

Inhibition of Experimental Metastasis of Human Fibrosarcoma Cells by Anti-recombinant 37-kDa Laminin Binding Protein Antibody

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The laminin binding protein of 37 kDa (37LBP) is regarded as a precursor protein of the high-affinity 67-kDa laminin receptor (67LR). Expression of 67LR/37LBP is well correlated with biological aggressiveness of cancer, particularly with invasive and metastatic potential. To investigate in detail the role of 37LBP in cancer cells, we synthesized recombinant 37LBP (r37LBP) as a fusion protein and generated an IgG-type polyclonal antibody P4G against r37LBP. Western blot analysis with P4G showed a single band of 67LR under both nonreducing and reducing conditions using cell extract of human fibrosarcoma cells HT1080. It was shown that P4G inhibited cell attachment to immobilized laminin in a dose-dependent manner. Further, the intravenous injection of HT1080 cells pretreated with P4G, compared with that of cells pretreated with normal rabbit serum, resulted in a reduced number of experimental metastases (3.3 ± 5.1 vs. 58.0 ± 38.0 nodules per mouse, respectively) ($P < 0.005$). These results suggest that P4G inhibits the colonization and growth of HT1080 cells in the lungs of mice, and that the blocking of r37LBP with the specific antibody P4G may offer a potential strategy for preventing cancer metastasis.

Key words: Laminin receptor — Fusion protein — Antibody — Cell attachment — Cancer metastasis

Laminin, a high-molecular-weight glycoprotein ($M_r = 850,000$ – $1,000,000$), is regarded as one of the main components of the basement membrane structure. Since laminin plays an important role in the control of cell functions through the various binding sites of extracellular matrix components (proteoglycans, heparin, and collagen), an interaction between laminin and laminin receptor/binding protein on the cell surface is crucial for cancer-related processes such as proliferation, migration, adhesion, phagocytosis, and metastasis.^{1,2)}

The 37-kDa laminin binding protein (37LBP) has been described as a precursor protein of the non-integrin 67-kDa laminin receptor (67LR).^{3–5)} Previous reports have demonstrated the up-regulated expression of 37LBP in various human cancer cells and tissues such as colon,⁵⁾ breast,⁶⁾ ovary,⁷⁾ and lung.⁸⁾ In these cancers, a direct posi-

tive correlation was demonstrated between the expression of 37LBP in the primary tumor and the metastatic potential of the lesion.^{9–11)} These findings suggest that increased expression of the 37LBP gene participates in the acquisition of the metastatic phenotype.

In the present study, we report a polyclonal antibody that was generated against recombinant 37LBP (r37LBP). The resultant antibody P4G immunoreacted with 67LR and inhibited cell adhesion of H1080, a cell line of human fibrosarcoma, to immobilized laminin in a dose-dependent manner. In addition, pulmonary metastases of HT1080 cells in Balb/c *nu/nu* mice were dramatically inhibited by pretreatment of the cells with P4G. These results demonstrate that 37LBP is the functional domain of 67LR and suggest that the blocking of 37LBP with a specific antibody may constitute a potential future therapy for cancer metastasis.

MATERIALS AND METHODS

Antibody against the 67LR peptide A polyclonal antibody P1 was raised against a 20-mer peptide (PTEDWSAQPTATEDWSAAPT, in single letter symbols), which corresponds to a partial amino acid sequence of human 67LR.¹²⁾

Plasmid construction for synthesis of fusion protein For expression of the fusion protein, the 3'-cDNA frag-

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Abbreviations: BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; EDTA, ethylenediaminetetraacetic acid; EHS, Engelbreth-Holm-Swarm; ELISA, enzyme-linked immunosorbent assay; E-MEM, Eagle's modified essential medium; FCS, fetal calf serum; GST, glutathione S-transferase; IgG, immunoglobulin G; IPTG, isopropyl- β -D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; REDOX, reduction-oxidation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ment (345 bp encompassing nucleotide sequence #584–#929) of pKN43¹³) was subcloned into the plasmid vector pGEX2T (Pharmacia, Uppsala, Sweden) at the *Bam*HI/*Eco*RI positions of the multiple cloning site. Plasmid vector pGEX2T is designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST.¹⁴ The resultant expression vector was designated pGEX4-1 (Fig. 1). The inserted cDNA lacks the 5'-sequence coding the hydrophobic region of the N-terminal sequence of the 37LBP, but does contain the laminin binding sequence (peptide G).^{15, 16}

