Overexpression of Endocan Induces Tumor Formation

Arnaud Scherpereel, Thibaut Gentina, Bogdan Grigoriu, Stéphanie Sénéchal, Anne Janin, Anne Tsicopoulos, François Plénat, David Béchard, André-Bernard Tonnell, and Philippe Lassalle


ABSTRACT

Endocan is a proteoglycan specifically secreted by endothelial cells. Through its glycan domains, endocan binds to hepatocyte growth factor and increases its mitogenic activity. Here, we show that human embryonic kidney 293 cells, which have been genetically engineered to overexpress endocan, form tumors when injected s.c. in SCID mice. Both the glycan and a phenylalanine-rich region of endocan are necessary for mediating tumor growth activity. Blocking the phenylalanine-rich region with a monoclonal antibody results in a marked reduction of tumor growth. Finally, we report that circulating levels of endocan are increased in mice with the endocan-expressing human embryonic kidney 293 cell tumors and in a series of adult patients with lung cancer. Taken together, these results suggest that (a) endothelial-derived endocan induces tumor growth, (b) antibodies to endocan may have therapeutic potential, and (c) circulating levels of endocan may eventually represent a novel marker for cancer.

INTRODUCTION

PGs play an important role in vascular biology (1–3). Most PGs that are associated with the vascular wall are synthesized by endothelial cells and localized to the subendothelial basement membrane of capillaries. These include perlecán (4), members of the syndecan family and glypican-1 (5, 6), small leucine-rich chondroitin/DS PG biglycan (7), and decorin (8, 9). Collectively, these PGs serve to stabilize the blood vessel wall, support migrating and proliferating endothelial cells, and induce angiogenesis by modulating the activity and bioavailability of growth factors (10, 11). We have recently identified a novel endothelial cell-specific PG, termed endocan (12, 13). Endocan contains a single chain of DS. In contrast to most PGs, endocan does not localize to the subendothelial basement membrane but rather circulates freely in the blood (14). Endocan has been shown to bind to LFA-1, thereby reducing LFA-1-mediated leukocyte activation (15). Moreover, we recently reported that endocan binds to and promotes the mitogenic activity of hepatocyte growth factor, a property that was mapped to the DS chain of the PG (13). On the basis of these observations, we hypothesized that endocan may be involved in mediating tumor growth.

MATERIALS AND METHODS

Cell Culture

Wild-type and transfected HEK 293 and HT29 cells were maintained in DMEM supplemented with 10% FCS, 1-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Endocan mutants were purified from stably transfected HEK 293 cells cultured in serum-free 293-SFM medium (Life Technologies, Inc., Cergy-Pontoise, France). MEP 08 and MEP 14 mAbs (14) were produced in hybridoma cell clones cultured in Hybridoma SFM medium (Life Technologies, Inc.). HUVECs were routinely provided as described previously (16). HS was bought from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Vectors, Mutagenesis, and Stable Transfection

The full-length endocan cDNA previously inserted into pcDNA3 expression vector was used as substrate for mutagenesis (12). The mutated endocan S137A (13), endocan/F115A, endocan/F116A, and endocan/F115A-F116A were obtained by PCR with the QuickChange site-directed mutagenesis kit, according to the manufacturer’s instructions (Stratagene, Cambridge, United Kingdom) and confirmed by sequencing on an ABI Prism 377 automated DNA sequencer (PE Biosystems, Courtabeuf, France). These constructs were transfected into HEK 293 and HT29 cell lines with Lipofectamine (Life Technologies, Inc.), followed by selection by G418 (300 and 500 μg/mL, respectively) and cloning by limited dilution.

Animals

CB-17 scid/scid homozygous SCID mice (male, 5–6 weeks of age) were obtained from Pasteur Institute of Lille. These mice were continuously housed in a germ-free laminar-airflow facility inside a temperature- and light-controlled room. To eliminate the NK cell activity, mice received an i.p. injection of anti-asialo GM1 antibody (Wako Chemicals, Neuss, Germany) 1 day before subcutaneous injection (dorsal, anterior-interscapular) of HEK 293- (106 cells/injection) or HT29-transfected cells (2 × 105 cells/injection). Tumor size was assessed once a week, and the animals were killed when the tumor diameter reached up to 1 cm. Tumor volume was calculated by the formula V = (4/3π) × abc/8 or its simplified form: abc/2, where a, b, and c are the orthogonal diameters. Mouse blood endothelial levels were measured by ELISA. The antibody treatment consisted of a weekly i.p. injection of 400 μg of purified MEP 08 or MEP 14 in 200 μL of endotoxin-free and sterile PBS.

