levels of the $\gamma2$ chain monomer were determined by the SEIA using KP2-LN5-8C2E 18-4 and KP2-LN5-8C2E 12-1 MoAbs (anti-LN $\gamma2$ chain). Purified LN5 was used as the assay standards for both SEIAs. The pancreatic carcinoma cell line KP-1 solely expresses the monomeric form of the $\gamma2$ chain.

Table 1 *Average levels of integrin subunit expression on cells of LN5-secreting or nonsecreting human pancreatic carcinoma cell lines*

	(A) LN5-secreting cells[a] (n = 6) Average (± SD)	(B) LN5-non-secreting cells[b] (n = 5) Average (± SD)	Expression ratio[c]	P[d]
α1	1.65 (± 0.54)	1.18 (± 0.38)	1.4	NS
α2	38.6 (±29.2)	38.9 (±23.7)	0.99	NS
α3	50.1 (±35.1)	34.8 (±20.7)	1.44	NS
α5	1.67 (± 0.53)	7.26 (± 3.26)	0.23	<0.002
α6	27.5 (±15.8)	12.4 (±14.6)	2.22	NS
αv	12.8 (± 6.52)	14.9 (±10.7)	0.86	NS
β1	80.4 (±33.2)	74 (±40.2)	1.09	NS
β3	2.62 (± 1.28)	5 (± 1.68)	0.52	NS
β4	19 (± 9.67)	2.32 (± 1.31)	8.19	<0.002

[a] Relative fluorescence intensity of integrin subunits on cells of each of the 6 human pancreatic tumor cell lines (including KP-2, KP-3, AsPC-1, HPAC, BxPC-3, and SUIT-2) was obtained from the flow cytometric analysis. The average levels were obtained by averaging these data from the 6 cell lines.

[b] Relative fluorescence intensity of integrin subunits on cells of each of the 5 human pancreatic tumor cell lines (including MIApaca-II, PANC-1, KP-1, KP-4, and PSN-1) was obtained from the flowcytometric analysis. The average levels were obtained by averaging these data from the 5 cell lines.

[c] The significance of integrin subunit expression on cells of the pancreatic cell lines secreting LN5 is represented by the expression index, shown as the average level on cells of the LN5-secreting cell lines (A) corrected for that on the nonsecreting cell lines (B).

[d] Statistically significant differences between (A) and (B) were analyzed by the test for equal variance. NS, not significant.

assay, none of the other MoAbs besides KP2-LN5-8C2E 12-1 and 18-4 was applicable for immunodetection of LN5 in the human circulation. These results revealed that the ECL two-site immunoassay using KP2-LN5-8C2E 12-1 as a detector and KP2-LN5-8C2E 18-4 as a capture antibody is the only method that enables measurement of the concentration of the LN5 antigen in the human circulation. The mean concentration of LN5 in the 10 human individuals in this study was 39.2 ± 10.3 ng/ml (mean ± SD).

Levels of Circulating LN5 in Nude Mice Transplanted with Human Carcinoma Cells. All 3 of the human pancreatic tumor cell lines were able to grow in the pancreas of nude mice. There was no detectable LN5 antigen in the circulation of mice transplanted with MIApaca-II cells, because they have no ability to express LN5 *in vitro* (Fig. 5*B*). Culture supernatants of HPAC cells contain high levels of LN5 (Fig. 3), and these cells also secrete a large amount of LN5 into the circulation of mice (Fig. 5*A*). LN5 or its γ2-derived fragments drastically increased, along with the growth of the primary tumor, in the circulation of nude mice bearing HPAC cells at the primary site of the pancreas (mean ± SD: 351 ± 33 ng/ml, 5 weeks after transplantation). Cells of the human pancreatic carcinoma cell line KP-1 were shown to secrete a small amount of LN γ2 subunit monomer solely (Fig. 4). Additionally, an increase in the monomeric γ2 chain or its degraded fragments was also investigated (87.9 ± 82 ng/ml, 5 weeks after transplantation) in the sera of nude mice transplanted with KP-1 pancreatic tumor cell lines (Fig. 5*C*). Statistically significant correlation was observed between the serum concentrations and the weights of the primary tumors in the mice bearing HPAC cells, as the correlation coefficient was 0.77 ($P < 0.01$). The similar correlation was found significantly in the KP-1-transplanted nude mice, as the correlation coefficient was 0.71 ($P < 0.02$). These results reasonably