Affinity purification of fusion protein The *Escherichia coli* strain JM109 transformed with pGEX4-1 was cultured as previously described¹⁴ with some modifications. IPTG (1 mM) was added to a bacterial culture 3 h before cell harvest. Cells were pelleted and resuspended in PBS [containing 1% Triton X-100 and 1 mM PMSF (Sigma, St. Louis, MO)], lysed on ice by mild sonication, and cen-

trifuged at 10,000g for 15 min at 4°C. The supernatant was loaded onto a glutathione Sepharose 4B column (Pharmacia) and the GST-fusion protein including the 37LBP was eluted with an elution buffer (5 mM glutathione in 50 mM Tris-HCl, pH 8.0). Further purification of the fusion protein was carried out with FPLC (Pharmacia). Fractions were monitored with ELISA using both P1 and anti-GST antibody.¹⁷ Purified fusion protein designated F4 was confirmed to be stained positively in a western blot with P1 (Fig. 2).

Antibody against F4 Rabbits were immunized by intramuscular injections of 100 µg of affinity-purified fusion protein F4 in Freund's complete adjuvant (DIFCO, Detroit, MI) and given boosters twice at an interval of 2 weeks. An IgG fraction was separated from the anti-sera using a protein-A column and was designated P4G.

Immunodetection of 67LR by antibody P4G The human fibrosarcoma cell line HT1080 was a generous gift from the Japanese Cancer Research Resources Bank (Tokyo). Cells (1×10⁵) were lysed in the lysis buffer containing detergents (1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) and protease inhibitors [20 mM EDTA, 2 µg/ml aprotinin (Wako, Osaka) and 100 µg/ml PMSF] and centrifuged, and an aliquot of the supernatant

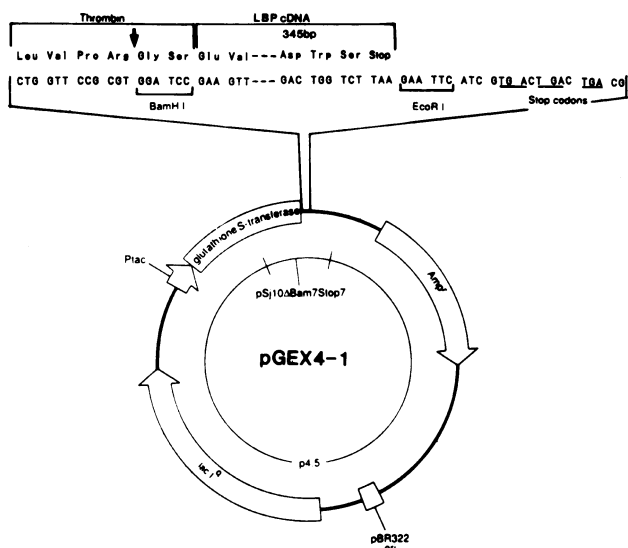


Fig. 1. Structure of the expression vector pGEX4-1. Plasmid pGEX4-1 consists of the 3'-terminal fragment of the 37LBP cDNA (345 bp) and the expression vector pGEX2T that is designed for inducible, high-level expression of the gene to be fused with GST. The inserted 3'-terminal cDNA fragment was obtained from the full-length cDNA of the 37LBP by the method of polymerase chain reaction and subcloned into pGEX2T using the *Bam*HI/*Eco*RI sites located immediately proximate to the *GST* gene. The thrombin recognition sequence is provided for cleavage of the GST domain from the fusion protein. The recombinant 13-kDa fragment of the 37LBP is, however, extremely unstable after separation by thrombin digestion. Thus, the whole fusion protein was used for immunization in this study. The molecular size of the fusion protein was expected to be around 40 kDa; a 13-kDa fragment of the 37LBP plus GST (26 kDa).