Animal experiments were carefully studied with the help of the newly formed Ethical Committee for the Use of Laboratory Animals in Saint-Louis Hospital. In vivo experiments have been limited to the part of experimental work that couldn’t be performed in vitro. The number of mice used has been strictly limited to the number necessary for the validation of statistical analysis and the controls. For ethical considerations, animals were sacrificed when the tumor reached 1 cm diameter and were considered as dead.

Pathological Analysis

Mice were killed and dissected with systematic macroscopic analysis of all organs. All tumoral localizations were photographed, measured, and systematically removed for additional microscopical analysis. Selected organ specimens were cut into three parts. One part was fixed in 4% buffered formaldehyde for 2 h and additionally processed for paraffin embedding. Three-μm thick paraffin sections were stained with H&E, Masson’s trichrome, and reticulin stain. The other part was fixed in 2% glutaraldehyde in cacodylate buffer and additionally processed for electron microscopic analysis. The third part was immediately snap-frozen and cryopreserved in liquid nitrogen for additional immunohistochemical studies or nucleic acid extractions. Immunohistochemical studies were performed either on cell pellets or on tumor section by an indirect immunoperoxidase method using the primary antibody directed against endocan, at a dilution of 1/200.
Counts of Microvessels, Apoptotic, Mitotic, and Inflammatory Cells

Microvessels were stained by an indirect immunoperoxidase method on paraffin sections, using antihuman CD31 (Novocastra, Newcastle, United Kingdom) as primary antibody, with controls by omitting the first antibody and by using an irrelevant antibody of identical isotype. Apoptotic bodies were detected using the terminal deoxynucleotidyltransferase-mediated nick end labeling assay on paraffin sections, treated with proteinase K (20 μg/ml) for 15 min at room temperature, with the ApopTag Peroxidase in situ Apoptosis Detection Kit (Intergen). Count of mitoses were performed on tumor cells in paraffin sections stained with H&E. Count of inflammatory cells took into account neutrophils and eosinophils, lymphocytes, plasma cells on H&E, and Muy Granulwald Giersma stains. Pathological quantitative data were obtained blindly by two pathologists on four different fields at magnification ×400 on an Olympus AX 70 microscope, with wide-field eyepiece number 26.5, providing a field size of 0.344 mm² at magnification ×400. Results were expressed as the mean number of microvessel sections, apoptotic cells, mitoses, and inflammatory cells counted in the four different fields observed at magnification ×400.

Purification of Endocan and Endocan Mutants

The nonglycanated endocan/S137A is purified in one-step affinity chromatography. Endocan/F115A, endocan/F116A, and endocan/F115A-F116A were purified by ion-exchange and affinity chromatographies exactly as previously described for endocan (13).

mAb Purification

The MEP 08 (IgG2a,K) and MEP 14 (IgG2a,K) hybridoma cell cultures conditioned in serum-free medium were applied to a column (1 × 8 cm) of protein G-Sepharose (Amersham Pharmacia Biotech, Saclay, France). The column was washed with 0.05 M sodium phosphate (pH 7) and eluted with 3 M MgCl₂, concentrated and dialyzed against PBS using an Ultrafree molecular weight cutoff membrane (Millipore, Bedford, MA).

Endocan ELISA

Specific ELISA for endocan was performed as previously described (14). The mutations did not modify the antibody reactivity in the sandwich assay.

Western Blotting

One-hundred nanograms purified of wild-type and mutated forms of endocan were analyzed by Western blot using MEP 08 or MEP 14 as described previously (13).

