# Novel monoclonal antibodies specific for CTLD-SSC and sialomucin domains of endosialin, a mural cell marker of tumor vasculature

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Abstract. Endosialin, alternatively named tumor endothelial marker or CD248, was originally discovered as an antigen selectively expressed in tumor blood vessels. Subsequent studies showed that it is confined to stromal fibroblasts and pericytes of tumor vasculature rather than to tumor endothelium. Endosialin levels are upregulated in different tumor types including those derived from the brain, colon and breast. Expression of endosialin is associated with tumor growth, progression and correlates with a pro-proliferative and promigratory phenotype. However, the function of endosialin and mechanisms of its regulation are still incompletely understood. To facilitate further study of endosialin in angiogenesis, its interaction with the potential binding partners and other aspects of endosialin function, we generated six new domainspecific anti-endosialin monoclonal antibodies. Two of them recognize the C-type lectin-like domain-Sushi/SCR/CCP and four antibodies are directed to the sialomucin domain. The antibodies are suitable for various immunodetection methods including immunoblotting and immunohistochemistry. They represent important tools for improving our understanding of endosialin regulation, biological role and contribution of its extracellular domains to the tumor phenotype.

## Introduction

Angiogenesis, the process of new vessel formation, was implicated in tumor biology already in 1940s (1). The formation of new blood capillaries from existing vessels is an important

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*Abbreviations:* TEM1, tumor endothelial marker; MAb, monoclonal antibody; GST, glutathione-S transferase

*Key words:* endosialin, monoclonal antibodies, angiogenesis, pericytes, cancer mechanism for supplying nutrients to cells that are distant from existing blood vessels (2). Angiogenesis is known to be principally driven by hypoxia in growing solid tumors and in developing embryos (3). When tumor grows to the size of about 2 mm<sup>3</sup>, cells are depleted of the nutrients and oxygen and this initiates a cascade of processes, which ultimately leads to the growth of new vessels. Under physiological conditions, blood vessels/capillaries are supported by mural cells (i.e. pericytes), which are embedded in the basement membrane and tightly interact with endothelial cells (4). The tumor vasculature differs from normal vessels and is characterized by a number of abnormalities, including irregular shape, blind ends, leakiness, and loose interaction between pericytes and endothelial cells (5). One of the features characterizing pericytes of the tumor vasculature is the expression of endosialin, also referred to as tumor endothelial marker 1 (TEM1) or CD248 (6).

Endosialin was first identified as a protein interacting with the monoclonal antibody raised against human fetal fibroblasts (7). Later, the protein TEM1 was found to be the most differentially expressed protein in human tumor tissue by SAGE (8). One year later, Christian et al (9) showed that endosialin and TEM1 is the same protein. The endosialin molecule was characterized as type I transmembrane protein of 757 amino acids encoded by an intronless gene. Its extracellular region is composed of four domains: C-type lectin-like domain (CTLD), Sushi/SCR/CCP (SSC) domain, three epidermal growth factor-like (EGF) domains, and sialomucin (MUC) domain. This domain structure makes endosialin a member of the C-type lectin-like receptor family, together with thrombomodulin and CD93 (9). Expression of endosialin in tumors is confined to capillaries, and is not found in the endothelium of larger vessels. In accord, endosialin co-localizes with the pericyte marker NG2 in breast cancer but not with the endothelial marker CD31 (10,11). The expression pattern is remarkably variable as expected for molecules participating in vascular reorganization (7). Endosialin expression correlates with the grade of brain tumors, where it was found strongly upregulated in aggressive high-grade gliomas - anaplastic astrocytoma and particularly glioblastoma multiforme (WHO grade III and IV), which are characterized by activation of the HIF pathway and massive neovascularisation (12-14). Stronger endosialin expression was similarly found in breast and colorectal tumors with nodal involvement when compared to node negative patients (15,16). Tumor cells generally lack endosialin, with the exception of soft tissue sarcomas of mesodermal origin (malignant fibrous histiocytomas, spindle-cell sarcomas, and liposarcomas) (7,17). In carcinomas, endosialin was detected in tumor capillaries and fibroblasts (17). In normal organism, endosialin is found in tissues with ongoing angiogenesis, such as embryonic tissues (11,18,19), corpus luteum, mesenchyme of the uterus, and granulation tissue during wound healing (8,17,18,20). Endosialin was shown to be dispensable in development, since endosialin-null mice develop normally, are fertile and have physiological wound healing (21). On the other hand, in vivo experiments suggested that endosialin is an active player in tumor development. Its role in angiogenesis is supported by the fact that abdominal implantation of tumor fragments in endosialin-null mice led to reduced tumor growth, invasiveness and metastasis compared to wild-type mice (21). It was proposed that the interaction between tumor cells and endosialin induces expression of other proteins, which promote tumor growth, cell migration and invasiveness. Alternatively, the expression of endosialin is required for maturation of tumor vessels (21). However, factors and pathways that control endosialin expression and tissue distribution have remained elusive. Endosialin was found to interact with components of the extracellular matrix, namely fibronectin and collagen type I and IV (22). Other studies have shown the importance of endosialin in regulating proliferation (6), tube formation on matrigel (23,24), and cell migration (23,6). Endosialin plays an essential role in securing the proliferation potential or preventing the differentiation of pericytes and is involved in the response of human pericytes to platelet-derived growth factor (PDGF-BB) (25). The cytoplasmic domain of endosialin is highly conserved and represents a potential site for cell signaling. The lack of cytoplasmic domain in transgenic mice results in reduced tumor growth with alteration in fibroblasts signaling via transforming growth factor  $\beta$  (TGF- $\beta$ ), PDGF-BB, and Notch pathway (26).

