[CANCER RESEARCH 63, 6084–6089, September 15, 2003]

Overexpression of Endocan Induces Tumor Formation[1]

Arnaud Scherpereel,[2] Thibaut Gentina,[2] Bogdan Grigoriu, Stéphanie Sénéchal, Anne Janin, Anne Tsicopoulos, François Plénat, David Béchard, André-Bernard Tonnel, and Philippe Lassalle[3]

INSERM U416, Institut Pasteur de Lille, 59019 Lille Cedex [A. S., T. G., B. G., S. S., A. T., A-B. T., P. L.]; Service de Pneumologie et d'Immuno-Allergologie, Hôpital Albert Calmette, Centre Hospitalier Régional Universitaire de Lille, 59037 Lille cedex [A. S., A-B. T.]; Laboratoire de Recherche de Pathologie, Université paris VII EA 2378 et INSERM ERIT-M, Hopital Saint Louis, 75 010 Paris, [A. J.]; Service d'Anatomie Pathologique, Hôpital Brabois Adulte, 54511 Vandoeuvre Cedex [F. P.]; and Endotis Pharma, Parc Eurasanté, 59120 Loos [D. B.], France

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[4] 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 perlecan (4), members of 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 PGs, 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, L-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, Courtaboeuf, France). These constructs were transfected into HEK 293 and HT29 cell lines with Lipofectamin (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 antiasialo GM1 antibody (Wako Chemicals, Neuss, Germany) 1 day before s.c. injection (dorsal, anterior-interscapular) of HEK 293- (10^6 cells/injection) or HT29-transfected cells (2×10^5 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 $V = (4\pi/3) \times (abc/8)$ or its simplified form: abc/2, where a, b, and c are the orthogonal diameters. Mouse blood endocan 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 immunoperoxydase method using the primary antibody directed against endocan, at a dilution of 1/200.

Received 4/19/02; revised 6/4/03; accepted 7/21/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded, in part, by PROGRES Research Program from INSERM and the Societé d'Enseignement et de Recherche en Pneumologie et Allergie.

[2] Both authors have contributed equally.

[3] To whom requests for reprints should be addressed, at E-mail: philippe.lassalle@pasteur-lille.fr.

[4] The abbreviations used are: PG, proteoglycan; DS, dermatan sulfate; LFA-1, leukotactic factor activity 1; mAb, monoclonal antibody; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; HS, heparan sulfate; FGF-2, fibroblast growth factor 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HGF/SF = hepatocyte growth factor/scatter factor.

Counts of Microvessels, Apoptotic, Mitotic, and Inflammatory Cells

Microvessels were stained by an indirect immunoperoxydase 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 Peroxydase *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 May Grunwald Giemsa 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 mm^2 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) hydridoma 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 $MgCl_2$, concentrated and dialyzed against PBS using an Ultrafree M_r 30,000 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[^{3}H]]thymidine uptake into HEK 293 cell clones. Cells were seeded at a density of 1×10^4/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[^{3}H]]thymidine/well for 16 h and [Methyl[^{3}H]]thymidine incorporation in to DNA was determined on a TopCount Microplate Scintillation Counter (Packard, Rungis, France). Assays were performed in quadruplicate.

HUVEC Proliferation Assay. The cell growth was determined by measuring [Methyl[^{3}H]]thymidine uptake and by the reduction of MTT in a MTT viability assay (17). Briefly, HUVECs were seeded at a density of 5×10^3/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[^{3}H]]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/10^6 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. 1*A*). Two additional HEK 293-endocan cell clones injected in 8 mice also formed tumors, clearly avoiding clonal selection artifacts. In keeping with its

