Sialyl Lewis X-dependent Lung Colonization of B16 Melanoma Cells through a Selectin-like Endothelial Receptor Distinct from E- or P-Selectin1

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Abstract

Endothelial carbohydrate binding proteins, E- and P-selectins, are thought to mediate sialyl Lewis A/X-dependent hematogenous cancer metastasis. We tested this hypothesis using sialyl Lewis X-dependent B16 melanoma lung targeting and its inhibition with selectin ligand mimicry peptide, IELLQAR. In E/P-selectin doubly deficient mutant mice, sialyl Lewis X-expressing B16 melanoma cells colonized the lung, and IELLQAR inhibited this colonization. However, tumors grown in E/P-selectin-deficient mice were significantly smaller than those grown in wild-type mice. These results indicate that the IELLQAR peptide receptor expressed in the lung vasculature plays a major role in sialyl Lewis X-dependent cancer cells targeting to the lung.

Introduction

Specific carbohydrate antigens serve as useful cancer markers (1, 2), which are exemplified by the sialyl Lewis antigen in colorectal and pancreas adenocarcinomas (3, 4), and the sialyl Lewis X antigen in breast carcinoma (5), colon (4), and lung adenocarcinomas (6). Clinicopathological studies of patients with colorectal carcinoma (7) and lung carcinoma (6, 8) show a positive correlation between sialyl Lewis A or sialyl Lewis X expression and poor prognosis, i.e., shorter survival rate and metastasis. Because both sialyl Lewis A and sialyl Lewis X antigens are recognized by E-selectin expressed on the surface of endothelial cells (9), E-selectin is considered to play an important role in hematogenous metastasis (10–13). Indeed, many human adenocarcinoma cells adhere to E-selectin-expressing cells in vitro in a sialyl Lewis A- and sialyl Lewis X-dependent manner (12, 13). Whereas E-selectin is not expressed in endothelial cells in vivo unless cells are stimulated by an inflammatory cytokine such as interleukin 1β or tumor necrosis factor α, E-selectin is expressed in response to cytokines secreted by tumor cells in cancer patients (9, 10). We found previously that a selectin ligand mimicry peptide IELLQAR inhibited sialyl Lewis X-dependent B16 melanoma cell colonization of the lung in the mouse (14). In this study, we demonstrate that this melanoma colonization is dependent on the IPR3 specifically expressed in the lung vasculature but not on E- and/or P-selectins. In addition, our study suggests that E-selectin and/or P-selectin promote tumor growth once metastatic colonies are established.

Materials and Methods

Peptides and Melanoma Cell Line. Phage displaying IELLQAR peptide was described previously (14). Synthetic multivalent (eight branched) IELLQAR peptide and its biotinylated form were purchased from AnaSpec (San Jose, CA). B16-FtIII-M, which expresses a moderate level of sialyl Lewis X antigen, was described previously (14). E-Selectin and P-Selectin Doubly Deficient Mutant Mouse Line. The mutant mouse line, SelectlmSnSelptm1Hyn lacking both E-selectin and P-selectin (15), was obtained from The Jackson Laboratory (Bar Harbor, ME). Propagation of E/P-selectin doubly deficient mutant mice was carried out under the C57BL/6 background.

Biotinylated Peptide Targeting in Vivo in the Mouse. Wild-type C57BL/6 or E/P-selectin double null mutant mice were anesthetized with avertin and biotinylated eight-branched IELLQAR peptide, 100 μg in 100 μl of PBS was injected i.v. through the tail vein (14). After 5 min, the mice were perfused through the heart with 10 ml of DMEM over 20 min. Mice were sacrificed by an overdose of avertin. The internal organs, i.e., lung, liver, kidney, and brain, were isolated and fixed with 2% paraformaldehyde in PBS at 4°C for 20 h. Paraffin sections were prepared, and biotinylated IELLQAR in the tissues was detected by staining the sections with peroxidase-conjugated avidin and aminomethyl carbazole substrate (Zymed Laboratories, San Francisco, CA). Counterstaining was performed with hematoxylin.

IPR Activity Assay. Cloned phage displaying the IELLQAR peptide (14) was amplified in K91kan bacteria in 1.5 ml of Luria broth containing kanamycin (100 μg/ml) and tetracycline (20 μg/ml). Phage protein was precipitated from the culture supernatant with 5% polyethylene glycol 8000 and 0.3 M NaCl. Phage was dissolved in PBS with 50% glycerol and stored at −20°C. In parallel, lung and liver were isolated from wild-type and E/P-selectin double null mutant mice, and homogenized in 25 mM HEPES buffer (pH 7.4) in a glass dounce homogenizer. After removing nuclei and cell debris by brief centrifugation, membranes were precipitated by centrifugation at 100,000 g for 1 h and suspended in 25 mM HEPES buffer (pH 7.4). Each well of an ELISA plate (Linbro/Titertek; ICN Biomedicals, Aurora, OH) was coated with 0.2 μg of monoclonal anti-Lewis A (clone 7LE) antibody in 100 μl of freshly cultured K91kan bacteria was added to the well. K91kan bacteria transformed by phage were plated on Luria broth agar containing kanamycin (100 μg/ml) and tetracycline (20 μg/ml), cultured at 37°C for 20 h, and the numbers of transformed colonies were counted. IPR activity was detected by a reduction in colony numbers.