In order to further study the role of endosialin in angiogenesis, its interaction with the potential binding partners and other aspects of endosialin function, we prepared a set of monoclonal antibodies. The new monoclonal antibodies described in this paper are domain-specific and represent important reagents for the study of functional contributions of extracellular endosialin domains where each of these domains can be associated with different aspect of cell function.

## Materials and methods

Cell culture. Human cervical carcinoma cells HeLa, glioblastoma cells 42-MG-BA (27) and hybridoma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml of penicillin, 100  $\mu$ g/ml streptomycin sulphate, and 0.25  $\mu$ g/ml amphotericin B (BioWhittaker, Verviers, Belgium) in humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The same culture conditions were

used for cultivation of cells expressing deletion variants of endosialin.

Preparation and production of recombinant endosialin protein. For prokaryotic expression, the cDNA fragment of endosialin was amplified using a set of specific primers: endosialin f2-ccgaattcTGGCTGCCCTTCGGCTCTGT and endosialin r2-ccctcgagttaACCAGCCTCCCCAGGGCT (lowercase sequences represent nucleotides inserted for the cloning purposes). The endosialin fragment encoding the amino acids 192-675 was cloned into pGEX-4T-1 plasmid (GE Healthcare, Buckinghamshire, UK). The expression of GST-endosialin fusion protein was induced in BL21-Codon plus(DE3)-RIPL cells with 0.4 mM IPTG at 30°C for 3.5 h. Recombinant protein was purified using glutathione sepharose 4B medium and eluted with 30 mM GSH (reduced glutathione). The obtained proteins were utilized either as immunogen (GST-endosialin) for immunization or as antigen for MAb testing.

Generation of endosialin-specific MAbs. BALB/c mice were immunized with four doses of 50  $\mu$ g recombinant endosialin-GST protein. Fusion of the spleen cells with NS0 myeloma cells was carried according to Kontsekova *et al* (28). Hybridomas were selected in DMEM-HAT medium containing hypoxanthine, aminopterin, and thymidine. Monoclonal antibodies produced by the hybridomas were screened for the specific reactivity towards the endosialin protein by ELISA and Western blotting. Hybridoma cultures were subcloned by limiting dilution, expanded, subjected to freezing-refreezing, repeatedly tested for reactivity to endosialin, and used for the MAb production. The protocol for the monoclonal antibody production was approved by the Slovak Veterinary Administration, in accordance with the European Union (EU) regulations.

*Construction of deletion mutants*. Expression of phCMV-TEM1 plasmid was described previously (29). Deletion mutants that were used to determine the epitopes of tested MAbs were constructed from this plasmid using reverse PCR. A set of primers flanking the region to be deleted was used to amplify the plasmid, the PCR product was ligated and transformed into *E. coli*. The deletion was verified by sequencing. Following sets of primers were used: ΔCTLD-SSC (deleted aa 28-236): FW 5'-TGCCTGGGGGACTGGCTGC-3', REV 5'-GGCACGGGGCTCAGAGC-3', ΔEGF (deleted aa 235-356): FW 5'-ATGGGTGCCCAGGCTTCC-3', REV 5'-GCCAGTCCCCAGGCACAG-3', ΔMUC (deleted 359-685 aa): FW 5'-CGGTGGCTGCTGGTGGCA-3', REV 5'-CTGGGCACCCATGGCCCC-3'.

