Therapeutic Antibodies to Human L1CAM: Functional Characterization and Application in a Mouse Model for Ovarian Carcinoma

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Abstract

Recent work has identified L1CAM (CD171) as a novel marker for human carcinoma progression. Functionally, L1CAM promotes tumor cell invasion and motility, augments tumor growth in nude mice, and facilitates experimental tumor metastasis. These functional features qualify L1 as an interesting target molecule for tumor therapy. Here, we generated a series of novel monoclonal antibodies (mAbs) to the L1CAM ectodomain that were characterized by biochemical and functional means. All novel mAbs reacted specifically with L1CAM and not with the closely related molecule CHL1, whereas antibodies to the COOH terminal part of L1CAM (mAb2C2, mAb745H7, pcytL1) showed cross-reactivity. Among the novel mAbs, L1-9.3 was selected and its therapeutic potential was analyzed in various isotype variants in a model of SKOV3ip cells growing i.p. in CD1 nude mice. Only therapy with the IgG2a variant efficiently prolonged survival and reduced tumor burden. This was accompanied by an increased infiltration of F4/80-positive monocytic cells. Clodronate pre-treatment of tumor-bearing animals led to the depletion of monocytes and abolished the therapeutic effect of L1-9.3/IgG2a. Expression profiling of tumor-derived mRNA revealed that L1-9.3/IgG2a therapy induced altered expression of cellular genes associated with apoptosis and tumor growth. Our results establish that anti-L1 mAb therapy acts via immunologic and nonimmunologic effector mechanism to block tumor growth. The novel antibodies to L1CAM could become helpful tools for the therapy of L1-positive human carcinomas.

Cancer Res; 70(6); 2504–15. ©2010 AACR.

Introduction

Monoclonal antibody-based therapy of non–Hodgkin’s lymphoma and a variety of solid tumors has proved to be rather successful (1). During the past two decades molecular biology has provided means to create chimeric, humanized, or fully human antibodies for the treatment of major malignant diseases (2). To date, nine antibodies are approved as cancer therapeutics and are on the market. They comprise unmodified antibodies as well as conjugates with toxins or radiouclides (3). The new reagents are able to interact with human effector molecules and thereby synergize with or even substitute for conventional chemotherapeutic regimens (4, 5). In breast and colorectal cancer, for example, two prominent members of the ErbB receptor family of tyrosine kinases are used as target molecules for antibody therapy (6). Trastuzumab (Herceptin), a humanized IgG1 antibody, binds to the HER2/neu antigen that is overexpressed in 25–30% of patients with mammary adenocarcinoma. It is approved for the treatment of HER2/neu-positive metastatic breast cancer and in addition to adjuvant chemotherapy (7, 8). Cetuximab (Erbitux), a chimeric IgG1 antibody, recognizes the epidermal growth factor receptor and is used for the treatment of advanced colorectal as well as head and neck cancer (9). Panitumomab (Vectibix) is a fully human IgG2 antibody derived from a transchromosomal mouse and was recently approved for metastatic colorectal cancer (10).

Two important aspects of antibody therapy have to be considered. Firstly, nearly all tumor antigens recognized by antibodies represent differentiation antigens that are over-expressed on certain tumor cells but are also present, although at lower density, on normal tissues. The binding to non-neoplastic tissue can cause side effects that, however, usually are much milder than those associated with chemotherapy (11). Secondly, there is accumulating evidence for the importance of the antibody Fc-part in terms of activation of immunologic effector functions. Besides direct antiproliferative
effects of therapeutic antibodies like interference with growth factors or their receptor and the induction of apoptosis, antibody-dependent cellular cytolysis (ADCC) has turned out to be of paramount importance for tumor cell destruction (12, 13).