Cell Proliferation Assays

HEK 293 Cell Proliferation Assay. The cell growth was determined by measuring [Methyl ³H]thymidine uptake into HEK 293 cell clones. Cells were seeded at a density of 1 × 10³/well in 96-well microplates and cultured in either complete medium, including 10% FCS or DMEM supplemented with transferrin, insulin, and 50 ng/ml recombinant human HGF/SF (R&D Systems, Abingdon, United Kingdom). Ten μg/ml purified mAbs MEP 08 and MEP 14 were added. After 56 h of culture, cells were pulsed with 0.5 μCi of [Methyl ³H]thymidine/well for 16 h and [Methyl ³H]thymidine incorporation into DNA was determined on a TopCount Microplate Scintillation Counter (Packard, Rungis, France). Assays were performed in quadruplicate.

HUVECs Proliferation Assay. The cell growth was determined by measuring [Methyl ³H]thymidine uptake and by the reduction of MTT in a MTT viability assay (17). Briefly, HUVECs were seeded at a density of 5 × 10³/well in fibronectin-coated 96-well microplates in basal medium containing RPMI 1640 plus 2% FCS supplemented for some experiments with 5 ng/ml FGF-2 (R&D Systems) and 1 μg/ml endocan or 1 μg/ml HS. After 18, 32, 56, and 70 h of cell culture, HUVECs were pulsed with 0.5 μCi of [Methyl ³H]thymidine/well for 16 h. The MTT viability assay was performed after 96 h culture.

Fluorescent Cell Sorter Analysis

Cell surface binding of endocan was evaluated by fluorescence-activated cell sorting as previously described (15), except that 24 h before fluorescence-activated cell sorting, adherent HEK 293 cells were enzymatically detached and cultured 24 h in 293-SFM II cell suspension medium (Life Technologies, Inc.) to reconstitute trypsin-sensitive cell membrane proteins.

Subjects and Patients

The study was conducted in the department of Pneumology of the University Hospital of Lille (France). Serum from patients with lung cancer was collected at the moment of diagnosis. Patients were included before any cancer treatment; on the contrary, patients with clinical symptoms or biological signs of infection were excluded. Sera were collected from each subject, spun down 15 min at 1500 × g, then kept at −20°C before assays. The protocol was approved by the local ethical committee.

RESULTS

HEK 293 Cells Overexpressing Endocan Form Tumors in SCID Mice. Full-length human endocan cDNA was cloned into the pcDNA3 vector, and the resulting construct was stably transfected into HEK 293 cells. The transfected cells were selected in G-418-containing medium, and resistant clones were assayed for endocan secretion by ELISA. Two separate clones that secreted 762 ± 211 ng endocan/day/10⁶ cells or control clones (transfected with vector alone) were injected s.c. into the flank of male SCID mice. Each of the two endocan-expressing HEK 293 cell clones developed into tumors at the site of injection, whereas the control clones had no such effect (Fig. 1A). Two additional HEK 293-endocan cell clones injected in 8 mice also formed tumors, clearly avoiding clonal selection artifacts. In keeping with its
function as a secreted PG, endocan was detected in the serum of mice that formed tumors (Fig. 1C). Importantly, circulating levels of endocan increased over time and correlated positively with tumor size ($r = 0.96$, $P < 0.001$, Spearman correlation test; Fig. 1E).

**Pathology.** Clinically, a round, s.c. tumor was found at the site of cell injection. When compared with mice having been injected with HEK cells, tumor-bearing mice looked leaner, but the comparison of weight curves did not show any significant difference because the weight of the developing tumor compensated the weight loss of the mice with tumors. At the dissection, the tumor did not adhere to the skin or to adjacent organs. The section showed a whitish, polylobulated tumor, with some necrotic areas. Additional dissection did not show lymph node involvement, nor metastatic dissemination, except for an isolated nodule in the kidney in 1 case. Microscopically, pelleted 293-endocan cells showed a high index of mitotic cells, some of them multinucleated or with irregular chromatin distribution (Fig. 2A). Histological analysis showed sheets of cells of irregular shape and distribution, with a high mitotic index and nuclear abnormalities. Vessels of small caliber could be found, intermingled with neoplastic cells, and the borders of the tumor were ill-defined (Fig. 2B). Quantitative analyses from six tumors showed $12.5 \pm 2.9$ mitotics cells/mm$^2$, $7.3 \pm 2.3$ apoptotic cells/mm$^2$, and $12.5 \pm 2.9$ vessels/mm$^2$), indicating a high degree of proliferation and tumor development contrasting with a virtually no leukocyte infiltrate ($1.4 \pm 1.4$ inflammatory cells/mm$^2$). Electron microscopic study of the tumors showed that all of them had neoplastic cells with desmosomes and tonofilaments, two characteristics of epithelial cells (Fig. 2C). Moreover, the vessels observed within the tumors had an endothelium and a basement membrane that could be clearly identified (Fig. 2D). Immunohistological analysis of HEK 293 cell patches showed that the expression of endocan within 293-endocan cells is only cytoplasmic (Fig. 2E). In the corresponding tumors, antiendocan antibodies only marked the cytoplasmic areas (Fig. 2F). Thus, transfection of cDNA of endocan in human kidney epithelial cells HEK 293 give them an oncogenic potential with occurrence of an epithelial carcinoma at the site of injections.