Stable transfection. HeLa cells were plated into 35 mm Petri dishes to reach approximately 70% confluence on the next day. Transfection was performed with 2  $\mu$ g of plasmid DNA using a GenePorterII reagent (Gelantis, San Diego, CA, USA) according to the manufacturer's recommendation. Two days after transfection, the cells were split into medium containing 600  $\mu$ g/ml geneticin (G418) (BioWhittaker). Outgrowing clones were transferred to multiwell plates and screened for the expression of endosialin and endosialin deletion mutants using the polyclonal antibody.

Spheroids in hanging drops. The spheroids have been preformed in 20  $\mu$ l hanging drops containing 600 cells each, during 3-4 day incubation in a humidified atmosphere on the lid of a 100 mm Petri dish. PBS (10 ml) was added into the dish to prevent drying of the drops. Resulting cell aggregates were carefully transferred to a 100 mm Petri dish with a nonadherent surface and cultivated in suspension for an additional 8-10 days with the medium exchange every third day. The spheroids were then quickly fixed in Carnoy's fluid to preserve their integrity and embedded in paraffin.

ELISA screening and isotypes determination. Screening of positive hybridomas was performed by sandwich ELISA. Microplate wells were coated overnight at 37°C with RIPA extract of endosialin-transfected HeLa cells (HeLa-TEM1) diluted 1:10 in PBS, and in parallel with HeLa-phCMV cells as a negative control and/or 10 ng per well of GST-endosialin, and GST as a control. The coated wells were, after blocking with 10% FCS in PBS, incubated with undiluted culture medium from individual hybridomas. Peroxidase-conjugated goat anti-mouse IgG (DAKO, Glostrup, Denmark) was used as a detector. MAb isotypes were determined using affinity purified rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA antibodies (Mouse Monoclonal Antibody Isotyping Reagents, Sigma, St. Louis, MO, USA) according to the instructions of the manufacturer.

Cell biotinylation and immunoprecipitation. Glioblastoma cells were grown in a monolayer to 70-80% confluence and washed with ice-cold buffer A (20 mM NaHCO<sub>3</sub>, 0.15 M NaCl, pH 8.0). A total of 1 mg of NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL, USA) was dissolved in 50 µl DMSO immediately before use, mixed with 4 ml buffer A, added to cells and incubated for 90 min at 4°C. Finally, the cells were washed 5 times with buffer A and lysed as described above. Tested MAbs (1 ml) in culture medium was bound to 40  $\mu$ l of 50% suspension of protein-G sepharose (Pharmacia, Uppsala, Sweden) for 2 h at room temperature. Biotinylated cell extract (200 µl) was precleared with 20  $\mu$ l of 50% suspension of protein-G sepharose and then added to the bound MAb. Collected immunocomplexes on protein-G sepharose were washed, boiled 5 min in Laemmli loading buffer and separated by SDS-PAGE gel (10%) electrophoresis. Afterwards, the proteins were transferred onto poly(vinylidene fluoride) (PVDF) membrane and revealed with peroxidase-conjugated streptavidin (1:1000; Pierce Biotechnology, Inc.) followed by enhanced chemiluminiscence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunohistochemistry. Spheroids were fixed in Carnoy's fluid (absolute ethanol-chloroform-glacial acetic acid in 6:3:1 of volume ratio) and embedded in paraffin according to the standard histological procedures. Four micrometer sections of spheroids were placed on polylysine-coated slides, dewaxed, and rehydrated. The tissue pretreatment procedure performed in Pascal pressure chamber (DakoCytomation, Carpinteria, CA, USA) at 125°C for 5 min in Tris/EDTA (pH 9.0) target retrieval solution was used for staining with the MAb VII-14. The immunostaining procedure was performed in Dako Autostainer using the DakoCytomation EnVision®+System-HRP (DAB) according to the manufac-

turer's instructions: (a) peroxidase and protein block (10 min each), (b) incubation for 1 h with the primary antibody (undiluted hybridoma medium) or PBS (negative control), (c) incubation for 30 min with the peroxidase-conjugated goat anti-mouse antibody diluted 1:1000 in antibody diluent (DakoCytomation). Staining was visualized with DAB solution for 1 min and samples were counterstained with Mayer's hematoxylin and mounted in DePeX (Serva, Heidelberg, Germany). The stained sections were examined with Leica DM4500B microscope (Leica, Mannheim, Germany) and photographed with Leica DFC480 camera (Leica).