LICAM (L1 cell adhesion molecule) is a 200- to 220-kDa transmembrane glycoprotein of the immunoglobulin (Ig) superfamily that initially was found to play an important role during the development of the nervous system (14). Subsequently, L1 was also found in human tumors and its expression was linked to poor prognosis (for review, see ref. 15). Meanwhile, the interference with L1 expression by genetic manipulation was found to be growth inhibitory in vitro (16, 17). Importantly, antibodies to L1 were shown to have therapeutic potential and can reduce cell proliferation in vitro and in vivo growth of SKOV3ip human ovarian carcinoma cells in a xenograft mouse model (18–20). Thus, a systematic evaluation of anti-L1 monoclonal antibodies (mAb) as potential tools for cancer treatment and a better understanding of the mode of action of anti-L1 antibodies are warranted.

Ovarian cancer is the leading cause of death from gynecologic malignancies in the United States and Europe. It represents the fifth most common cause of cancer mortality in women (21). Most of the patients already present with peritoneal metastases at diagnosis and suffer from recurrent malignant ascites formation causing severe symptoms. Because only few palliative strategies are available at advanced stages of ovarian cancer, new therapeutic options, as antibody therapy, are urgently needed. Here we describe the generation and preclinical evaluation in vitro and in vivo of a series of novel anti-L1 mAbs with different epitope specificity, avidity, and IgG isotype for the treatment of ovarian adenocarcinoma.

Materials and Methods

Cells lines. The ovarian tumor cell lines OVMz and SKOV3ip and the stably transfected cell lines CH0-hL1 were described before (22, 23). J558-L1 cells were obtained from Vance Lemmon (University of Miami). All cell lines were cultivated in DMEM supplemented with 10% FCS at 37°C, 5% CO2, and 95% humidity.

Antibodies and chemicals. Antibodies to the ectodomain of human L1 (L1-11A, L1-14.10, L1-9.3) were described earlier (20, 22). Further mAbs against L1 were obtained after immunization of mice with human L1-Fc protein comprising the ectodomain of L1 or using SKOV3ip cells for immunization. All mAbs were produced and purified by In vitro Biotech Services. mAb huL1-9.3/F was produced by Celltrion. The L1-mAb 5G3 was obtained from Pharmingen (BD Biosciences). mAb 745H7 and pcytL1 (22) to the COOH terminal part of L1 were a gift from V. Lemmon (Miami, Florida). mAb 2C2 was obtained from Abcam. The antibody to human CHL1 was from R&D Systems. mAbs to mouse leukocyte cell surface antigens were FITC-F4/80 (Serotec) and APC-Gr1 (BD Pharmingen). Clodronate-loaded liposomes or PBS-control liposomes were a gift from clodronateliposomes.org (Dr. Nico van Rooijen, Amsterdam, the Netherlands; ref. 24). CI2MDP (clodronate) was a gift from Roche.

Immunohistochemistry. This method has been described in previous publications (25, 26).

ELISA. This method has been described before (27).

Biochemical analysis. SDS-PAGE and Western blot analysis using enhanced chemiluminescence detection have been described previously (28).

ADCC assay. SKOV3ip as target cells were 51Cr-labeled and preincubated with respective mAb concentrations for 30 min at room temperature. Peripheral blood mononuclear cells (PBMC) were enriched by Ficoll-Hypaque gradient centrifugation and used as effector cells in a standard 4 h Cr-release assay. Percentage cytotoxicity was calculated as (test cpm – spontaneous cpm) / (total cpm – spontaneous cpm) × 100. Spontaneous release was determined from supernatant of labeled target cells incubated in assay medium alone. Total release was determined from labeled cells lysed with 10% Triton X-100. Data are expressed as the mean of triplicate wells.

Flow cytometry. The staining of cells with mAbs and PE-conjugated secondary antibodies has been described (22). Cells were analyzed on a FACS Canto II flow cytometer (Becton Dickinson) using FlowJo 6.4 software (TreeStar).

Tumor model and therapy. For survival experiments pathogen-free, female athymic CD1 nu/nu mice (Charles River) were inoculated with 5 × 106 human SKOV3ip cells into the peritoneal cavity at day 0. Therapeutic L1 mAbs were diluted in sterile PBS to 1 mg/mL. After 2 d, random groups of animals were formed and treated thrice weekly with 200 μL antibody (10 mg/kg bodyweight per application or 200 μL vehicle, PBS). Tumor-bearing animals were kept until ascites was visible or a weight loss of >15% combined with behavioral signs of distress was observed. At this end point, animals were euthanized.