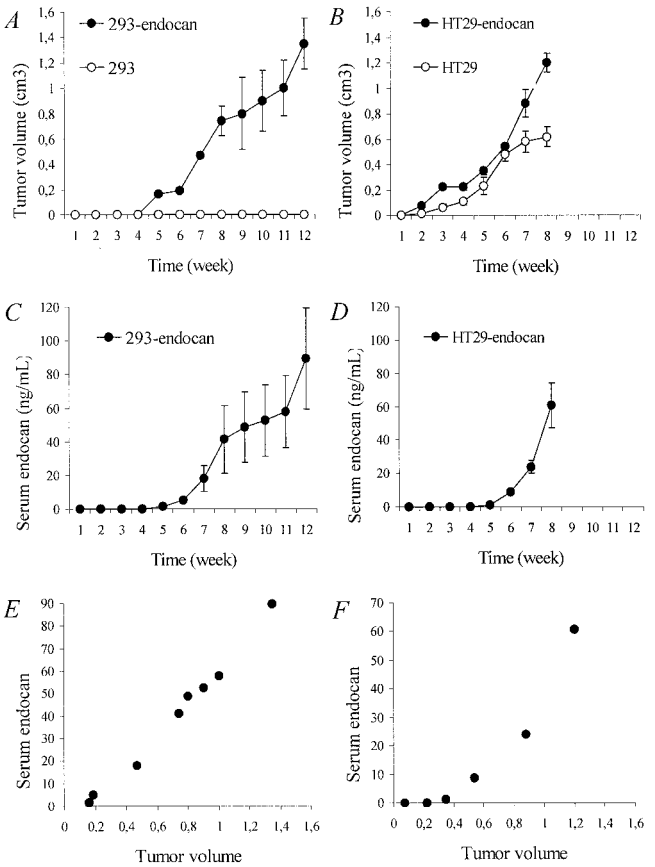

Fig. 1. Kinetics of tumor growth and serum endocan levels. In *A*, kinetics of 293-endocan cell growth (mean ± SE of 13 tumors). In *B*, kinetics of HT29-endocan cell growth (mean ± SE of 10 tumors). In *C*, kinetics of serum endocan in 293-endocan cell tumors. In *D*, kinetics of serum endocan in HT29-endocan cell tumors. In *E* and *F*, relationships between serum endocan value and tumor volume with 293-endocan cells (*E*) and in HT29-endocan cells (*F*).