**Endocan-Promoting Tumor Growth Is Not Specific to HEK 293 Cells.** In the next set of experiments, HT29 cells were stably transfected with the endocan cDNA construct, and G418-resistant cell clones were selected for their ability to secrete endocan. HT29 do not normally express endocan and, in contrast to HEK 293 cells, are spontaneously tumorigenic in vivo. HT29-endocan cell clones that secreted $34 \pm 7$ ng endocan/day/10$^6$ cells or control vector-transfected clones were injected s.c. into SCID mice. The HT29-endocan clones exhibited greater tumor growth, compared with control clones (Fig. 1B). Macroscopic analysis of tumor HT29-endocan showed a whitish nonadherent nodule, with necrotic areas. No satellite lymph node involvement and no metastatic dissemination could be found. Again, serum endocan levels increased with time (Fig. 1D) and correlated with tumor size ($r = 0.72$, $P < 0.01$; Fig. 1F). Taken together with the HEK 293 results, these data demonstrate that endocan induces tumor growth of otherwise nontumorigenic cells and increases the growth potential of tumorigenic cells.

**The Glycan from Endocan Is Required for Tumor Cell Growth.** To determine whether the glycan chain plays a role in mediating tumor growth, we generated a nonglycanated form of endocan by mutagenesis of serine 137 to alanine (endocan/S137A). HEK 293 cell clones expressing this cDNA mutant were selected and screened for mutant endocan secretion. Each of two endocan/S137A clones that secreted $2023$ ng/ml $\pm 360$ ng endocan/day/10$^6$ cells was injected s.c. into groups of 4
SCID mice in three separate experiments and systematically compared with the positive control group receiving endocan-expressing HEK 293 cells developing consistently a tumor (cumulative frequency of 32 tumors among 34 mice in eight distinct experiments). Endocan/S137A-expressing clones did not give rise to tumors (0 tumor/12 mice). These results are consistent with a previous study in which the S137A mutation abrogated the positive effect of endocan on HGF/SF-mediated proliferation of cultured HEK 293 cells (13). These findings suggest that the glycan moiety of endocan is necessary for tumor cell proliferation under both in vitro and in vivo conditions.

To examine the efficiency of MEP 08 on macroscopically

is critically involved in mediating tumor growth.

of these results suggest that MEP 08 recognizes an epitope that is

development. To determine the effect of the antibody on the kinetics of tumor growth, we recorded weekly the tumor size of mice under treatment with MEP 08. In groups 0 and 2, the tumors appeared 2 weeks later than in the control group (Fig. 3C). The delayed appearance of the tumors correlated with a delayed detection of serum endocan (data not shown). At the ninth week, we observed a reduced mean tumor volume in groups 0, 2, and 4 (Fig. 3C). Taken together, these findings suggest that MEP 08 mAb exerts an antitumor activity on xenografted tumors of small sizes.

No Effect of MEP 08 on HEK 293-Endocan Cell Proliferation.