Results and Discussion

Lung Colonization of B16 FT-III-M Cells in E- and P-Selectin-deficient Mice. We have demonstrated previously that the parental B16 melanoma cells lacking sialyl Lewis X produced a negligible

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5 The abbreviations used are: IPR, IELLQAR peptide receptor; TBS, Tris-buffered saline; [20 mM Tris- HCl (pH 7.4) containing 0.15 M NaCl]; FITC-III, fluorescein isothiocyanate III.
number of lung tumor foci, whereas B16-FT-III-M cells expressing sialyl Lewis X produced many lung tumor foci on i.v. injection into wild-type mice (16). We also demonstrated that the lung tumor foci formation with B16-FT-III-M cells was completely inhibited when B16-FT-III-M cells were preincubated with anti-sialyl Lewis X antibody before injection (16). Thus, these studies established that the lung tumor formation with B16-FT-III-M cells is dependent on sialyl Lewis X expressed on these cells.

To determine whether sialyl Lewis X-dependent tumor cell colonization requires the expression of E-selectin or P-selectin, we injected B16-FT-III-M cells i.v. through the tail vein. Fourteen days after B16-FT-III-M cells injection, melanoma foci found in E/P-selectin-deficient lungs were smaller in sizes than those found in wild-type mice. However, the numbers of melanoma foci found in E/P-selectin-deficient mouse lungs were only slightly reduced compared with those seen in wild-type (Fig. 1A). These results indicate that E-selectin and/or P-selectin play an important role in promoting tumor growth, whereas these selectins may not play a significant role in lung targeting in this model.

We found previously that a selectin ligand mimicry peptide IELLQAR inhibits the binding of sialyl Lewis X and sialyl Lewis A oligosaccharides to E-selectin (14). We also showed that the binding of the IELLQAR peptide to selectins is calcium dependent as is the binding of sialyl Lewis X to selectins (14). These results strongly suggest that the IELLQAR peptide binds to selectins as ligand mimicry. In the same study, we also demonstrated that IELLQAR peptide inhibits B16-FT-III-M cell colonization of the wild-type mouse lung (14). In this study, we conducted similar experiments using E/P-selectin-deficient mice. Therefore, E/P-selectin-deficient mice were i.v. injected with IELLQAR peptide, followed by an injection of B16-FT-III-M cells. After 24 days, many melanoma foci were found in the E/P-selectin-deficient mouse lungs, whereas no or only a few foci were found in IELLQAR preinjected mice (Fig. 1B), the results comparable with those obtained previously in wild-type mice (14).

In a similar approach to ours, Kim et al. (17) reported that lung metastasis of sialyl Lewis X-expressing colorectal adenocarcinoma LS180 cells was drastically reduced in P-selectin-deficient mutant mice. Many colon carcinoma cells, including LS180 cells, express P-selectin glycoprotein ligand 1 and bind to P-selectin (18). In contrast, B16-FT-III-M cells do not express P-selectin glycoprotein ligand 1 and do not bind to P-selectin. LS180 cells i.v. injected into wild-type mice formed aggregates with platelets (17), whereas B16-FT-III-M cells found in the wild-type lung were mostly isolated (14, 16). Such differences in reactivity of cancer cells to P-selectin may explain the apparently different results obtained in the present study from that reported by others (17).

Absence of Inflammation and Angiogenesis in Wild-type and E/P-Selectin-deficient Mice. Histological examination of lung tissues revealed that metastatic colonizations largely occurred in the subpleural and peribronchiolar alveoli in wild-type and E/P-selectin-deficient mice (Fig. 2). Preexisting vessels from which tumor cells extravasate to the alveolar space were frequently found in the area of tumor colonization in both mice. Extrapleural invasion of the tumor cells was occasionally seen in E/P-selectin-deficient mice (Fig. 2F, see the cells between arrowheads), and such an extrapleural invasion was also observed in wild-type mice (not shown). Although both E- and P-selectin promote inflammation, and E-selectin induces angiogenesis (19), wild-type and E/P-selectin-deficient mutant mice exhibited no significant differences in inflammation or angiogenesis (Fig. 2). In E/P-selectin-deficient mice, focal lymphocytic aggregations occurred in the lung (shown by arrowheads in Fig. 2B), which may be related to the elevation of neutrophils, monocytes, and eosinophils in E/P-selectin-deficient mice (15). Lack of inflammation suggests that inflammatory cytokines may not be a cause for promoting tumor growth in wild-type mice. These observations suggest a yet undisclosed role of E-selectin and/or P-selectin on tumor cell growth in vivo.