For tumor mass analysis CD1 nu/nu mice or BALB/c nu/nu mice (Charles River) were inoculated i.p. with 6 × 106 luciferase-tagged SKOV3ip cells. mAb therapy proceeded as described above. After the respective time of treatment, mice were sacrificed and weighed and tumor mass was determined. For macrophage depletion experiments, mice were injected i.p. with clodronate 2 d before tumor inoculation followed by repeated clodronate injection every 6 d.

In vivo images were acquired with the IVIS charge-coupled device camera system 100 (Caliper Life Science) and analyzed with the LivingImage 2.11 software package (Caliper Life Science). Mice were anesthetized using 2.5% to 3.5% isoflurane and i.p. injected with Luciferin solution (30 mg/mL, in PBS) or 3 mg/mouse. Light emission of the Luciferin reaction through luciferase activity was detected as indicator for the tumor growth in vivo.

Tumor single-cell suspension. For preparation of single-cell suspensions, fresh tumor tissue samples were digested for 15 min with DNase I (Applichem Darmstadt) and collagenase IV (PanBiotech GmbH) in PBS/3% FCS at 37°C. Pelleted cells were suspended in PBS/3% FCS for staining.

Statistical analysis. For the analysis of statistical significance the Student’s t test was used. For survival analysis log-rank (Mantel-Cox) test was performed using GraphPad Prism software (V 5.01).
Results

Production and characterization of novel anti-L1 mAbs.
We produced mouse mAb to the ectodomain of human L1 by using recombinant L1-Fc or SKOV3ip cells as immunogen. Selected mAbs were characterized by fluorescence-activated cell sorting (FACS) analysis on L1-positive ovarian carcinoma cells (OVmz, SKOV3ip) and stably transduced CHO-L1 cells. The novel mAbs were specific for L1 as they reacted with L1-expressing cells but not with untransfected Chinese hamster ovary (CHO) cells (Fig. 1A). When tested by Western blot analysis on cell lysate, the mAbs reacted with the full-length 220-kDa form of L1 (Fig. 1B). There were some differences noted in the signal intensity between transfected CHO-L1 cells and endogenously L1-expressing carcinoma lines. This was mostly apparent for mAb L1-38.12 (see below).

Several mAbs also reacted with paraffin-embedded ovarian carcinoma sections in immunohistochemical staining. In addition to the previously described mAbs L1-14.10 and L1-11A (29), the mAbs L1-9.3, L1-35.9, and L1-OV52.24 (not shown) worked well in immunohistochemistry (Fig. 1C). Interestingly, L1-38.12 did not stain ovarian carcinoma sections but worked well in immunohistochemistry (Fig. 1C). Interestingly, also L1-38.12 did not stain ovarian carcinoma sections but was positive on peripheral nerves that served as internal control (Fig. 1C). This observation suggested that L1-38.12 recognized preferentially the neural isoform of L1 in the signal intensity between transfected CHO-L1 cells and endogenously L1-expressing carcinoma lines. This was most apparent for mAb L1-38.12 (see below).

Novel anti-L1 mAbs react with different epitopes. To determine the epitope specificity, we constructed a series of L1-Fc proteins carrying distinct Ig domains (Supplementary Fig. S1A and B). For fine mapping we also applied recombinant V5-tagged L1 fragments that were described recently (30). The recombinant proteins were used in ELISA or in Western blot analysis for epitope mapping. The mAbs L1-9.3 (Supplementary Fig. S1C), L1-14.10, L1-OV5366.1, and L1-OV 543.18 clearly reacted with a fusion protein consisting of the first Ig domain (L1-Ig-L1-Fc; Fig. 1C). Interestingly, also L1-38.12 recognized preferentially the neural isoform of L1 in transfected CHO-L1 cells that was, however, not abundant in tumors and tumor cell lines.