Tissue specimens were obtained from Tampere University Hospital (Tampere, Finland). All specimens were formalinfixed and paraffin-embedded according to standard histological procedures. Five micrometer thick sections were deparaffinised, rehydrated and stained in Lab Vision Autostainer 480 (Thermo Fisher Scientific, Runcorn, Cheshire, UK) using Power Vision+TM Histostaining Kit (ImmunoVision Technologies, Brisbane, CA, USA). The staining procedure was: (a) peroxidase block 5 min, (b) protein block 10 min, (c) incubation with primary antibody for 30 min (undiluted or diluted 1:5), (d) postblock 20 min, (e) secondary antibody 30 min. Staining visualization by DAB (5 min) was intensified using CuSO<sub>4</sub> (5 min) and samples were counterstained with hematoxylin for 1 min. Stained sections were examined with Leica DM4500B microscope and photographed with Leica DFC480 camera.

Western blotting. Proteins were extracted from cells with cold RIPA buffer (1% Triton X-100, 0.1% sodium deoxycholate, and 1X Complete protease inhibitor cocktail (Roche, Mannheim, Germany) in PBS) for 15 min at 4°C, and total protein concentration in the extract was determined by BCA assay (Pierce Biotechnology, Inc.). Samples of 50-80  $\mu$ g were separated by SDS-PAGE and blotted onto the PVDF membrane (Amersham Pharmacia Biotech). The membrane was treated with the monoclonal antibodies in undiluted hybridoma medium followed by the peroxidase-conjugated goat-anti mouse immunoglobulins (DAKO) and developed using ECL kit (Amersham Pharmacia Biotech).

*Immunofluorescence*. For immunofluorescence studies, cells were grown on glass coverslips, fixed in ice-cold methanol for 10 min at 4°C, washed in PBS and blocked in 1% BSA for 30 min at 37°C. Blocked cells were incubated with antiendosialin monoclonal antibodies for 1 h at 37°C, washed four times with 0.05% Tween<sup>®</sup> 20 in PBS and incubated with the secondary goat anti-mouse antibody Alexa Fluor-488 (Invitrogen/Molecular Probes, Carlsbad, CA, USA) diluted 1:1000 in 1% BSA in PBS for 1 h at 37°C. Finally, cells were washed and mounted using Fluorescein-FragEL mounting medium (Calbiochem, Darmstadt, Germany). The samples were subjected to fluorescence analysis using Leica DM4500B microscope and photographed with Leica DFC480 camera.

## Results

*Production and characterization of monoclonal antibodies.* In order to facilitate future functional studies of endosialin, we prepared and characterized a panel of monoclonal antibodies

MAb M78.MAb	Isotype	Epitope	WB	IP	ELISA	IF	IHC
II-50	IgG1	MUC	+	+	++	++	+
III-15	IgG2a	CTLD-SSC	+	+	++	+	NT
VI-71	IgG1	MUC	++	+	++	+	NT
VII-14	IgG2a	CTLD-SSC	(+)	+	++	+	+
VIII-16	IgG1	MUC	++	+	++	++	+
XIII-70	IgG1	MUC	++	++	++	++	_
M78	IGM	MUC	++	_	++	++	+

Table I. Summary of the reactivity and isotypes of novel endosialin-specific antibodies in comparison with previously described.

