Carboxymethyl Benzylamide Dextran Blocks Angiogenesis of MDA-MB435 Breast Carcinoma Xenografted in Fat Pad and Its Lung Metastases in Nude Mice¹

Rozita Bagheri-Yarmand,² Yamina Kourbali, Ana Maria Rath, Roger Vassy, Antoine Martin, Jacqueline Jozefonvicz, Claudine Soria, He Lu, and Michel Crépin


Abstract

We previously showed that carboxymethyl benzylamide dextran (CMDB7) prevents tumor growth and tumor angiogenesis by binding to angiogenic growth factors, thereby preventing them from reaching their receptors on tumor or stromal cells (Bagheri-Yarmand et al. Br. J. Cancer, 78: 111–118, 1998; Bagheri-Yarmand et al. Cell Growth Differ., 9: 497–504, 1998). In this study, CMDB7 inhibited neovessel formation within the fibroblast growth factor 2–enriched matrigel in mice, and its anticancer effect was then tested in a metastatic breast cancer model. Human MDA-MB435 cells were injected into the mammary fat pad of nude mice, and breast tumors developed within a week; all of the mice had lung metastases at 12 weeks. CMDB7 treatment (50, 150, or 300 s.c. or 300 i.v. mg/kg/week for 10 weeks) reduced the incidence of lung metastases to 12%. Histological analysis showed markedly less tumor neovascularization in the CMDB7-treated mice. Pulmonary metastasis incidence was strongly dependent on the intratumoral neangiogenesis in primary tumors.

Introduction

Tumor-associated neovascularization is a prerequisite of rapid tumor growth and metastasis (1, 2). Increased vascularity may allow for not only enhanced tumor growth but also a greater chance of hematogenous tumor embolization. Thus, it is hypothesized that inhibiting tumor angiogenesis will halt tumor growth and decrease metastatic potential. Weidner et al. (3) demonstrated a statistically significant correlation between the incidence of metastases and microvessel counts in invasive breast carcinoma. Thus, antiangiogenic agents targeting the tumor vasculature are expected to block neovascularization and thereby prevent metastasis (4).

We have previously reported (5) that CMDB7 displays an in vitro growth-inhibitory activity in breast tumoral cells. A positive correlation was found between the inhibition of cell proliferation and the overall content of benzyamide (5). Growth inhibition was associated with a decrease in the proportion of S-phase cells and an accumulation of G1 phase cells (5). CMDB7 specifically inhibited the mitogenic effect and receptor binding of angiogenic growth factors, such as FGF-2, FGF-4, platelet-derived growth factor BB, and transforming growth factor β1, by forming complexes with them (6–8) and prevented endothelial cell proliferation and migration (7). In vivo studies demonstrated that growth of MCF-7 (8) and FGF-4-transfected HBL100 cell (7) tumors in nude mice was blocked by CMDB7 treatment, and histological analysis showed much less neovascularization in these tumors.

We have now investigated the effect of CMDB7 on FGF-2–induced angiogenesis in a matrigel angiogenic assay. Estrogen receptor–negative MDA-MB435 breast carcinoma cells were xenografted into nude mice to determine whether CMDB7 might have therapeutic utility in the prevention or treatment of metastatic breast cancer. After injection into m.f.p., these cells gave rise to metastases in lung organ and thus provided an experimental model for the metastasis of a highly aggressive human breast carcinoma (9).

Materials and Methods

Dextran Derivative Preparation. A water-soluble dextran derivative (CMDB7) was prepared from dextran T40, as described previously (10), by a statistical substitution of dextran in two steps: carboxymethylation, followed by the coupling of benzyamide. This derivative was equilibrated and purified by ultrafiltration and then lyophilized. The chemical composition was determined by acidimetric titration and elementary analysis of nitrogen (dextran, 0%; carboxymethyl, 70%; benzyamide, 30%; mass apparent molecular weight = 80,000 g/mol).

Cell Line and Cell Cultures. MDA-MB435 is an estrogen receptor–negative cell line isolated from the pleural effusion of a patient with breast carcinoma (11). The cells were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 2 mM l-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO2. Cells were routinely passed once a week at a 1:10 split ratio.

Murine Angiogenesis Assay. Angiogenesis was assayed as the growth of blood vessels from s.c. tissue into a solid gel of reconstituted basement membranes containing the test sample (12). Matrigel (11.46 mg/ml; Becton Dickinson Labware, Bedford, MA) in liquid form at 4°C was mixed with FGF-2 (1 μg) with or without different concentrations of CMDB7 and injected into the abdominal s.c. tissue of five mice/group. At body temperature, matrigel rapidly solidified, thereby trapping the factor, assuring its slow release, and prolonging exposure of surrounding tissues. Mice were killed 2 weeks later, and the matrigel plugs were exposed for photography. NIH image analysis (see “Image Analysis”) was applied to quantitate the vascularization in each tissue section, the extent of which is expressed as the percentage area of labeled endothelial cells ± SE on each slide.