Novel anti-L1 mAbs do not cross-react with human CHL1.
CHL1 (close homologue to L1) is a neural cell recognition molecule and a member of the L1 gene family. Human CHL1 is located on chromosome 3p26.1 in contrast to L1 being localized on the X chromosome. To test whether the novel anti-L1 mAbs cross-reacted with CHL1, we produced a Fc-fusion protein containing the ectodomain of human CHL1 (CHL1-Fc). When analyzed by ELISA, none of the novel mAbs to L1 showed cross-reactivity with CHL1-Fc (Fig. 2A). In contrast, the Fc protein was readily detected by an antibody to the NH2 terminus of CHL1 (Fig. 2A).

In contrast to the ectodomain, the cytoplasmic parts of L1 and CHL1 have much higher homology and there is sequence identity in some parts (Fig. 2B). To extend our analysis, we tested whether mAbs reactive with the cytoplasmic tail of L1 (2C2 and 745H7, pcytL1) might cross-react with CHL1. Indeed, all antibodies reacted with glutathione S-transferase (GST)-fusion proteins generated from the cytoplasmic portion of L1 and CHL1 in ELISA (Fig 2C) and in Western blot (not shown).

The epitope for 745H7 was mapped by the sequence SEARPMKDETTGEY in the L1 cytoplasmic part (33). We investigated whether 2C2 could bind to the same region. Indeed, both mAbs reacted with the immobilized peptide in ELISA (Fig. 2D). We concluded that the novel mAbs to the cytoplasmic domain of L1 did not cross-react with CHL1. In contrast, the established mAbs 2C2, 745H7, and pcytL1 to the cytoplasmic part of L1 are cross-reactive with human CHL1.

Inhibition of L1 homophilic binding by novel mAbs. We investigated whether the novel anti-L1 mAbs could block L1-L1 homophilic binding using a cellular binding assay with the established L1 mAb 5G3 (31) and CHL1 as substrate (32). To study inhibition, the cells were preincubated with the respective purified mAb (10 μg/mL) and allowed to bind to the substrate. The mAbs to the first Ig domain (L1-9.3 and L1-14.10) could inhibit homophilic binding, whereas other mAbs were inactive (Supplementary Fig. S3; data not shown). Similar blocking results were reported before for the L1 mAb 5G3, which also binds to the first Ig domain (32).

Analysis of selected mAb in the SKOV3ip ovarian carcinoma model on survival. Our previous work has shown that mAbs to L1 are effective in preventing tumor growth in xenotransplanted mice (18). In this therapy model, LacZ-labeled SKOV3ip ovarian carcinoma cells were implanted i.p. into nude mice followed by multiple injections of mAb over 28 days. Therapy with anti-L1 mAb in contrast to isotype controls leads to substantially reduced abdominal tumor burden (18). We examined whether mice receiving therapy would also survive longer. On the basis of highest affinity and antibody isotype, we selected the mAbs L1-9.3, L1-14.10, and L1-11A (18) for comparison. Surprisingly, mice treated with L1-9.3 or L1-11A showed only little prolonged survival, which was not statistically significant (Fig. 3A). In contrast, L1-14.10 treatment resulted in a strong and significant survival benefit (*P < 0.001; Fig. 3A).

We reasoned that the different efficacy might be due to the isotype of the therapeutic mAb. To analyze this question, we performed isotype switching of L1-9.3 (IgG1) using cell sorter technology. By enrichment for rare hybridoma variants that have undergone rearrangement of the heavy chain constant regions, we were able to select hybridoma sublines secreting L1-9.3 as IgG1, IgG2a, or IgG2b (Fig. 3B). To test whether the isotype selection procedure had altered the ability to bind to L1 at the cell surface, we carried out cytofluorographic analysis of SKOV3ip cells. The fluorescent staining intensity was very similar for all L1-9.3 isotype variants (Fig. 3C). Using isotype-specific secondary antibodies, the correct isotype of each variant was confirmed (Fig. 3C).
Figure 1. Characterization of novel mAb to L1. A, cytofluorographic analysis of mAb binding to cell lines expressing endogenous L1 (SKOV3ip, OVMz) or after cDNA transfection (CHO-L1). Untransfected CHO cells (CHO-wt) served as control. B, Western blot analysis of cell lysate from the indicated cells. C, immunohistochemical staining of human ovarian carcinoma tissues with anti-L1 mAbs. D, analysis of binding to the first Ig domain of L1. The purified Fc protein was coated, and mAb binding was analyzed by ELISA.
To test the effect of isotype switching on therapy efficacy, we performed survival experiments with all isotype variants and L1-OV198.5 was included as control. As shown in Fig. 3D, both IgG2a mAbs showed a significant prolongation of survival ($P < 0.05$). However, the other isotype forms of L1-9.3, i.e., the IgG1 and IgG2b, failed to be effective.