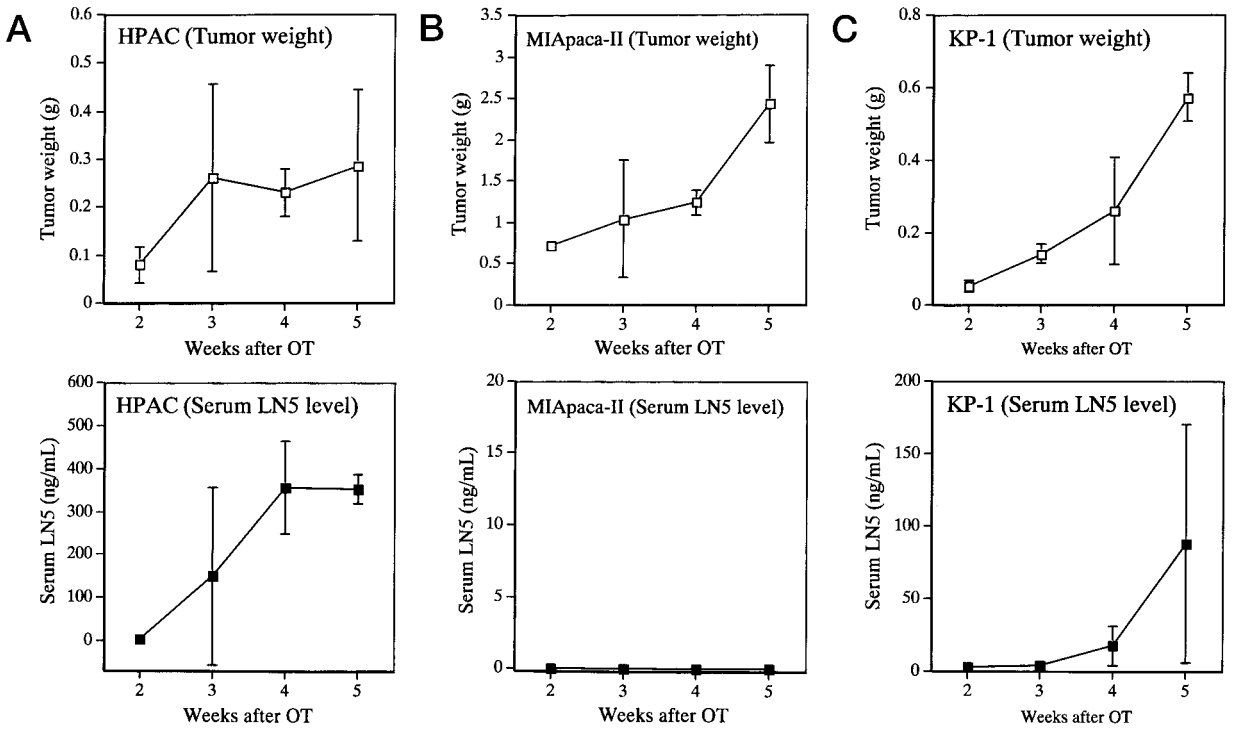

Fig. 5. Primary tumor growth and circulating levels of LN γ2 in nude mice orthotopically transplanted with human pancreatic carcinoma cell lines. Human pancreatic carcinoma cell lines including HPAC (*A*), MIApaca-II (*B*), and KP-1 (*C*) were orthotopically transplanted into nude mice. Blood collection, tumor dissection, and the ECL assay for LN γ2 levels in serum samples were performed at 2, 3, 4, and 5 weeks after implantation, as described in "Materials and Methods." Each *square mark* designates the average levels of data obtained from three animals; *bars*, ± SD. The circulating levels were significantly correlated with the weights of the primary tumors in the mice transplanted with HPAC ($r = 0.77$; $P < 0.01$) and in those transplanted with KP-1 ($r = 0.71$; $P < 0.02$). OT, orthotopic transplantation.

explain that the elevated levels of circulating LN5 detected by this ECL assay are the result of increases in heterotrimers $\alpha3\beta3\gamma2$ or monomeric $\gamma2$ production in the primary sites of growing carcinomas.