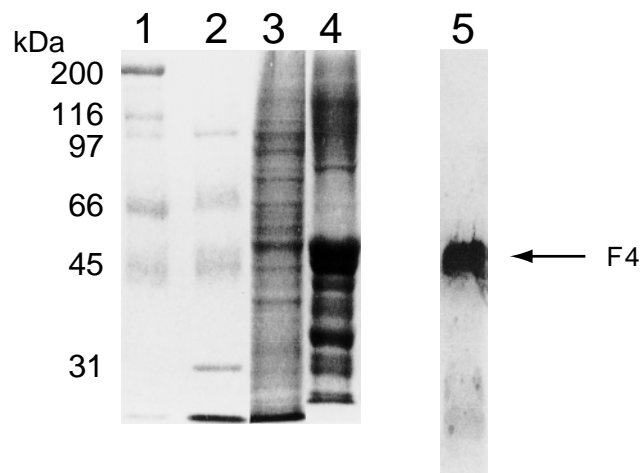


Fig. 2. Expression and purification of the fusion protein in JM109 transformed with pGEX4-1. Cell lysate of JM109 transformed with pGEX4-1 or affinity-purified fusion protein was subjected to 15% SDS-polyacrylamide gel under reducing conditions. The recombinant fusion protein composed of the 13-kDa fragment of the 37LBP and 26-kDa GST was stained by Coomassie Brilliant Blue as a band of 40 kDa (lane 4). Affinity-purified fusion protein F4 was immunodetected with P1 (lane 5). Lane 1, high-molecular-weight markers (BIORAD, Richmond, Ca); lane 2, low-molecular-weight markers (BIORAD); lane 3, cell lysate of JM109 without transformation; lane 4, cell lysate of JM109 transformed with pGEX4-1; lane 5, western blot of the affinity-purified fusion protein F4 (10 µg).

was subjected to 7.5% SDS-PAGE by the method of Laemmli.¹⁸⁾ Western blots were carried out with P4G or anti-GST antibody in the ordinary manner with or without 2-mercaptoethanol. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Seikagaku Kogyo, Tokyo) was used as the second antibody. Coloring reaction was carried out using the BCIP/NBT phosphatase substrate system (KPL, Gaithersburg, MD).

Cell attachment to immobilized laminin Human fibrosarcoma HT1080 cells (1×10^6 /ml) were incubated in a medium (10% FCS/E-MEM) supplemented with various concentrations (1 to 10^{-3} dilution) of P4G (original concentration; 1.1 mg/ml) for 60 min at 37°C under 5% CO₂. Anti-human albumin antibody (IgG fraction, Seikagaku Kogyo) and anti-GST antibody were used for a control study. After having been washed twice with PBS, cells suspended in E-MEM were plated into EHS laminin-coated 24-well culture plates (Falcon, Lincoln Park, NJ) at the density of 1×10^6 /ml and were cultured for 20 min. After removal of detached cells, laminin-attached cells were further cultured for 4 h in conditioned medium containing 10% (v/v) AlamarBlue (Alamar Biosciences, Sacramento, CA), a fluorometric/colorimetric indicator of cellular REDOX.¹⁹⁾ Absorbance of the culture supernate was monitored at 570 nm and 600 nm according to the method of the supplier.

In vitro assessment of cytotoxicity by P4G antibody HT1080 cells (4×10^3) were seeded in 96-well plates (Falcon), and incubated with P4G (100 μ g/ml and 10 μ g/ml), normal rabbit IgG (NRS, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 100 μ g/ml and 10 μ g/ml) or medium only for up to 4 days. Cell counts and viability were assessed by trypan blue assay.