Animal Model for Metastases. Female athymic nude mice (nu/nu), 3 weeks old, were obtained from Janvier Laboratory (Le-Genest-St-Isle, France). Animals were kept in a temperature-controlled room on a 12 h/12 h light/dark schedule with food and water ad libitum. MDA-MB435 cells were grown in DMEM supplemented with 10% FCS in T150 plates and harvested at 80% confluence. The m.f.p. has been shown to be a more favorable graft site for the growth of mouse mammary tumors—because of its good blood supply as compared with the subcutis—and also for the dissemination of metastases from the m.f.p. tumors (9). Mice were anesthetized with Metofane, and a 5-mm incision was made in the skin over the lateral thorax to expose the m.f.p. The inoculum (106 cells/0.1 ml) was injected into the tissue taking care to avoid the s.c. space. All of the mice developed tumors after about 1 week. CMDB7...
treatment was initiated 2 weeks after cell inoculation, when tumors were well established (approximately 0.34 cm³). Mice (n = 42) were arbitrarily assigned to receive 0.1 ml of PBS s.c. (controls, n = 10) or CMDB7 injected s.c. close to the tumor at 50, 150, or 300 mg/kg/week (n = 8) or i.v. at 300 mg/kg/week (n = 8) for 10 weeks. Tumors were measured along two major axes with calipers. Tumor volume was calculated as follows:

\[ V = \frac{4}{3} \pi R_1^2 R_2 \]

where \( R_1 \) is radius 1, \( R_2 \) is radius 2, and \( R_1 < R_2 \).

Tissue Preparation and Immunohistochemical Analysis. Immediately after surgical resection, primary tumor specimens were weighed and cut into small pieces; fragments were fixed with 4% formalin processed to paraffin in the usual way, and 4-mm sections were stained with H&E. Endothelial cells were specifically stained with GSL-1 (Vector Laboratories, Burlingame, CA). The GSL-1 lectin binds specifically to galactosyl residues and thus labels the vascular endothelium in mice (13). Sections were deparaffinized and rehydrated. Endogenous peroxidase was inactivated with 3% H₂O₂ and washed in TBS (pH 7.6) followed by preincubation in FCS for 30 min at room temperature. The sections were then incubated for 45 min with biotinylated GSL-1 (0.01 mg/ml), washed with TBS, and treated for 30 min with avidin-peroxidase (Vector Laboratories), and washed again with TBS. The peroxidase was visualized by incubation for 10 min in 0.1M acetate buffer (pH 5.2) containing 3% H₂O₂ and 3% 3-amino-9-ethylcarbazole. Finally, the slides were washed in distilled water and tap water, counterstained with hematoxylin, dehydrated, and coverslipped with Permount.

Image Analysis. For each GSL-1-labeled section of control and CMDB7-treated tumors, five fields containing exclusively viable tumoral cells, as indicated by the hematoxylin stain, were selected randomly for analysis. Image analysis was performed on a Power Macintosh computer 8500/120 using the public domain NIH program. The endothelial cell area in each section was calculated as the ratio of the labeled area to the total viewed area × 100; these values were then averaged for untreated (control) and treated-CMDB7 tumors.

Statistical analysis. The results are presented as means ± SE. Multiple statistical comparisons were performed using ANOVA and the Mann-Whitney U tests in a multivariate linear model.

Results

CMDB7 Inhibits Angiogenesis Induced by FGF-2 in Matrigel.

In the murine angiogenic assay, FGF-2 mixed with matrigel was injected s.c. into 20 mice. The mice were killed 2 weeks later for evaluation. Each group contained 5 animals, and a representative animal from each group is shown. Matrigel containing: 1 μg of FGF-2 (A); 1 μg of FGF-2 and 2 mg of CMDB7 (B); 1 μg of FGF-2 and 4 mg of CMDB7 (C); and 1 μg of FGF-2 and 8 mg of CMDB7 (D). E, the antiangiogenic effect of CMDB7 was quantitated by NIH image analysis, and data are expressed as the mean percentage areas ± SE (bars) of endothelial cells on day 14 (n = 5). *, significantly different from controls (P < 0.001).

Fig. 1. The inhibition by CMDB7 of FGF-2-induced angiogenesis in mice. Mice received s.c. injections of 300 μl of a matrigel mixture containing FGF-2 with or without CMDB7. The animals were killed and dissected 2 weeks later, and the matrigel plugs were exposed and photographed. Each group contained 5 animals, and a representative animal from each group is shown. Matrigel containing: 1 μg of FGF-2 (A); 1 μg of FGF-2 and 2 mg of CMDB7 (B); 1 μg of FGF-2 and 4 mg of CMDB7 (C); and 1 μg of FGF-2 and 8 mg of CMDB7 (D). E, the antiangiogenic effect of CMDB7 was quantitated by NIH image analysis, and data are expressed as the mean percentage areas ± SE (bars) of endothelial cells on day 14 (n = 5). *, significantly different from controls (P < 0.001).