WB, Western blot; IP, immunoprecipitation; IF, immunofluorescence; IHC, immunohistochemistry



Figure 1. Reactivity of the new monoclonal antibodies in immunoblotting and immunoprecipitation. (A) Western blotting analysis of the new monoclonal antibodies for their specific reactivity with the endosialin expressed ectopically in the transfected HeLa-TEM1 cells compared to the mock-transfected control HeLa cells. The figure shows representative blot obtained with selected monoclonal antibodies. (B) Immunoprecipitation of endosialin from the extract of biotinylated glioblastoma cell line 42-MG-BA using selected monoclonal antibodies.

against recombinant human endosialin. Antibody M78 previously prepared in our laboratory (29) is of an IgM isotype and is thus not suitable for all techniques used in our laboratory. To obtain a new set of antibodies, we used a different strategy to prepare the antigen as well as a different fusion protocol. Altogether we screened 1277 hybridomas, which led to six viable clones that were also stable after freezing and refreezing. Four of these antibodies were of the IgG1 and two of the IgG2a isotype (Table I).

The antibodies were first tested for their specificity in ELISA using HeLa-TEM1 cells expressing endosialin as the source of the antigen. All antibodies detected endosialin in these cells but not in the control HeLa cells that were transfected with the empty vector. Next, the antibodies were tested for their activity in Western blotting (Fig. 1A) and immunoprecipitation (Fig. 1B). Western blotting of lysates of HeLa-TEM1 cells demonstrated that all six MAbs recognize denatured form of endosialin. While ELISA and Western blotting were performed on the cell lysates from HeLa cells expressing endosialin under the control of the cytomegalovirus (CMV) promoter, the higher sensitivity of immunoprecipitation enabled us to detect endogenous endosialin naturally expressed in glioblastoma cell line 42-MG-BA. All six antibodies were able to immunoprecipitate the endosialin protein from biotinylated cell extract. In both assays, we were able to detect the fully matured, glycosylated form of the endosialin (165 kDa) as well as the core protein (90 kDa). The data characterizing the obtained monoclonal antibodies are summarized in Table I.

Reactivity of the monoclonal antibodies in immunohistochemistry. An important attribute of monoclonal antibodies is their capability to specifically react with relevant antigen in paraffinembedded tissues sections. In order to test the endosialin-specific monoclonal antibodies in routine imunohistochemistry, we first prepared spheroids from HeLa-TEM1 cells. Spheroids were generated in hanging drops from a mixture of clones with different levels of endosialin expression. The spheroids were fixed in Carnoy's fluid, paraffin-embedded, sectioned and stained for endosialin. Of the four antibodies tested, two gave positive staining (II-50, VIII-16), one (VII-14) gave positive staining after demasking with antigen retrieval buffer (Tris/ EDTA, pH 9.0), and one (XIII-70) did not stain the spheroid sections. The staining with these MAbs produced typical signal localized to membranes. The representative slides are shown in Fig. 2.

Previous studies have shown strong upregulation of endosialin in brain tumors (30), especially in aggressive high-grade gliomas (12-14). Thus, we stained sections of paraffin-embedded samples of brain tumors to validate the expression of endosialin using two new monoclonal antibodies, MAbs II-50 and VIII-16. We studied several types of brain tumors including glioblastoma multiforme, anaplastic



Figure 2. Immunohistochemistry of endosialin in multicellular spheroids. The spheroids were prepared from a mixture of HeLa-TEM1 clones with different levels of endosialin expression. The monoclonal antibodies II-50, VIII-16 gave positive staining, VII-14 MAb required demasking with Tris/EDTA antigen retrieval buffer (pH 9.0). The MAb XIII-70 did not stain the spheroid sections. The results show the plasma membrane staining of endosialin.



Figure 3. Immunohistochemical analysis of endosialin expression pattern in brain tissue sections using MAb VIII-16. Tissue sections: (A) normal brain; (B) anaplastic astrocytoma; (C) and (D) glioblastoma multiforme; (E) gliosarcoma. Arrows represent blood vessels.



Figure 4. Schematic illustration of endosialin structure and deletion mutants. The scheme illustrates a domain composition of endosialin with the corresponding amino acid sequences indicated below the scheme. Deletion mutants are shown in the lower part:  $\Delta$ CTLD-SSC mutant lacks the C-type lectin-like and Sushi/SCR/CCP domains (aa 28-236),  $\Delta$ EGF mutant lacks the three EGF-like repeats (aa 235-356),  $\Delta$ MUC mutant lacks the sialomucin domain (aa 359-685). Binding regions of the characterized monoclonal antibodies are marked on the top.