**Isotype switch variant IgG2a efficiently inhibits tumor growth in vivo.** The L1-9.3/2a antibody not only extended survival rate of mice but also reduced tumor growth. Using luciferase-tagged SKOV3ip cells, we found a significant reduced tumor load in L1-9.3/IgG2a–treated mice compared with the isotype control–treated group (not shown). Single-cell suspensions were prepared to determine F4/80-positive monocytic cells at the tumor site (Fig. 5A). Indeed, F4/80-positive cells were increased from 2% to $\sim 6\%$ in L1-9.3–treated mice ($P < 0.05$). In contrast, there were no differences observed in the Gr-1–positive granulocyte compartment. Immunohistochemical staining of tumors and ascites revealed these findings. Tumors from L1-9.3/IgG2a–treated mice showed increased infiltration of leukocytes (Fig. 5B, a–d). Furthermore, these tissues revealed the presence of necrosis sites and increased sites of vascularization. Paraffin-embedded ascites from antibody-treated mice contained only a few tumor cells but a high degree of infiltration with immune cells. By contrast, ascites from control mice showed many clumps of tumor cells and just a few inflammatory cells (Fig. 5B, e and f).
Figure 3. Characterization of L1-9.3 isotype switch variants. A, survival experiment using the indicated mAbs. Only L1-OV198.5–treated mice showed significant survival benefit compared with the control. B and C, screening and verification of isotype switch variants by ELISA. L1-9.3 hybridoma cells were FACS sorted to isolate isotype variants. ELISA assay (B) and flow cytometry (C) were used to verify isotype switch variants. D, survival experiment using isotype variants of mAb L1-9.3. Mice treated with the IgG2a variant showed significantly extended survival compared with mice injected with other variants.
To show that the monocytic infiltrate was of therapeutic relevance, we depleted monocytes by clodronate liposomes (24). As expected, CD1 mice treated with clodronate liposomes showed a substantial reduction of F4/80-positive cells both in the spleen, in the tumor (Fig. 5C), and in the blood (not shown). To maintain the depletion status, clodronate liposomes were applied every 6 days. Indeed, whereas in control liposome-treated mice the expected therapy effect was observed, this effect vanished in mice treated with L1-9.3/IgG2a or a nonbinding IgG2a isotype control mAb. L1-9.3/IgG2a–treated mice showed a significant (P = 0.025) reduction of tumor growth after 26 d. C, the ascites volume of mice after therapy with antibody was significantly (P = 0.032) reduced. D, normal body weight (loss caused by cachexy) was maintained (P = 0.015) after mAb therapy.