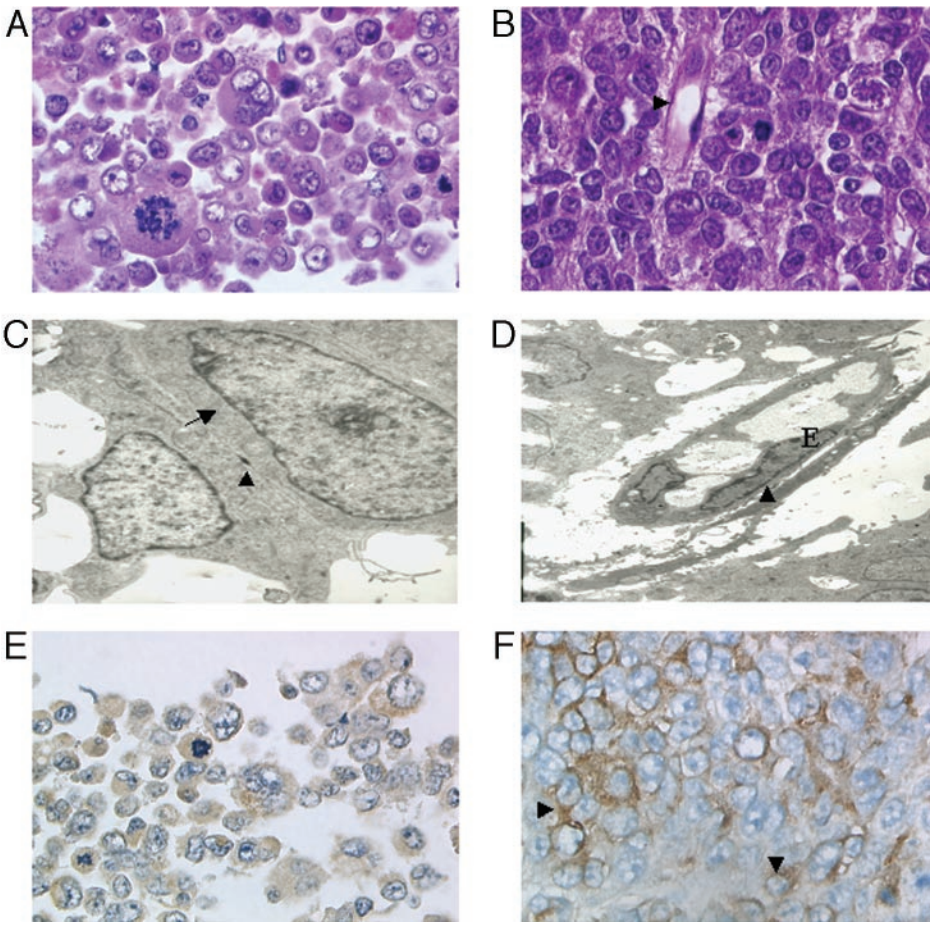

Fig. 2. Pathological analysis of 293-endocan tumors. In *A*, histological section of a pellet of 293-endocan cells stained with H&E: presence of tumoral cells with high nuclear/cytoplasmic ratio, multinucleated cells and abnormal mitoses, ×400. In *B*, histological section of the tumor showing a nontumoral well-differentiated capillary (*arrowhead*), ×400. In *C*, electron microscopic view of tumoral cells showing intracytoplasmic filaments (*arrows*) and a junctional structure (*arrowhead*). In *D*, electron microscopic view of a capillary within the tumor, with well-defined basement membrane (*arrowhead*) and normal endothelial cells (*E*). In *E*, histological section of a pellet of 293-endocan cells marked with an antibody directed against endocan: the staining is distributed throughout the whole cytoplasmic area. Indirect immunoperoxydase, ×400. In *F*, indirect immunoperoxydase with MEP 08 in a tumor section: staining within tumor cells (*arrowheads*), ×400.

function as a secreted PG, endocan was detected in the serum of mice that formed tumors (Fig. 1*C*). Importantly, circulating levels of endocan increased over time and correlated positively with tumor size ($r = 0.96$, $P < 0.001$, Spearmann correlation test; Fig. 1*E*).

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. 2*A*). 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 neoplasic cells, and the borders of the tumor were ill-defined (Fig. 2*B*). Quantitative analyses from six tumors showed 12.5 ± 2.9 mitotics cells/mm^2, 7.3 ± 2.3 apoptotic cells/mm^2, and 12.5 ± 2.9 vessels/mm^2), indicating a high degree of proliferation and tumor development contrasting with a virtually no leukocyte infiltrate (1.4 ± 1.4 inflammatory cells/mm^2). Electron microscopic study of the tumors showed that all of them had neoplasic cells with desmosomes and tonofilaments, two characteristics of epithelial cells (Fig. 2*C*). Moreover, the vessels observed within the tumors had an endothelium and a basement membrane that could be clearly identified (Fig. 2*D*). Immunohistological analysis of HEK 293 cell patches showed that the expression of endocan within 293-endocan cells is only cytoplasmic (Fig. 2*E*). In the corresponding tumors, antiendocan antibodies only marked the cytoplasmic areas (Fig. 2*F*). Thus, transfection of cDNA of endocan in human kidney epithelial cells HEK 293 give them an oncogenetic 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 ± 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. 1*B*). 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. 1*D*) and correlated with tumor size ($r = 0.72$, $P < 0.01$; Fig. 1*F*). 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 ± 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.