To elucidate the antitumor effect of MEP 08, we examined if MEP 08 could bind to HEK 293 cells or influence HEK 293 cell proliferation. Using fluorescent analysis on cell sorter, cell surface-bound endocan could not be detected on either parental HEK 293 cells charged with endocan or HEK 293-endocan cells, even in the presence of divalent ions (data not shown). In addition, high doses of MEP 08 did not change the rate of [3H]thymidine incorporation by HEK 293 cell clones expressing the control vector, endocan, or endocan/S137A (Table 1), which was confirmed by the MTT cytotoxicity assay. These results indicated that the antitumor effect of MEP 08 does not appear to be mediated by direct cytostatic or cytotoxic activities on tumor cells.

No Effect of MEP 08 on Endothelial Cell Proliferation. Another question was to study if MEP 08 possesses antiproliferative effect on HUVECs. FGF-2 is a well-known angiogenic factor that induces proliferation of HUVEC, and glycans are known to greatly influence FGF-2 activity. The fact that endothelial cells secrete endocan could therefore influence the net FGF-2 effect on HUVECs. Surprisingly,
membrane nor to the extracellular matrix but released in a soluble form from the other PGs is that endocan is neither associated to the cell previously cloned and characterized a novel PG called endocan that is – these PGs can stimulate or inhibit tumor cell growth (18).

PGs consist of a core protein to which are attached one or more glycosaminoglycan chains. They are mainly found associated to the cell membrane and the extracellular matrix (1–3). There is now evidence that these PGs can stimulate or inhibit tumor cell growth (18–24). We previously cloned and characterized a novel PG called endocan that is specifically synthesized by endothelial cells. One distinguishing point from the other PGs is that endocan is neither associated to the cell membrane nor to the extracellular matrix but released in a soluble form that circulates into the bloodstream (14). We previously showed that endocan binds to HGF/SF and promotes HGF-induced [3H]thymidine uptake by HEK 293 cells (15). Here, we demonstrate the capacity of endocan to promote tumor growth. To establish our hypothesis, we engineered human tumor cells to express human endocan and used them in a tumor xenograft mouse model. We took advantage of the fact that endocan is a secreted molecule, reducing thus the importance of its cellular origin. Importantly, endocan is expressed locally, within the tumor, produced in our mouse model by the transfected cells.

Consistent with our in vitro results, the glycan moiety of endocan was necessary for mediating tumor formation. A more surprising finding was that the protein core of endocan is also an important determinant of tumor growth. Specifically, the F115 and F116 residues in the phenylalanine-rich region were shown to support binding of endocan to phenyl-Sepharose and to mediate tumor growth. These findings were additionally supported by the observation that MEP 08, an antiendocan mAb, specifically directed against the phenylalanine-rich region, blocked the effect of endocan on tumor growth.

The fact that the polypeptide itself is not involved in the modulation of the mitogenic effects of growth factors but is indispensable for tumor growth suggests that it functions in vivo through another host component. One host receptor candidate is represented by the leukocyte integrin LFA-1. LFA-1 (CD11a/CD18) is a heterodimeric transmembrane molecule constituted of β2 and αL chains. LFA-1 plays an important role in leukocyte adhesion, migration and activation. Leukocytes from mice lacking CD11a displayed selective impairments in alloantigen triggered T-cell proliferation, cytotoxicity and tumor rejection (25–27). Previously, endocan was shown to bind to LFA-1 and to reduce LFA-1-ICAM-1 interactions (15), so endocan may inhibit infiltration and activation of leukocytes into the tumor xenograft, as suggested by the absence of leukocyte infiltrates in the tissue sections of tumors.

An important finding was that therapy with MEP 08 antibody reduced

addition of recombinant endocan in HUVEC cultures did not modify the FGF-2-induced [3H]thymidine incorporation (Fig. 4A) despite doses 100-fold greater than that secreted. By contrast, addition of HS still increased [3H]thymidine uptake (Fig. 4A). On the other hand, addition of 10 μg/ml MEP 08 or MEP 14 has no cytotoxic effect on HUVECs and did not modify cell proliferation induced by FGF-2 as judged by MTT cytotoxicity assay (Fig. 4B).

Serum Endocan Is Increased in Patients with Lung Cancer. We explored the hypothesis that serum endocan may be increased in human cancer. Fifty patients admitted in the hospital for lung cancer were studied and compared with 25 healthy volunteers. Endocan’s blood level was increased in patients (3.34 ± 0.37; mean ± SE) as compared with healthy subjects (0.63 ± 0.06, P < 0.0001, Mann-Whitney test; Fig. 4C).