**Antibody treatment leads to altered gene expression.** To examine the influence of antibody therapy on gene expression, we performed an expression profile analysis. Two tumors of each group of anti-L1 mAb–treated and control mice were homogenized, and mRNA was isolated for DNA Chip analysis. Out of 48,803 human transcripts, we could identify 147 genes (72 upregulated and 75 downregulated) from which 116 were >1.5 times regulated (selected through greater than thrice SD; see Supplementary Table S1). These genes were analyzed for hierarchical clustering and visualized by heat map (Supplementary Fig. S4A). In addition, we analyzed the 147 regulated genes by DAVID (database for annotation, visualization, and knowledge base) to identify potential biological pathways and functional groups.
Figure 5. L1-9.3/IgG2a treatment results in infiltration of inflammatory cells. Mice were treated with anti-L1/IgG2a mAbs (L1-OV198.5 or L1-9.3/IgG2a) or a nonbinding IgG2a isotype control. Mice were sacrificed after 33 d, and tumor weights were determined. Therapy resulted in a reduced tumor mass (OV198.5, $P = 0.022$; L1-9.3/IgG2a, $P = 0.005$). A, cytofluorometric analysis showed a significant (OV198.5, $P = 0.041$; L1-9.3/IgG2a, $P = 0.036$) increase of infiltrated F4/80-positive immune cells at the tumor site. Presented data show a representative analysis. B, H&E staining of tumor tissues of mice treated with mAb L1-9.3/IgG2a. Note the increased sites of inflammation, vascularization, and necrosis compared with the control group as indicated by open or solid arrowheads. C, depletion of F4/80-positive monocytes after treatment with clodronate, but not control liposomes. Depletion in the spleen was measured 2 d after i.p. injection. D, clodronate pretreatment of mice abolishes the therapeutic effect of L1-9.3/IgG2a treatment. Clodronate liposomes were applied every 6 d to maintain the depletion status.
integrated discovery) for functional clustering. On hundred thirty-three of 147 genes could be identified and divided into 32 clusters. Twelve cluster groups result in significant at $P < 0.05$ (Supplementary Fig. S4B). Seventeen genes could be identified for apoptosis function ($P = 2.3 \times 10^{-5}$), e.g., interleukin-6 (IL-6), DDIT, TNFSF10, APOE, BNIP3L, ANGPTL4, CXCR4, and CRYAB. Seven genes were clustered for chemotaxis ($P = 3.2 \times 10^{-4}$) and seven for blood vessel morphogenesis ($P = 5.7 \times 10^{-4}$; transglutaminase 2, IL-8, ANGPT1A, CXCR4, ApoD1, hemoxygenase 1, and thrombospondin 1). Further significant gene clusters were found for regulation of protein kinase activity, calcium ion homeostasis, cation homeostasis, and inflammatory response (Supplementary Fig. S4B). We verified the altered expression by reverse transcription–PCR (RT-PCR) using selected genes, and representative data are shown in Supplementary Fig. S4C. Collectively, these data show that mAb L1 therapy can lead to altered gene expression pattern in the targeted tumor cells.

Chimerization and humanization of L1 9.3 mAb. We produced chimerized (chiL1-9.3) and fully humanized (huL1-9.3) versions of mAb L1-9.3 containing a human IgG1 constant part. The chimerized mAb was manufactured by homologous recombination of Ig genes in hybridoma cells.5 Lüttgau and colleagues, submitted for publication.
formats showed similar binding capacity when compared with the parental mouse mAb as tested by flow cytometry (Fig. 6A) or by plasmon resonance on immobilized L1-Fc (Supplemental Fig. S2A). We compared the therapeutic efficacy of the mAbs ch19.3 and hu9.3 in the SKOV3ip model. There was a significant ($P < 0.005$) reduction of tumor size in both mAb-treated groups compared with the control group (Fig. 6B).

The ADCC function of humanized mAbs has been shown to be of paramount importance for the immunotherapy of cancer (12). We used freshly isolated human PBMC as effector cells and SKOV3ip as target cells in an ADCC assay. The fully humanized L1 mAb was produced either in CHO cells (huL1-9.3) or in F2N cells (huL1-9.3/F), a human Namalwa B-cell hybrid. Interestingly, neither ch1L1-9.3 nor huL1-9.3 showed a specific lysis. In contrast, mAb huL1-9.3/F gave good dose-dependent lysis of SKOV3ip cells (Fig. 6C) and other L1-positive target cells (data not shown).

**Discussion**

L1 qualifies as a novel, promising target molecule for antibody-based therapy, as it fulfills several criteria: (a) L1 seems to be a biologically important molecule for tumors as its expression drives cell motility, invasion, and metastatic formation and promotes growth; (b) L1 is highly expressed in cancers, such as ovarian and pancreatic carcinoma that have a bad prognosis with conventional chemotherapy and for which appropriate mAb-based therapies are not available; (c) L1 has a rather favorable tissue distribution in the adult organism, as it is expressed only in peripheral nerves and in kidney tubular cells (as detected by immunohistochemistry). Weak expression is also seen on subpopulations of leukocytes (detected only by fluorescent methods). Moreover, preliminary experiments using weekly L1 mAb application in mice (antimouse L1 mAb 555) even for prolonged time have not shown adverse side effects. This prompted us to develop novel mAbs to human L1 and to explore the therapeutic potential in a model system of ovarian carcinoma.