Assay of in vivo efficacy of P4G in nude mice The inhibitory effect of the P4G on lung colonization was assessed as previously described.²⁰⁾ HT1080 human fibrosarcoma cells (1×10^6) in 0.1 ml of PBS containing 12.5 μ g of the P4G or NRS were incubated at 37°C for 1 h. Cell viability was evaluated with trypan blue assay, then the cells were injected into the cervical veins of mice (male 6-week-old Balb/c *nu/nu* mice). Four mice were used for each group. Twenty-eight days later, the mice were killed under anesthesia with tribromoethanol. Lungs were removed, and the colonies were visualized by injecting India ink into the trachea as previously described.²¹⁾ Colonies were counted over the whole lung surface.

RESULTS

Synthesis of the GST/37LBP fusion protein As shown in Fig. 2, *E. coli* JM109 transformed by the plasmid pGEX4-1 expressed the recombinant fusion protein (13-kDa fragment of 37LBP fused with 26-kDa GST) (lane 4). Western blot analysis indicated that the affinity-puri-

fied fusion protein F4 reacted with the antibody P1 raised against a synthetic peptide corresponding to a partial sequence of 67LR (lane 5).

Antibody against F4 Polyclonal antibody P4G was generated against purified F4. ELISA showed significant reactivity at up to 10^{-4} dilution of P4G. Western blot analysis using cell extract of human fibrosarcoma HT1080 was performed with P4G or anti-GST antibody (Fig. 3). Under both nonreducing (lane 1) and reducing (lane 2) conditions, one immunoreactive band corresponding to 67LR was detected with P4G. No immunoreactive band was indicated with anti-GST antibody (lanes 3 and 4).

Inhibition of cell attachment to immobilized laminin To investigate the function of P4G, we added P4G to HT1080 to block cell attachment to the laminin-coating plate. As a result, P4G prevented HT1080 cells from binding to immobilized laminin in a dose-dependent manner, while neither anti-albumin antibody nor anti-GST antibody interfered with cell-laminin interaction (Fig. 4). This result indicates that the 37LBP is actually associated with a cell surface protein that functions as a laminin receptor.

Estimation of in vitro cytotoxicity of P4G On the basis of the data indicating that the P4G antibody blocked HT1080 attachment to a laminin substrate (Fig. 4), we

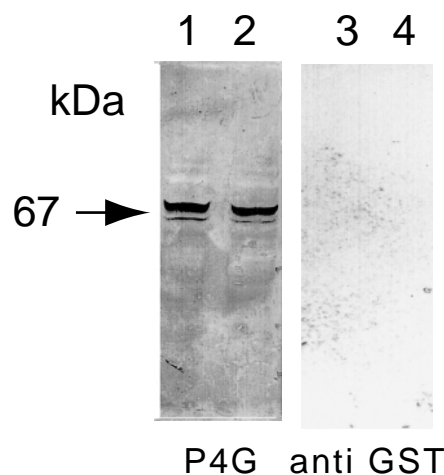


Fig. 3. SDS-PAGE and immunoblot analysis of the cell lysate of HT1080. Cell lysate of HT1080 (1×10^5) was subjected to 7.5% SDS-PAGE. Bands were transferred to a nitrocellulose membrane, and immunodetection was performed with P4G antibody ($\times 20$) or anti-GST antibody ($\times 1000$). Proteins were visualized by alkaline phosphatase staining. A band of around 70 kDa corresponding to 67LR was immunodetected with P4G raised against F4 under both reducing and nonreducing conditions (lanes 1 and 2). On the other hand, no immunoreactive band was seen with the anti-GST antibody (lanes 3 and 4). Lanes 1 and 3: nonreducing conditions. Lanes 2 and 4: reducing conditions.