DISCUSSION

Some basement membrane constituents are well known to be degraded into small domains or peptide fragments by several proteases from invasive carcinoma cells (28–30). Soluble degradation products of LNs in human biological fluids have been studied extensively and applied to clinical diagnostics for certain cancers (31, 32). We have already investigated that the NH_2-terminal fragments of the LN $\gamma1$ chain, initially designated as the B2 chain, are present in the human circulation and excreted frequently into the urine of lung cancer patients (23). The present study showed that G2Fs are also proteolytically processed *in vivo* and released into the human circulation.

LN $\gamma1$ chain is proposed to be assembled typically with each $\beta1$ or $\beta2$ chain in the first step, and secondly with either the $\alpha1$, $\alpha2$, $\alpha4$, or $\alpha5$ chain, resulting in the intracellular formation of at least 8 types of LN heterotrimers (2). On the other hand, the $\gamma2$ chain is complexed with the $\beta3$ chain into the heterodimer, which is also associated with the $\alpha3$ chain, finally resulting in the LN5 heterotrimer (33). Considering these distinct chain-assembly processes for LN isoforms, it is presumed that the NH_2-terminal fragments of the $\gamma1$ chain in the human circulation are derived from the total of these different LN isoforms expressed in several tissues throughout the body (1, 2). Therefore, circulating G2Fs form a remarkable contrast with those of the $\gamma1$ chain in the following characteristics. First, we can positively indicate that the $\gamma2$ chain fragments must be generated solely from LN5 molecules expressed specifically in epithelial tissues. Second, the released levels of G2Fs into the circulation may reflect new production of the LN $\gamma2$ chain by the epithelial cells, rather than proteolytic degradation of the endogenous ECM containing LN $\gamma2$ chains. We are reasonably certain that the increase in the circulating levels of G2Fs reflects newly deposited LN5 into the ECM *in vivo*.

We revealed that cells of the human pancreatic tumor cell line BxPC-3 actually secrete G2Fs with apparent molecular weights of 50 kDa and 85 kDa (Fig. 2). We also found that the molecular weights of the G2Fs secreted from cells of the KP-2 or HPAC cell lines were quite similar to those from BxPC-3 cells (data not shown), suggesting that these human carcinoma cells generate the identical processing proteases that cleave the NH_2-terminal region of LN $\gamma2$. The previous report demonstrated that most of the NH_2-terminal processing of the $\gamma2$ chain is mediated by cellular MT1-MMP rather than by the other MMPs (34). Therefore, we hypothesized that G2Fs with molecular weights of 50 and 85 kDa can be simultaneously generated in these pancreatic tumor cell lines only by MT1-MMP-mediated processing (7).

We observed that the LN5 heterotrimer could be secreted from cells of some human tumor cell lines used in this study (Fig. 3). No apparent production of LN5 was detected in any of the breast carcinoma cell lines (Fig. 3). This result corresponds well with previous data suggesting the diminished expression of LN5 mRNA in the breast tumor cell lines or tissues (35). Cells of prostatic or lung carcinoma cell lines secreted high or moderate amounts of LN5 extracellularly *in vitro* (Figs. 3 and 4), offering the excellent prospect for their high potential to express LN5 *in vivo*. It is very interesting that active secretion of LN5 was observed only in 6 of 11 lines of human pancreatic carcinoma cells used here, indicating that carcinomas in human pancreatic cancer patients can be clinicopathologically classified into distinct groups of LN5-producing types or nonproducing types. Such a pathological classification has been performed already for some human cancers (12–15), and circulating markers for monitoring LN5 production *in vivo* have been needed for a long time. Our present data showed that immunodetectable LN5 in the circulation of nude mice originates from the implanted human tumor cell lines and that increases in the level possibly indicate enhanced production of LN $\gamma2$ by the pancreatic tumors. The elevation of LN $\gamma2$ concentrations in mouse sera was significantly correlated with the growth of the primary tumor from HPAC- or KP-1-transplanted cells (Fig. 5, *A* and *C*).