Figure 5. Immunofluorescence detection of endosialin and its deletion mutants. Endosialin and  $\Delta$ CTLD-SSC,  $\Delta$ EGF,  $\Delta$ MUC mutants were expressed in HeLa cells. The antibodies revealed plasma membrane staining of endosialin as well as its deletion mutants. HeLa cells transfected with the empty vector served as a negative control. The monoclonal antibody III-15 recognizes the CTLD-SSC region of endosialin, the MAb VIII-16 recognizes the mucin domain. Immunofluorescence staining of cells: (A) HeLa-TEM1 stained with the MAb III-15; (B) HeLa-phCMV stained with the MAb III-15 (negative control); (C) HeLa  $\Delta$ CTLD-SSC stained with the MAb III-15; (E) HeLa  $\Delta$ MUC stained with the MAb III-15; (F) HeLa  $\Delta$ MUC stained with the MAb III-15; (G) HeLa  $\Delta$ EGF stained with the MAb III-15; (H) HeLa  $\Delta$ EGF stained with the MAb VIII-16.

astrocytoma, gliosarcoma, and diffuse astrocytoma. Positive reaction was observed in both neural tissues and blood vessels of specimens. The highest intensity of staining was observed in gliosarcoma, however, the number of samples with this type of tumor was very low, so we could not statistically evaluate the data. Overall staining intensity was slightly more intense with anti-endosialin antibody VIII-16 (Fig. 3). The staining intensity was generally stronger in blood vessels than in nervous tissues with both antibodies. Moreover, the staining intensity was the strongest in blood vessels stained with the antibody VIII-16. Blood vessels were stained well in samples of glioblastoma multiforme. Both nerve tissue and blood vessels showed the most intense immunostaining reaction with both endosialin antibodies in anaplastic astrocytoma, grade 3.

Determination of target domains of MAbs using deletion mutants of endosialin. To determine the domain specificity of the produced monoclonal antibodies we performed immunofluorescence analysis of HeLa cells expressing deletion mutants of endosialin. The antibodies were first examined for their reactivity in indirect immunofluorescence using HeLa cells overexpressing the full-length endosialin. Specific cell surface staining was observed with all antibodies, suggesting that they recognize endosialin in this assay.

Next, we used three different deletion mutants of endosialin (Fig. 4). The  $\Delta$ CTLD-SSC mutant was lacking the aa 28-236 including the C-type lectin-like and Sushi/SCR/ CCP domains, the  $\Delta$ EGF mutant was lacking the aa 235-356 containing three EGF-like repeats, and the mutant  $\Delta$ MUC was lacking the aa 359-685 containing the sialomucin domain. These mutants were expressed in HeLa cells under the control of the CMV promoter and subjected to immunofluorescence analysis. Representative samples of each type of the mutant are shown in Fig. 5. Two of the monoclonal antibodies, namely III-15 and VII-14, recognized the CTLD-SSC region of endosialin, while the rest of the antibodies - II-50, VI-71, VIII-16, XII-70 recognized the MUC domain of this protein. In addition, we found that all of the endosialin mutants lacking part of the extracellular domain were correctly localized to the cytoplasmic membrane. The domain specificity of prepared antibodies was further confirmed by Western blotting (Fig. 6).



Figure 6. Immunoblotting of endosialin deletion mutants. Immunoblotting was performed on HeLa cells stably transfected with cDNA of endosialin (HeLa-TEM1) and its deletion mutants (HeLa- $\Delta$ CTLD-SSC, HeLa- $\Delta$ EGF, HeLa- $\Delta$ MUC) under the control of the CMV promoter. The deletion mutants  $\Delta$ CTLD-SSC and  $\Delta$ EGF as well as endosialin are recognized by the MAb VIII-16. The  $\Delta$ MUC deletion mutant was detected using the MAb III-15.

The MAb VIII-16 was able to recognize the  $\Delta$ CTLD-SSC,  $\Delta$ EGF endosialin deletion mutants as well as the full length endosialin but not the  $\Delta$ MUC deletion mutant. The  $\Delta$ MUC deletion mutant was detected using the MAb III-15. The MAb VIII-16 recognized both the core and glycosylated form of endosialin deletion mutants  $\Delta$ CTLD-SSC and  $\Delta$ EGF, while the  $\Delta$ MUC deletion mutant was detected as a single ungly-cosylated band. Similar results were obtained using other anti-endosialin MAbs.