A Phenylalanine-rich Region of Endocan Polypeptide Is Required for Tumor Growth. To determine the role of the protein core of endocan in mediating tumor growth, we mutated the phenylalanine at positions 115 and/or 116 to alanine. Expression of these mutants in HEK 293 cells resulted in fully glycanated forms of endocan but with reduced ability to bind to phenyl-Sepharose. Endocan/F115A and endocan/F116A demonstrated half normal binding, whereas the combined mutation (endocan/F115A-F116A) did not bind at all. We established several HEK 293 cell clones expressing these mutants (665–1545 ng endocan/day/10^6 cells), and we injected these cells s.c. into SCID mice, as described above. All SCID mice grafted with HEK 293-endocan/F115A cells developed tumors at the site of inoculation (4 tumors/4 mice and 4 of 4). Serum endocan levels increased in parallel with tumor growth. Surprisingly, the HEK 293 cells, which expressed endocan/F116A or endocan/F115A-F116A, did not form any tumor despite the presence of the glycan (0 of 4 and 0 of 4 with HEK 293-endocan/F116A; 0 of 5 and 0 of 5 with HEK 293-endocan/F115A-F116A). Taken together, these results suggest that in addition to the glycan moiety, the endocan polypeptide plays a significant role in promoting tumor growth. The phenylalanine at position 116 is particularly critical for this effect.

MEP 08 mAb Directed against the Phenylalanine-rich Region Blocks the Tumor-Promoting Activity of HEK 293 Cells Overexpressing Endocan. To confirm the role of the endocan polypeptide in mediating tumor growth, we tested antiendocan mAbs as blocking agents. We focused on two mAbs, one that recognizes the region spanning the F116A (MEP 08) and one that recognizes the COOH-terminal portion of endocan (MEP 14). Mice were injected with 400 μg of purified mAb on a weekly basis. Serum antibodies were still detectable in large excess 7 days after i.p. injection of mAb. In a first series of experiments, mice were inoculated with HEK 293-endocan cells and received weekly injections of MEP 08 or MEP 14 starting at week 2 and continuing until week 12. Of the 13 mice that received MEP 08 in three separate experiments, 5 did not develop a tumor and 7 survived (Fig. 3*A*), which represents a clearly different feature from that of untreated groups of mice that develop tumors, except for 1 case (4 of 4, 4 of 4, and 3 of 4). Of the mice that received MEP 14, 1 did not develop a tumor, whereas the remaining 9 died from tumor formation. Administration of MEP 14 at the moment of the tumor cell inoculation did not change the survival 12 weeks after (1 of 6 and 0 of 4). These results suggest that MEP 08 recognizes an epitope that is a critical determinant for tumor growth. As MEP 08 epitope encompasses the phenylalanine-rich region, the data suggest that this region is critically involved in mediating tumor growth.

MEP 08 Prevents Tumor Appearance and Slows Tumor Growth. To examine the efficiency of MEP 08 on macroscopically established tumors, we initiated a second series of experiments, starting MEP 08 injections either on the same day of tumor cell inoculation (group 0), or 2, 4, or 6 weeks after tumor cell inoculation (groups 2, 4, and 6, respectively). Sixty percent of mice in groups 0 and 2 survived, whereas 31 and 22% of groups 4 and 6 survived, respectively, as compared with 13% in the untreated control group (Fig. 3*B*). Thus, the MEP 08 is most effective in the early stages of tumor 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. 3*C*). 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. 3*C*). Taken together, these findings suggest that MEP 08 mAb exerts an antitumor activity on xenografted tumors of small sizes.