We show here that nine new mAbs to human L1 could be generated using conventional mAb technology in mice. Five of nine of the L1 mAbs were directed against the first Ig domain, whereas others bound to the third Ig domain (L1-14.10), to the sixth Ig domain (L1-35.9), or to the FNIII repeat domains (L1-549.20, L1-52.24). No mAbs were generated against the fourth or fifth Ig domain. This failure could be due to either low immunogenicity or low conformational accessibility of these Ig domains. Despite having good $K_d$ binding values and clear reactivity in Western blot, L1-14.10 and L1-35.9 showed week staining by FACS analysis, suggesting that at the cell surface the respective epitopes were somewhat hidden. Several of the novel anti-L1 mAbs worked also in immunohistochemistry on paraffin sections.

We also investigated the cross-reactivity of the novel anti-L1 mAb to CHL1. A recent report has shown that this molecule is overexpressed in serous ovarian carcinoma (34), although this was not analyzed at the protein level. Because L1 is expressed by serous ovarian carcinoma and is strongly stained by L1 mAb in immunohistochemistry, it was of interest to exclude any cross-reactivity with CHL1. We found that none of our novel anti-L1 mAbs cross-reacted with CHL1-Fc in ELISA. However, we found that three antibodies to L1 that bind to the intracellular part (2C2, 745H7, pcytL1) and have been used before to detect L1 in ovarian carcinoma and normal ovarian epithelium by immunohistochemistry (35) are cross-reactive with CHL1 and are therefore not specific for L1. In this context it is important to note that, in contrast to ovarian cancers, we have been unable to detect L1 in normal ovarian epithelium ($n = 4$) using our novel panel of anti-L1 mAb and standard immunohistochemical staining protocols.

An important aspect of our work was to investigate the therapeutic potential of the novel L1 mAbs in the SKOV3ip model. Despite our initial findings that anti-L1 mAbs could prevent tumor growth and have functional blocking abilities (18, 20), we were surprised that antibody therapy did not result in a robust survival benefit. The observation that L1-OV198.5 (IgG2a) was performing much better than L1-9.3 (IgG1; both mAbs bind to the first Ig domain and block homophilic binding) prompted us to consider the importance of the Fc part. To address this question, we carried isotype switching of the parental IgG1 hybridoma to other IgG variants (i.e., IgG2a, IgG2b). We observed a clear-cut improvement of therapeutic efficacy in the IgG2a format that resulted in longer survival time, significantly reduced tumor burden, and smaller ascites volume. In contrast, the IgG2b format was not better than the IgG1 version.

The superior therapeutic performance of the IgG2a mAb was not unexpected. Nimmerjahn and colleagues had shown before that distinct subclasses of IgG display substantial differences in their ability to mediate effector responses (36). In a model of melanoma cell clearance from the lungs of tumor-bearing mice with the help of an antimalanoma cell surface mAb, the IgG2a variant was much more efficient than the IgG1 or IgG2b variants of the same mAb (36). It was argued that IgG2a triggers activating Fc receptors on immune cells that shift the balance between the inhibitory and activating function of Fc receptors (13, 36). In our therapy model in CD1 nu/nu mice (deficient in mature lymphocytes), we observed an accumulation of F4/80-positive monocytes at the tumor site. The depletion of monocytes by clodronate treatment abolished the therapeutic effect. It is therefore possible that Fc receptor–positive monocytes are essential for the therapeutic effect in the SKOV3ip system. Similar conclusions were recently drawn in another mAb tumor therapy model in which the therapeutic potential of a mAb to CD40 in mice was significantly dependent on antibody-dependent phagocytosis and cytotoxicity by macrophages (37).