According to the international classification, the highest levels of serum endocan were found in patients with extended forms of cancer. These findings were additionally supported by the observation that MEP 08, an antiendocan mAb, specifically directed against the phenylalanine-rich region, blocked the effect of endocan on tumor growth.

<table>
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<tr>
<th>Serum Endocan Is Increased in Patients with Lung Cancer.</th>
<th>Table 1 Effect of MEP 08 and MEP 14 on [3H]thymidine incorporation by HEK 293 cell clones</th>
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<td>Cells</td>
<td>Medium</td>
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<tr>
<td>293</td>
<td>+MEP08</td>
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<tr>
<td>293 clone 1</td>
<td>+MEP14</td>
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<tr>
<td>293 clone 2</td>
<td>+MEP08</td>
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<tr>
<td>293-endocan clone 1</td>
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<td>293-endocan clone 3</td>
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<td>293-endocan clone 8</td>
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<tr>
<td>293-endocan/S137A clone 2</td>
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<tr>
<td>293-endocan/S137A clone 4</td>
<td>+MEP08</td>
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<td>293-endocan/S137A clone 5</td>
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DISCUSSION
PGs consist of a core protein to which are attached one or more glycosaminoglycan chains. They are mainly found associated to the cell membrane and the extracellular matrix (1–3). There is now evidence that these PGs can stimulate or inhibit tumor cell growth (18–24). We previously cloned and characterized a novel PG called endocan that is specifically synthesized by endothelial cells. One distinguishing point from the other PGs is that endocan is neither associated to the cell membrane nor to the extracellular matrix but released in a soluble form that circulates into the bloodstream (14). We previously showed that endocan binds to HGF/SF and promotes HGF-induced [3H]thymidine uptake by HEK 293 cells (15). Here, we demonstrate the capacity of endocan to promote tumor growth. To establish our hypothesis, we engineered human tumor cells to express human endocan and used them in a tumor xenograft mouse model. We took advantage of the fact that endocan is a secreted molecule, reducing thus the importance of its cellular origin. Importantly, endocan is expressed locally, within the tumor, produced in our mouse model by the transfected cells.

Consistent with our in vitro results, the glycan moiety of endocan was necessary for mediating tumor formation. A more surprising
ENDOCAN PROMOTES TUMOR GROWTH

growth of macroscopic tumors. Interestingly, optimal antitumor activity was observed when MEP 08 was injected 2 weeks after tumor cell inoculation, indicating a potent curative effect. Several findings argue against a direct cytotoxic role of MEP 08. First, endocan is not expressed at the surface of HEK 293 cells. Second, MEP 08 does not bind to the surface of HEK 293-endocan cells. Third, MEP 08 has no effect on endocan-mediated proliferation of HEK 293 cells under in vitro conditions. Thus, one surprising point is that the antiproliferative activity of MEP 08 antibody does not require specific recognition of tumor cells. Another point is that the MEP 08 antitumor activity does not depend on angiostatic effects under in vitro conditions. First, addition of blocking (MEP 08) or nonblocking (MEP 14) antibodies exhibited no cytotoxic nor cytosstatic activities on cultured HUVECs pulsed with FGF-2. Interestingly, antisense inhibition of endocan mRNA does not block vasculogenesis in vitro, despite its increase level of synthesis (28). Generally, a nonspecific antitumor activity of antibodies requires Fc receptor engagement (29–31). Interestingly, two different endocan mAbs of the same isotype exhibited different antiproliferative capacity, which is not supporting a role for Fc receptor in MEP 08 activity. In these conditions, the MEP 08 antitumor activity may be more related to the blockade of a specific and functionally important domain within the endocan molecule, resulting in a reduction of tumor growth.

Finally, we observed a clear increase of serum level of endocan in patients with a diagnosis of lung cancer. The cellular origin of endocan has to be discussed: in our mouse tumor model, endocan expression in the mammary gland. J. Mammary Gland Biol. Neoplasia, 6: 253–273, 2001.


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