This report also revealed for the first time that cellular expression of $\beta4$ integrin on cells of the established human tumor cell lines is significantly correlated with their ability to produce LN5 heterotrimers. It is well known that $\beta4$ integrins are complexed only with $\alpha6$ subunits to form the heterodimeric integrin receptors specific to LN5, concentrated in the hemidesmosome structures of epithelium (2, 4, 19). Intracellular signaling by the $\alpha6\beta4$ integrin is known to stimulate carcinoma cell invasion, and this detailed function involves the remodeling of the LN5-rich basement membranes and hemidesmosome turnover, leading to the acquisition of a motile, invasive phenotype (19, 36). In the present study, we explored the possibility that cells of the human pancreatic ductal carcinoma cell line HPAC express the cell surface $\alpha6\beta4$ integrins (data not shown), and actively secrete LN5 *in vitro* and *in vivo* (Fig. 3; Fig. 5*A*). Cells of the human pancreatic ductal carcinoma cell line AsPC-1 are known to be highly invasive in nude mice when they are orthotopically transplanted (37), and here we showed that they can express high amount of LN5 (Fig. 3). We preliminarily found that the circulating levels of G2Fs drastically increased in all of the mice orthotopically transplanted with AsPC-1 cells (data not shown). HPAC cells, as well as AsPC-1 cells, showed extensive peritoneal dissemination in the transplanted mice (data not shown). The primary tumor weights did not drastically increase in the nude mice transplanted with HPAC cell lines 4 and 5 weeks after transplantation, as compared with those in the mice transplanted with MIApaca-II or KP-1 (Fig. 5, *A–C*), because growing HPAC cells frequently form the cell spheroidal islands and migrate into the peritoneum from the primary sites. Therefore, the serum G2F concentration in the HPAC-transplanted mice appeared to be elevated simultaneously with primary tumor peritoneal dissemination. These data revealed that an increase in the level of circulating G2Fs indicates the progression of the invasive pancreatic carcinoma in the host (Fig. 6).

We mentioned that the monomeric LN $\gamma2$ chain is apparently secreted from cells of the KP-1 pancreatic carcinoma cell line (Fig. 4). This cell line is novel in that it secretes only the monomer of the $\gamma2$ chain, without expression of the $\alpha3$ or $\beta3$ subunits. In the data presented in Fig. 4, relatively elevated levels of the $\gamma2$ chain were observed in the culture supernatants of the tumor cell lines secreting LN5, as compared with the levels of the heterotrimer. In particular, the elevation of the $\gamma2$ chain concentration is most significant in the culture of KATO-III (Fig. 4). Possible interpretation on this phenomenon is that the LN $\gamma2$ monomeric form or its degraded fragments are constitutively expressed by almost all of these carcinoma cells, except for the heterotrimeric form of LN5 (schematically represented in Fig. 6). Some stomach carcinoma cells have been shown to express the monomeric $\gamma2$ and the heterotrimer ($\alpha3\beta3\gamma2$) simultaneously (16). Clinical significance of circulating G2F as an invasion marker is also supported by the previous data demonstrating the expression of the monomeric form of the LN $\gamma2$ chain in human malignant tissues, not associated with $\alpha3$ or $\beta3$ chains, is so prominent as to be recognized as an important histochemical marker for epithelial carcinoma invasiveness (16, 17, 38). Additionally, we could hardly immunodetect the heterotrimer ($\alpha3\beta3\gamma2$) or the heterodimer ($\beta3\gamma2$) form in human sera (data not shown), suggesting that circulating G2F level is solely effective in estimating the invasive ability of epithelial tumors by using sera from the patients.