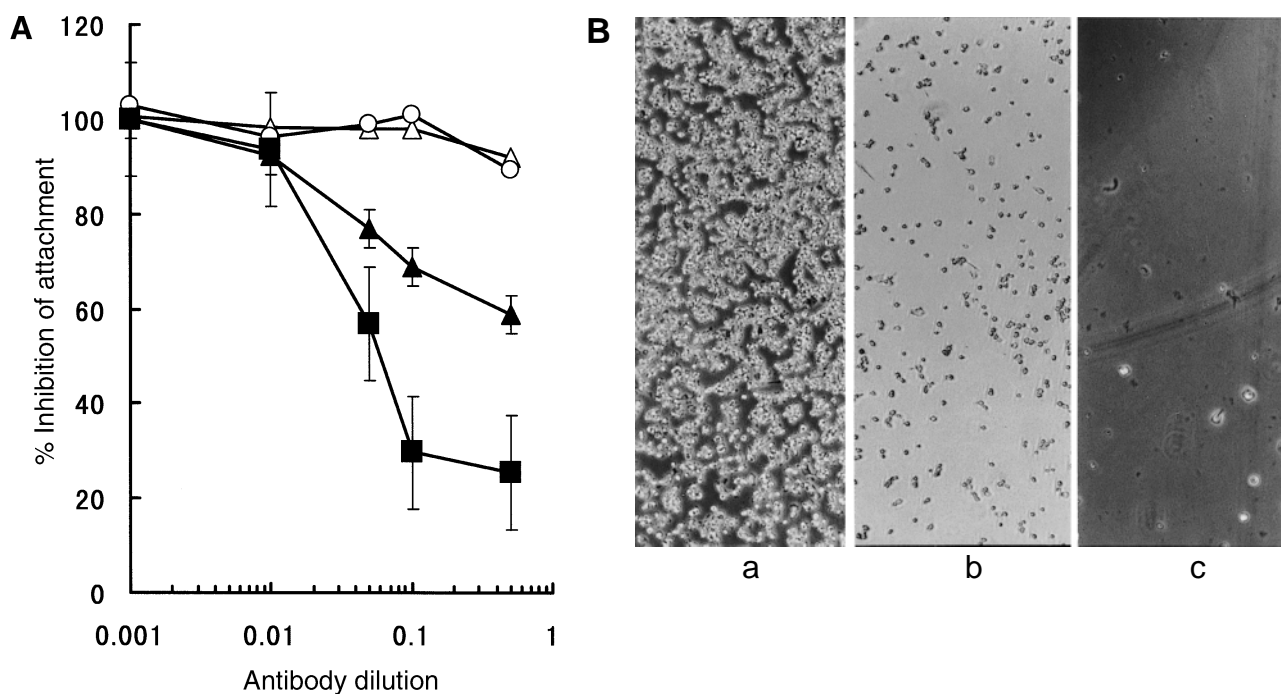


Fig. 4. Inhibition of cell attachment of HT1080 to immobilized laminin in the presence of P4G. A: After incubation with serially diluted antibodies (original concentration; 1.1 mg/ml) for 60 min at 37°C, HT1080 cells were resuspended in E-MEM, plated and attached to a laminin-coating culture dish for 20 min at 37°C. After removal of detached cells, the inhibition of cell attachment was monitored by addition of AlamarBlue (a REDOX indicator) to the culture medium at a concentration of 10% (v/v). The experiment was carried out in triplicate and the percentage of attachment is shown as the mean±standard deviation. Inhibition of cell attachment to laminin was observed in proportion to the P4G (■) and P1 (▲) concentrations in the culture medium. On the other hand, neither anti-human albumin antibody (△) nor anti-GST antibody (○) inhibited cell-binding to laminin. B: Phase contrast microscopy of HT1080 cells on a laminin-coated plate (×100). Conditions of culture were the same as given in the legend to Fig. 4A. a, no P4G added; b, 20 μg/ml; c, 200 μg/ml.

examined the toxicity of P4G towards HT1080 cells. After 1 h incubation, cell viability and count were assessed by trypan blue assay, and no significant difference was demonstrated between the cells incubated with P4G and those incubated with NRS as a control (data not shown). In addition, we incubated cells with P4G for up to 4 days. There was no significant difference in proliferation rate between those treated with NRS and those with P4G (Fig. 5), confirming that P4G is not toxic to HT1080 cells.

In vivo inhibitory effect of P4G on lung colonization of tumor cells in Balb/c nu/nu mice Next, we used a model of lung metastasis to determine whether P4G inhibits the colonization of HT1080 cells in the lungs of nude mice. As shown in Fig. 6, on day 28 after tumor cells had been injected following antibody incubation, the mean number of nodules on the surface of the lungs was 3.3±5.1 in the P4G pre-treated group, compared with 58.0±8.0 in the NRS pre-treated group ($P<0.005$). These results suggest that P4G inhibits the colonization and growth of HT1080 cells in the lungs of mice.