The homologous isotype for mouse IgG2a is human IgG1 (13). It has been pointed out that nearly all therapeutic mAbs currently in clinical use are of this isotype and that activation of the immune system is an essential part of the therapeutic success (12). In this respect, it was important to note that the humanized huL1-9.3/F was capable of supporting ADCC when tested on L1-positive tumor targets. The failure of CHO cell

$^6$ M. Fogel, unpublished results.
produced huL1-9.3 to show ADCC may be related to the known importance of mAb glycosylation for ADCC function.

Beside immune system–mediated antitumor effects, the binding of therapeutic mAb can perturb the function of the targeted receptor. The relative contribution of such nonimmunologic effector functions to the immune-mediated effects are often unclear. We observed before that mAbs to L1 can inhibit tumor cell invasion and migration and interfere with the expression of selected genes (18, 20, 38). Here we have carried out a genome-wide expression profiling of residual tumors isolated from the mice and found that mAb L1-9.3 therapy led to altered gene expression. One hundred forty-seven genes were identified that were altered by mAb L1 therapy, and these changes were confirmed by RT-PCR.

When functionally grouped, the selected genes were involved in apoptosis regulation, angiogenesis, and/or chemotaxis. For example, we noted a downregulation by anti-L1 mAb therapy of the following genes involved in apoptosis function: DDIT4 and APP. DDIT4 (REDD1) overexpression was found to promote cell proliferation and anchorage-independent growth of immortalized human ovarian epithelial cells (39). APP was found to promote androgen-dependent prostate cancer growth (40). The extent of the changes in gene expression that contribute to the therapeutic effect remains to be investigated.

We reported before that anti-L1 mAb can inhibit cell proliferation and extracellular signal-regulated kinase (erk) phosphorylation in vitro (18, 20). We have carried out similar assays with the novel anti-L1 mAb but found the results often poorly reproducible. We concluded that the inhibition of cell proliferation and effects on erk phosphorylation were not robust enough assays to assess the therapeutic potential of anti-L1 mAb.

In summary, our results suggest that anti-L1 mAb, when analyzed in the appropriate format, are powerful tools to control the tumor growth and ascites formation in the SKOV3ip model system. Our results highlight the essential role of the IgG2a isotype to obtain optimal therapeutic effects and advocate that in future preclinical testing of novel targets the mAb format should be IgG2a, as it is most similar in function to the human IgG1. Anti-L1 mAb could be used unconjugated as shown here or as radiolabeled mAb as already successfully shown before (19). The use of L1 mAb could help to improve the therapy of patients with L1-positive cancers, such as ovarian carcinoma.

Disclosure of Potential Conflicts of Interest

S. Wolterink, G. Moldenhauer, M. Fogel, I. Moebius, and P. Altevogt are patient holders for L1 mAb therapy. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Falk Nimmerjahn for helpful discussions, Tatjana Schmidt and Elvira Hallauer for excellent technical assistance, Nico van Rooijen for liposomes, Myung-Sam Cho at Celltrion for production of mAb huL1-9.3/F, and Nick Pumphrey for plasmon resonance measurements.

Grant Support

Deutsche Krebshilfe (Schwerpunktprogramm: Invasion and Migration), EU-FP6 framework program OVCAD project PE-14034 (P. Altevogt), financial support from Helmholtz Alliance Immunotherapy of Cancer (G. Moldenhauer), and Medigene, Inc. (Munich) collaborative research grant (P. Altevogt and G. Moldenhauer).

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Received 10/08/2009; revised 12/09/2009; accepted 01/07/2010; published OnlineFirst 03/09/2010.

References


Cancer Res; 70(6) March 15, 2010

Cancer Research
Correction: Online Publication Dates for Cancer Research April 15, 2010 Articles

The following articles in the April 15, 2010 issue of Cancer Research were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:


Dudka AA, Sweet SMM, Heath JK. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res 2010;70:3391–401. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3033.


Published OnlineFirst 05/11/2010.
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doi: 10.1158/0008-5472.CAN-10-1347
# Therapeutic Antibodies to Human L1CAM: Functional Characterization and Application in a Mouse Model for Ovarian Carcinoma

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*Cancer Res* 2010;70:2504-2515. Published OnlineFirst March 9, 2010.

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