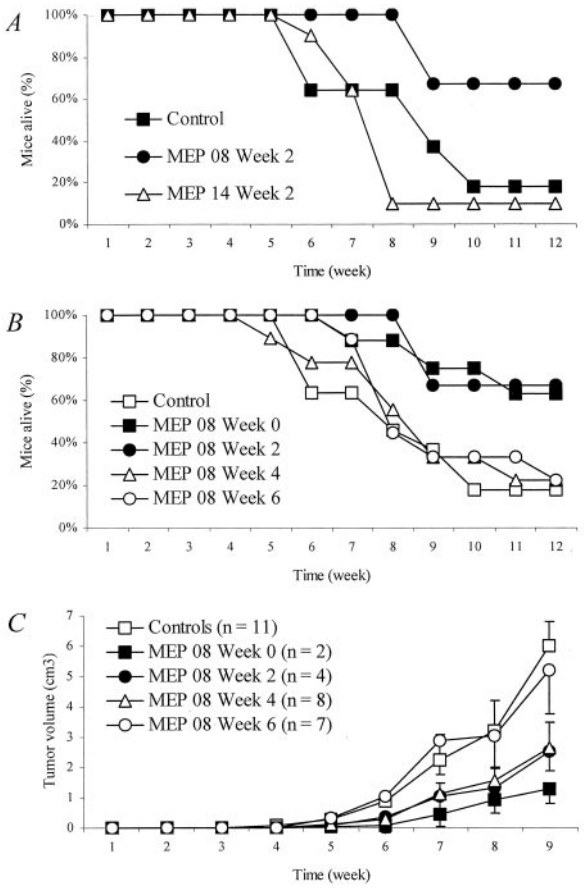

Fig. 3. Treatment with antiendocan mAb MEP 08 reduces tumor incidence and delays tumor appearance. In *A*, kinetics of survival of mice not treated (□) or treated with MEP 08 mAb (●) or MEP 14 mAb (▲). In *B*, relationships between survival and the beginning of the mAb treatment. In *C*, MEP 08 therapy slows down tumor growth (mean ± SE of tumor volume).

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 [^{3}H]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,

Table 1 Effect of MEP 08 and MEP 14 on [^{3}H]thymidine incorporation by HEK 293 cell clones

Cells	Medium	cpm
293	-	327 ± 60
	+MEP08	297 ± 69
	+MEP14	300 ± 55
293 clone 1	-	522 ± 87
	+MEP08	630 ± 198
	+MEP14	540 ± 117
293-clone 2	-	667 ± 83
	+MEP08	710 ± 153
	+MEP14	671 ± 123
293-endocan clone 1	-	1443 ± 387
	+MEP08	1209 ± 588
	+MEP14	1095 ± 666
293-endocan clone 3	-	3841 ± 484
	+MEP08	3066 ± 721
	+MEP14	3279 ± 1026
293-endocan clone 8	-	873 ± 162
	+MEP08	1020 ± 342
	+MEP14	1074 ± 120
293-endocan/S137A clone 2	-	1551 ± 485
	+MEP08	1924 ± 377
	+MEP14	1654 ± 293
293-endocan/S137A clone 4	-	970 ± 170
	+MEP08	1194 ± 357
	+MEP14	1182 ± 513
293-endocan/S137A clone 5	-	2688 ± 985
	+MEP08	3704 ± 1019
	+MEP14	2973 ± 839

addition of recombinant endocan in HUVEC cultures did not modify the FGF-2-induced [^{3}H]thymidine incorporation (Fig. 4*A*) despite doses 100-fold greater than that secreted. By contrast, addition of HS still increased [^{3}H]thymidine uptake (Fig. 4*A*). 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. 4*B*).

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. 4*C*). According to the international classification, the highest levels of serum endocan were found in patients with extended forms of cancer. These results suggest that endocan may represent a marker of human cancer.

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 [^{3}H]thymidine uptake by HEK 293 cells (13). 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