IELLQAR Peptide Targets the Restricted Lung Vasculature in the Mouse. Our published study (14) and results shown in Fig. 1B suggest that IELLQAR peptide inhibited adhesion of B16-FT-III-M cells to the lung endothelial cells in both wild-type and E/P-selectin-deficient mutant mice. To visualize in vivo targeting of IELLQAR peptide, we injected biotinylated IELLQAR peptide and examined the distribution of this peptide in mouse organs by histochemistry. Wild-type mice injected with biotinylated IELLQAR peptide exhibited strong peroxidase-avidin staining in the lung (Fig. 3A), whereas injection with control peptide, made entirely with d-amino acids, produced no signal in the lung (Fig. 3B). Positive signals were restricted to small blood vessels (Fig. 3, A and C). No staining was observed in the large blood vessels (data not shown). Staining was visible at the luminal surface of endothelial cells as well as in the cytoplasm of endothelial cells (Fig. 3C), suggesting that the peptide was internalized in the endothelial cells after binding on the cell surface. No signals were apparent in the liver (Fig. 3E), spleen (Fig. 3F), kidney (data not shown), or brain (data not shown) of the same animal injected with this peptide.

Histochemistry of the E/P-selectin-deficient mutant mouse injected with biotinylated IELLQAR peptide revealed a pattern (Fig. 3D) similar to that seen in wild-type mice (Fig. 3A). These results strongly suggest that IELLQAR peptide binds to lung endothelial cells through a yet unidentified endothelial cell receptor or IPR, which is distinct from E-selectin or P-selectin.
IPR Activity Determined by in Vitro Assays. We developed a semiquantitative IPR activity assay using phage clones displaying IELLQAR peptide (see “Materials and Methods”). Phage (1 × 10^3 colony-forming units) and mouse lung membranes (protein 0.1~100 µg) were incubated together, and this mixture was added to an ELISA plate coated with monoclonal anti-Lewis A antibody (clone 7LE), originally used to screen the peptide-displaying phage library (14). Wild-type mouse lung membranes inhibited phage binding to the 7LE antibody in a dose-dependent manner, whereas membranes prepared from wild-type mouse liver showed no inhibitory activity (Fig. 4A). Membranes prepared from the lungs of wild-type mouse and E/P-selectin-deficient mutant mouse showed no quantitative differences in this assay (Fig. 4B). This result is consistent with the histochemistry, which did not reveal significant differences in IELLQAR bindings to the lung vasculatures between wild-type and E/P-selectin-deficient mutant mice (Fig. 3, A and D). This suggests that IELLQAR peptide binds to IPR but not to E- or P-selectins in wild-type mice.

Because E-selectin and P-selectin are barely expressed in unstimulated endothelial cells (9, 11, 15), it is likely that in our previous study of wild-type mice (14, 16), IPR rather than E- or P-selectins mediated sialyl Lewis X-dependent tumor cell colonization. This hypothesis is supported by the current results, which show that the numbers of melanoma foci formed in E/P-selectin-deficient lungs are not significantly lower than those seen in wild-type mice and that preinjected IELLQAR peptide almost completely suppresses B16-FT-III-M cell colonization in E/P-selectin-deficient mutant mice (Fig. 3). IPR may be a carbohydrate binding protein, which shares ligand specificity with selectins, as IPR binds to the selectin ligand mimic peptide IELLQAR.
Previous studies suggest strongly that a backbone structure carrying sialyl Lewis A/X antigens correlates with the prognosis for human cancer patients (2, 4, 7, 8, 20). Thus, highly metastatic colorectal carcinomas present the sialyl Lewis X structure on an extended polylactosamine core structure, whereas sialyl Lewis X is attached to a short core in poorly metastatic cells (20). The expression of core 2 β1,6-N-acetylglucosaminyltransferase, which extends polylactosaminyl O-glycan, is positively correlated with the progression of colon carcinoma and lung carcinoma (2, 7, 8). It is possible that a carbohydrate binding protein, such as IPR, prefers a sialyl Lewis X attached to the polylactosamine core structure. Taken together, the present study suggests that IPR is largely responsible for carbohydrate-dependent metastasis of cancer cells to the lung. Identification of the IPR will be required to additionally define the hematogenous route of carbohydrate-dependent cancer cell metastasis.

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