Expression levels of the LN $\gamma2$ monomer in KP-1 pancreatic

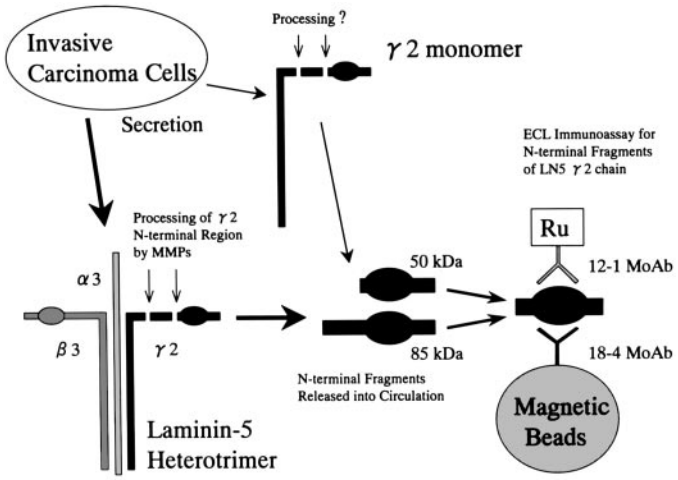

Fig. 6. Schematic representation of tumor cell secretion and proteolytic cleavage of G2Fs, and the detection process by the sandwich ECL immunochemical techniques. Invasive carcinomas actively secrete LN5 heterotrimers and shed the NH_2-terminal region of the γ2 chain. Some of these tumors can express the monomeric γ2 chains, which may be processed proteolytically. As a result, 50 and 85 kDa fragments may be released into circulation, and detected by the sensitive ECL immunoassay constructed in the present study.

carcinoma cells were found to be relatively lower *in vitro* and *in vivo* (Fig. 4; Fig. 5*C*), as compared with those in HPAC cell lines (Fig. 5*A*). The *in vitro* expression potency for soluble LN5 or γ2 chains of HPAC cell lines was shown to be ~25 times higher than that of KP-1 cell lines (comments in Results and Fig. 4). Four weeks after orthotopic transplantation, the average circulating level for LN γ2 in mice bearing HPAC cells was quantified (mean ± SD; 355.3 ± 105.7 ng/ml) and defined to be ~20 times higher than that (17.7 ± 13.3 ng/ml) in the KP-1-transplanted mice (Fig. 5, *A* and *C*). Because the dissected tumor weights of HPAC and KP-1 were found to be quite similar (0.23 ± 0.049 g and 0.258 ± 0.148 g, respectively) at that time, we are confident that cells of the HPAC cell line have an enhanced productivity for LN5 also in the primary site of the nude mice, as well as in the *in vitro* cultured condition. These results positively suggested that serum concentrations of LN γ2 can accurately reflect *in vivo* expression of LN5 or monomeric γ2 chains in the primary carcinomas.

Although detailed cellular functions and the frequency of the monomeric γ2 chain expression in human epithelial carcinomas are still unclear, it should be mentioned that the present ECL assay is of great physiological value, with the ability to detect the cellular expression of the LN γ2 monomer *in vivo* (Fig. 6). The NH_2-terminal region of LN γ2 chain can bind to type VII collagen, heparin, sulfatide, nidogen-1, fibulin-1C, or -2 (39–41), and these γ2-associated molecules may function in any processes of epithelial migration. Some additional pathophysiologic and cell biology studies are required to understand the relationship between LN γ2 production and tumor invasiveness.

In conclusion, we suggest that the circulating G2F level has the potential to be a surrogate marker (instead of clinicopathologic analysis quantifying tumor cell malignancy) when it is used in diagnostics for human cancers. In the mRNA analysis published previously, the normal pancreas is reported to be one of the human organs expressing little LN5 molecule (27). On the contrary, human pancreatic carcinomas secrete large amounts of LN5, and prefer to adhere and migrate on the newly deposited LN5 in their ECM (42). This phenomenon apparently agrees with the previous immunohistochemical data describing the prominent LN γ2 deposition in human pancreatic carcinomas (43). The clinical diagnostic performance of this analysis will be described in our future reports.

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Laminin γ2-Chain Fragment in the Circulation: A Prognostic Indicator of Epithelial Tumor Invasion

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