DISCUSSION

The laminin binding protein of 37 kDa (37LBP) has been reported to be a multifunctional protein. Its amino acid sequence is highly homologous to that of the p40 ribosomal protein, which plays a part in translation.²² Other studies have demonstrated that 37LBP functions as a receptor for the sindbis virus,²³ elastin¹¹ or prion protein,²⁴ and also as a positional marker for differentiation of the fetal eye.²⁵ Although the gene for 37LBP has been cloned recently,²⁶ the functions of 37LBP are not yet fully understood.

In the present study, we found that the anti-r37LBP antibody inhibits both cell attachment to laminin *in vitro*, and blood-borne pulmonary metastasis of HT1080 cells in an animal model. The recombinant 37LBP fusion protein described here includes the sequence of peptide G,¹⁵ a high-affinity laminin-binding site in 67LR, so that P4G may selectively interfere with tumor cell attachment to the vascular endothelium via the laminin-67LR pathway. This

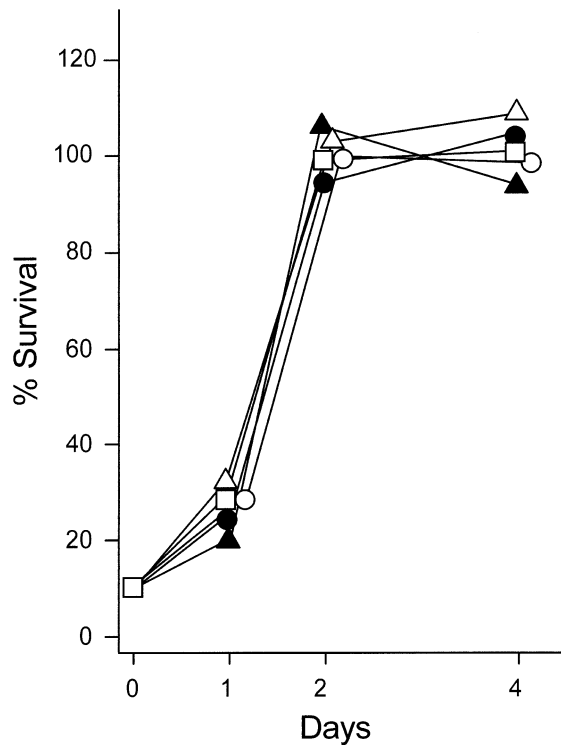


Fig. 5. *In vitro* evaluation of the cytotoxicity of anti laminin binding protein (LBP) antibody (P4G) against HT1080 cells. HT1080 cells (4×10^3) were seeded in 96-well plates (Falcon), incubated with P4G (\circ 100 $\mu\text{g}/\text{ml}$, \bullet 10 $\mu\text{g}/\text{ml}$), normal rabbit IgG (NRS; Δ 100 $\mu\text{g}/\text{ml}$, \blacktriangle 10 $\mu\text{g}/\text{ml}$) or medium only (\square) for up to 4 days. Cell count and viability were assessed by trypan blue assay. There was no significant difference in cell proliferation rate between cultures treated with NRS and with P4G.

hypothesis is supported by the fact that the antibody P1 raised against a different peptide from peptide G also inhibits cell attachment to laminin, albeit less effectively than P4G does, as shown in Fig. 4A.