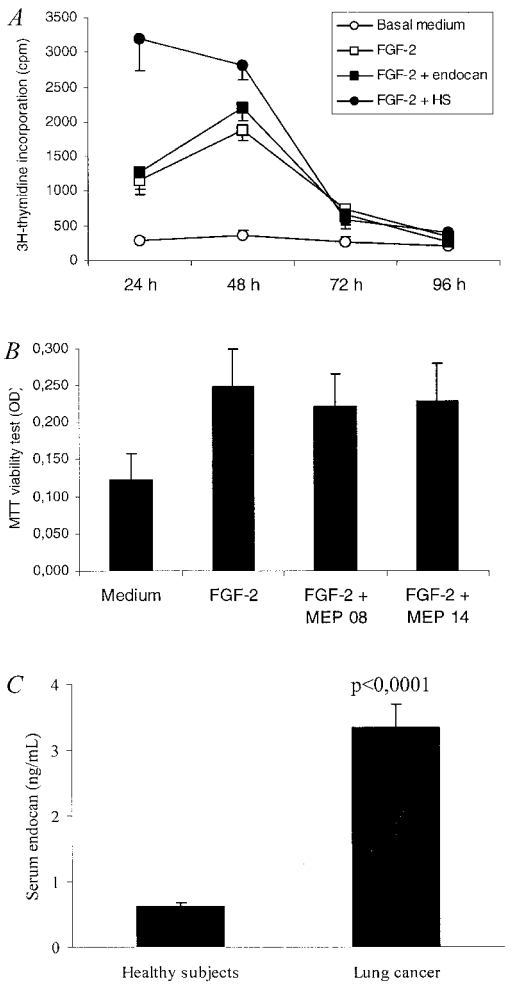

Fig. 4. No effects of endocan and MEP 08 on FGF-2-induced HUVEC proliferation. In *A*, kinetics of [methyl]^{3}H thymidine uptake in HUVEC treated with 5 ng/ml FGF-2 (□), FGF-2 + 1 μg/ml endocan (■), FGF-2 + 1 μg/ml heparan sulfate (●). Data are mean ± SE of three experiments. In *B*, MTT viability test of HUVECs treated for 96 h with 5 ng/ml FGF-2, FGF-2 + 10 μg/ml MEP 08, and FGF-2 + 10 μg/ml MEP 14 (mean ± SD of one representative experiment of two done in quadruplicate). In *C*, increased levels of serum endocan in patients with lung cancer (mean ± SE of 50 patients *versus* 25 healthy individuals, $P < 0.0001$, Mann-Whitney test).

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 cytostatic 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 antitumoral activity of antibodies requires Fc receptor engagement (29–31). Interestingly, two different antiendocan mAbs of the same isotype exhibited different antiproliferate 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 HEK 293 cells is under the control of the strong cytomegalovirus enhancer/promoter, explaining why serum endocan levels are increased during the development of the tumor. In this situation, serum levels of endocan were directly correlated with the size of the tumor. In human beings, we know at present that endocan is mainly produced by the vascular endothelial cells (12, 14). It seems logical to postulate that the increased serum endocan in patients with lung cancer might both reflect the activation of tumor vascular bed and perhaps the tumoral proliferation. However, it is too early for considering that circulating endocan may already represent an useful marker for cancer in humans. Additional investigations are needed to evaluate this point.

In conclusion, our results suggest that: (*a*) endocan promotes tumor growth; and (*b*) its activity involves interactions with growth factors through the glycan and with the effector cells of the immune response through the polypeptide. Taken together these data raise the concept that in response to angiogenic factors, the vascular endothelial cells from solid tumors may secrete endocan, which, in turn, may result in a powerful control of the tumor development. So endocan might represent in the future an original and a novel target for anticancer therapy and additionally a marker for some kinds of solid tumors.

ACKNOWLEDGMENTS

We thank Gwenola Kervoaze for her assistance in cell culture, Genevieve Marchandise for endocan ELISA, and Philippe Marquillies for his help in animal facility.