### Discussion

Angiogenesis is a crucial process that restores oxygen homeostasis in an oxygen-deprived tissue (31). Without access to vascular system, tumors cannot grow beyond a critical size or metastasize to another organ (32). Release of pro-angiogenic factors by hypoxic cancer cells is a critical stimulus for the formation of new blood vessels (33,34). Previously we have shown that the endosialin gene transcription is induced by hypoxia predominantly through a mechanism involving cooperation of hypoxia inducible factor 2 (HIF2) and Ets-1 transcriptional factors (29). The angiogenic response involves changes that occur in endothelial cell interactions with the extracellular matrix, as well as changes in cell-cell interactions. Possible function of endosialin has been predicted with respect to its structure. Based on the structure of the extracellular domain of endosialin, a role in cellto-cell interaction or cell-to-extracellular matrix interaction has been suggested (35). However, the exact mechanism and function of endosialin still remains elusive. In order to further study endosialin and its function in angiogenesis, we prepared a set of new monoclonal antibodies.

In the first attempt, we prepared the MAb M78 (29). This MAb is not suitable for the further studies because of its IgM isotype. Thus, we prepared a panel of new anti-endosialin antibodies that are characterized in this study. Unlike other antibodies previously described and used for detection of endosialin, here we described domain-specific monoclonal antibodies that recognize the CTLD-SSC and MUC domains of endosialin. Altogether, we prepared six MAbs that recognize denatured endosialin protein in immunoblotting, can be used for immunoprecipitation, ELISA, and produce only negligible background. In addition, all prepared antibodies recognize the native conformation of endosialin and are suitable for immunofluorescence studies. The antibodies were tested in immunohistochemistry and were found to specifically react with the relevant antigen in sections of paraffin-embedded tissues. First, the MAbs were tested for their ability to recognize endosialin in sections from multicellular spheroids generated from mixture of clones with different levels of endosialin expression. Of the four antibodies tested, two gave positive staining (II-50, VIII-16), one (VII-14) gave positive staining after demasking, and one (XIII-70) did not stain the spheroid sections. Then, tissue sections from glioblastoma multiforme, anaplastic astrocytoma, gliosarcoma, and diffuse astrocytoma were analyzed using two MAbs. The highest intensity of staining was observed in gliosarcoma coinciding with previously published data (12-14,30). A positive reaction was detected in nerve tissues as well as in blood vessels of specimens. This suggested possible usefulness of the new MAbs for immunohistochemical studies of tissue sections.

The domain specificity of the new MAbs was determined using deletion mutants of endosialin. Two of these MAbs (III-15, VII-14) recognize the CTLD-SSC domain and the rest of MAbs (II-50, VI-71, VIII-16, XII-70) recognize the MUC domain. These antibodies represent important reagents for the study of functional contributions of extracellular endosialin domains. Each of these domains can be associated with different aspect of cell function. It has been suggested that endosialin may block normal cell-to-cell interactions by interacting with one of the cell surface molecules, or endosialin-expressing cells may more intensively interact with proteins of extracellular matrix. Tomkowicz et al (22) described the interaction of CTLD with extracellular matrix proteins fibronectin and collagen I and IV. They have also shown that the expression of endosialin in cells increases cell migration through the extracellular matrices as well as their adhesive properties. Endosialin is a highly sialylated cell surface protein that contains a rich O-glycosylated sialomucinlike domain. It has been described that the overexpression of sialomucin can interfere with the function of cell adhesion molecules by steric blocking of the interaction of the cell surface molecules. In breast cancer cells, the overexpression of similarly glycosylated MUC1 protein suppresses cell adhesion properties by interfering with the functions of E-cadherin and other cell adhesion molecules (36-38). Sialomucins may also be involved in the invasion of the basement membrane by modulating of the cell-matrix attachment (39-42). Even though these data suggest possible role of endosialin, direct mechanisms of its regulation remain still unclear.

In conclusion, the new monoclonal antibodies characterized in this paper are specific for the CTLD-SSC and sialomucin-like domains of endosialin. Endosialin, as a marker of pericytes strongly upregulated in tumor vasculature, represents an attractive therapeutic opportunity. However, the biological role and mechanisms of endosialin regulation are still incompletely understood. These novel domain-specific MAbs provide a powerful experimental tool to further study of the biological role of endosialin in angiogenesis.

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