Previous studies suggested that 67LR/37LBP may play a role in the attachment and migration of tumor cells *in vitro* to laminin-coated surfaces and *in vivo* during lung colonization by intravenously injected tumor cells. Thus, 67LR/37LBP may provide the basis for new anticancer therapeutic strategies. Wewer *et al.* first indicated that anti-67LR/37LBP antisera raised against a 20-mer peptide partial sequence blocked the surface interaction of melanoma cells with endogenous laminin, resulting in the inhibition of laminin-mediated cell attachment and migration.¹²⁾ Rahman *et al.*²⁷⁾ have reported that anti-67LR/37LBP antibody-coupled liposomes encapsulating an anticancer drug selectively killed breast cancer cells. Iwamoto *et al.*²⁸⁾ demonstrated that synthetic YIGSR peptide,

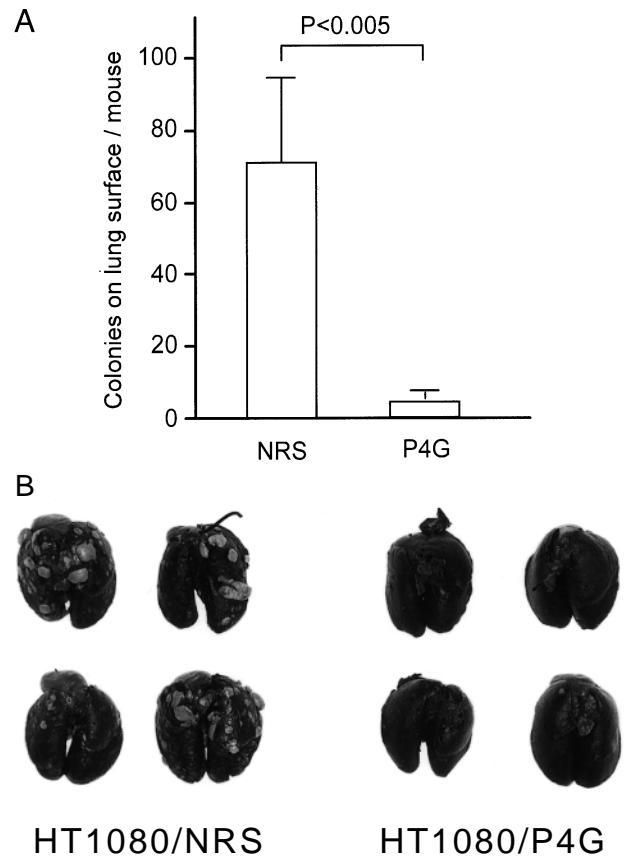


Fig. 6. Blocking effect of anti laminin binding protein (LBP) antibody (P4G) on HT1080 cell colonization in the lung. Balb/c *nu/nu* mice were given an intravenous injection of 1×10^6 HT1080 human fibrosarcoma cells which had been pre-incubated with anti LBP antibody (P4G) or with normal rabbit IgG (NRS) at 37°C for 1 h, respectively. Twenty-eight days later, the colonies on the whole lung surface were counted. A: Data are numbers of colonies on the lung surface (mean \pm standard deviation of four recipient mice). Significant suppression of pulmonary metastases was caused by the treatment with P4G ($P < 0.005$ as assessed with the unpaired two-tailed Student's *t* test). B: Lungs stained with India ink demonstrate the reduced pulmonary metastases of HT1080 cells pre-treated with P4G (right panel) compared with those of HT1080 cells pre-treated with NRS as a control (left panel).

derived from the sequence of the laminin B1 chain and reported to be a binding site for 67LR/37LBP,²⁹⁾ inhibited experimental metastasis of B16 melanoma. Recently, it has been shown that a multimeric form of the YIGSR sequence reduced angiogenesis, tumor growth, and experimental metastasis of HT1080 more effectively than a monomeric form did.²⁰⁾ The sequence of YIGSR has also been reported to induce apoptosis of HT1080 cells.³⁰⁾

We have carried out a preliminary cell attachment inhibition experiment using anti-integrin $\alpha 6$ antibody. The $\alpha 6$ antibody could also inhibit cell attachment of HT1080 (approximately 60% inhibition), indicating that the laminin receptor (belonging to the integrin family) contributes to HT1080 cell attachment to laminin as well as 37LBP. Taken together, these findings suggest that interference with laminin binding by the antibody P4G directed against the r37LBP sequence including peptide G may offer a potential strategy for prevention of cancer metastasis, as may the use of antibody against $\alpha 6$ integrin subunit.^{32, 33)}

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