REFERENCES

1. Kjellen, L., and Lindahl, U. Proteoglycans: structures and interactions. Annu. Rev. Biochem., *60:* 443–475, 1991.
2. Iozzo, R. V. Matrix proteoglycans: from molecular design to cellular function. Annu. Rev. Biochem., *67:* 609–652, 1998.
3. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem., *68:* 729–777, 1999.
4. Saku, T., and Furthmayr, H. Characterization of the major heparan sulfate proteoglycan secreted by bovine aortic endothelial cells in culture. Homology to the large molecular weight molecule of basement membranes. J. Biol. Chem., *264:* 3514–3523, 1989.
5. Mertens, G., Cassiman, J. J., Van den Berghe, H., Vermylen, J., and David, G. Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. J. Biol. Chem., *267:* 20435–43, 1992.
6. Kojima, T., Shworak, N. W., and Rosenberg, R. D. Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. J. Biol. Chem., *267:* 4870–4877, 1992.
7. Jarvelainen, H. T., Kinsella, M. G., Wight, T. N., and Sandell, L. J. Differential expression of small chondroitin/dermatan sulfate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. J. Biol. Chem., *266:* 23274–81, 1991.
8. Jarvelainen, H. T., Iruela-Arispe, M. L., Kinsella, M. G., Sandell, L. J., Sage, E. H., and Wight, T. N. Expression of decorin by sprouting bovine aortic endothelial cells exhibiting angiogenesis *in vitro*. Exp. Cell Res., *203:* 395–401, 1992.
9. Nelimarkka, L., Salminen, H., Kuopio, T., Nikkari, S., Ekfors, T., Laine, J., Pelliniemi, L., and Jarvelainen, H. Decorin is produced by capillary endothelial cells in inflammation-associated angiogenesis. Am. J. Pathol., *158:* 345–353, 2001.
10. Nelson, R. M., Venot, A., Bevilacqua, M. P., Linhardt, R. J., and Stamenkovic, I. Carbohydrate-protein interactions in vascular biology. Annu. Rev. Cell. Dev. Biol., *11:* 601–631, 1995.
11. Delehedde, M., Lyon, M., Sergeant, N., Rahmoune, H., and Fernig, D. G. Proteoglycans: pericellular and cell surface multireceptors that integrate external stimuli in the mammary gland. J. Mammary Gland Biol. Neoplasia, *6:* 253–273, 2001.
12. Lassalle, P., Molet, S., Janin, A., Heyden, J. V., Tavernier, J., Fiers, W., Devos, R., and Tonnel, A. B. ESM-1 is a novel human endothelial cell-specific molecule expressed in lung and regulated by cytokines. J. Biol. Chem., *271:* 20458–64, 1996.
13. Bechard, D., Gentina, T., Delehedde, M., Scherpereel, A., Lyon, M., Aumercier, M., Vazeux, R., Richet, C., Degand, P., Jude, B., Janin, A., Fernig, D. G., Tonnel, A. B., and Lassalle, P. Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. J. Biol. Chem. *276:* 48341–9, 2001.
14. Bechard, D., Meignin, V., Scherpereel, A., Oudin, S., Kervoaze, G., Bertheau, P., Janin, A., Tonnel, A., and Lassalle, P. Characterization of the secreted form of endothelial-cell-specific molecule 1 by specific monoclonal antibodies. J. Vasc. Res., *37:* 417–425, 2000.
15. Bechard, D., Scherpereel, A., Hammad, H., Gentina, T., Tsicopoulos, A., Aumercier, M., Pestel, J., Dessaint, J. P., Tonnel, A. B., and Lassalle, P. Human endothelial-cell specific molecule-1 binds directly to the integrin CD11a/CD18 (LFA-1) and blocks binding to intercellular adhesion molecule-1. J. Immunol., *167:* 3099–3106, 2001.
16. Lassalle, P., LaGrou, C., Delneste, Y., Sanceau, J., Coll, J., Torpier, G., Wietzerbin, J., Stehelin, D., Tonnel, A. B., and Capron, A. Human endothelial cells transfected by SV40 T antigens: characterization and potential use as a source of normal endothelial factors. Eur. J. Immunol., *22:* 425–431, 1992.
17. Sladowski, D., Steer, S. J., Clothier, R. H., and Balls, M. An improved MTT assay. J. Immunol. Methods, *157 (1–2):* 203–207, 1993.
18. Alexander, C. M., Reichsman, F., Hinkes, M. T., Lincecum, J., Becker, K. A., Cumberledge, S., and Bernfield, M. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. Nat. Genet., *25:* 329–332, 2000.
19. Kleeff, J., Wildi, S., Kumbasar, A., Friess, H., Lander, A. D., and Korc, M. Stable transfection of a glypican-1 antisense construct decreases tumorigenicity in PANC-1 pancreatic carcinoma cells. Pancreas, *19:* 281–288, 1999.
20. Lin, H., Huber, R., Schlessinger, D., and Morin, P. J. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res., *59:* 807–810, 1999.
21. Santra, M., Mann, D. M., Mercer, E. W., Skorski, T., Calabretta, B., and Iozzo, R. V. Ectopic expression of decorin protein core causes a generalized growth suppression in neoplastic cells of various histogenetic origin and requires endogenous p21, an inhibitor of cyclin-dependent kinases. J. Clin. Investig., *100:* 149–157, 1997.
22. Mathiak, M., Yenisey, C., Grant, D. S., Sharma, B., and Iozzo, R. V. A role for perlecan in the suppression of growth and invasion in fibrosarcoma cells. Cancer Res., *57:* 2130–2136, 1997.
23. Marchisone, C., Del Grosso, F., Masiello, L., Prat, M., Santi, L., and Noonan, D. M. Phenotypic alterations in Kaposi's sarcoma cells by antisense reduction of perlecan. Pathol. Oncol. Res., *6:* 10–17, 2000.
24. Sharma, B., Handler, M., Eichstetter, I., Whitelock, J. M., Nugent, M. A., and Iozzo, R. V. Antisense targeting of perlecan blocks tumor growth and angiogenesis *in vivo*. J. Clin. Investig., *102:* 1599–608, 1998.
25. Schmits, R., Kundig, T. M., Baker, D. M., Shumaker, G., Simard, J. J., Duncan, G., Wakeham, A., Shahinian, A., van der Heiden, A., Bachmann, M. F., Ohashi, P. S., Mak, T. W., and Hickstein, D. D. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J. Exp. Med. *183:* 1415–1426, 1996.
26. Shier, P., Otulakowski, G., Ngo, K., Panakos, J., Chourmouzis, E., Christjansen, L., Lau, C. Y., and Fung-Leung, W. P. Impaired immune responses toward alloantigens and tumor cells but normal thymic selection in mice deficient in the $\beta 2$ integrin leukocyte function-associated antigen-1. J. Immunol. *157:* 5375–5386, 1996.
27. Shier, P., Ngo, K., and Fung-Leung, W. P. Defective CD8+ T-cell activation and cytolytic function in the absence of LFA-1 cannot be restored by increased TCR signaling. J. Immunol. *163:* 4826–4832, 1999
28. Aitkenhead, M., Wang, S. J., Nakatsu, M. N., Mestas, J., Heard, C., and Hughes, C. C. Identification of endothelial cell genes expressed in an *in vitro* model of angiogenesis: induction of ESM-1, βig-h3, and NrCAM. Microvasc. Res., *63:* 159–171, 2002.
29. Clynes, R., Takechi, Y., Moroi, Y., Houghton, A., and Ravetch, J. V. Fc receptors are required in passive and active immunity to melanoma. Proc. Natl. Acad. Sci. USA, *95:* 652–656, 1998
30. Isaacs, J. D., Greenwood, J., and Waldmann, H. Therapy with monoclonal antibodies. II. The contribution of Fc γ receptor binding and the influence of C(H)1 and C(H)3 domains on *in vivo* effector function. J. Immunol., *161:* 3862–3869, 1998.
31. Clynes, R. A., Towers, T. L., Presta, L. G., and Ravetch, J. V. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. Nat. Med. *6:* 443–446, 2000.

Overexpression of Endocan Induces Tumor Formation

Arnaud Scherpereel, Thibaut Gentina, Bogdan Grigoriu, et al.

Cancer Res 2003;63:6084-6089.