Fig. 2. CMDB7 treatment does not affect tumor growth of MDA-MB435 carcinoma cells. MDA-MB435 tumor cells (10⁶ cells/site) were injected into the m.f.p. When tumor volume reached 0.34 cm³ (n = 42), CMDB7 was administered s.c. at 50, 150, or 300 mg/kg/week or i.v. at 300 mg/kg/week for 10 weeks. Tumor growth was measured for up to 10 weeks, and the results are presented as the mean volumes ± SE (bars) of tumor obtained from 10 control mice and from 8 mice in each treated group.

morphometric analysis of matrigel plugs. Plugs containing FGF-2 (1 μg) were bright red and often contained superficial blood vessels (Fig. 1A). Growth of blood vessels from s.c. tissue and their penetration into the solidified matrigel was totally dependent on FGF-2 (data not shown). When 2, 4, or 8 mg of CMDB7 were added to the matrigel, angiogenesis was greatly inhibited compared with the control (Fig. 1 B-D). Image analysis quantification showed that neovascularization was 16 and 85% significantly lower when the matrix contained 2 and 4 mg of CMDB7, respectively, and was almost completely inhibited in the presence of 8 mg of CMDB7 (Fig. 1E).

**CMDB7 Inhibition of Tumor Angiogenesis and Lung Metastasis of MDA-MB435 Human Breast Carcinomas.** After injection of 10⁶ MDA-MB435 cells into the m.f.p.; all of the mice had developed tumors by the end of the first week and CMDB7 treatment was initiated 1 week later, when the tumors were well established. CMDB7 treatment (50, 150, or 300 mg/kg/week s.c. and 300 mg/kg/week i.v.) did not affect significantly primary tumor growth as compared with untreated control tumors (Fig. 2). At 12 weeks, all of the mice were killed, and the numbers and locations of lung metastases were recorded. Although all (10 of 10) of the untreated mice bearing primary breast carcinomas had microscopic lung metastases; lung metastases developed in only 1 of the 8 mice in each CMDB7-treated group (Fig. 3). No differences were found in the numbers of metastatic foci and their locations between lung metastasis in untreated and CMDB7-treated groups.

GSL-1 selectively labeled endothelial cells (Fig. 4, A and B) and thus enabled the relative density of endothelial cells (percentage of area occupied by endothelial cells) to be determined based on the image analysis of the detection of the label. The mean percentage of endothelial cell area in viable fields of control tumors (4.89 ± 0.9; 50 fields in 10 tumors) are compared with those in tumors treated with CMDB7 at 50, 150, or 300 mg/kg/week s.c. or 300 mg/kg/week by i.v., respectively: 1.71 ± 0.76, 1.5 ± 0.6, 1.28 ± 0.51 (120 fields in 24 tumors) and 0.53 ± 0.21 (40 fields in 8 tumors; Fig. 4C). The endothelial cell densities in all of the CMDB7 treatment groups were significantly lower (P < 0.001). Similar results were obtained with CD31 antibody but with nonspecific cell staining (data not shown). In each treated group, 1 tumor escaped CMDB7-induced inhibition and had a high density of endothelial cells (Fig. 3). These results indicate that the prevention of angiogenesis in primary tumors was responsible for this antimitastatic effect.

**Dependence of the Incidence of Lung Metastases on Primary Tumor Angiogenesis.** The mean ± SE percentage of endothelial cell area for 28 nonmetastatic tumors was significantly lower than that for micrometastasized 14 tumors: 0.8 ± 0.5 and 4.82 ± 2.7, respectively (P < 0.001). We showed that lung micrometastases developed when the percentage area of endothelial cells reached a threshold of 2% (Fig. 3). This finding confirmed the relationship between microvascular density in primary tumor and the development of metastases.

**No Toxicity of CMDB7 in Nude Mice.** The body weight of the inoculated mice (s.c. or i.v. injections) was not affected by CMDB7 at all of the doses tested after 10 weeks of treatment (data not shown). CMDB7 administrated i.v. or s.c. at 300 mg/kg produced no signs of toxicity such as diarrhea, infection, weakness, and lethargy. All of the 32 treated mice were alive at the end of 10 weeks.

**Discussion**

In this study, we assessed the antimitastatic activity of CMDB7 against estrogen receptor-negative MDA-MB435 xenografted tumors in nude mice. This model—based on tumor cell injection into m.f.p. followed by metastasis to the lung—mimics the clinical situation, in which tumors become estrogen receptor-negative and develop resistance to the antiestrogen tamoxifen after some duration of treatment.
This study confirmed that the injection of 10^6 viable MDA-MB435 cells yielded a 100% tumor-take rate in the m.f.p. (9).

This study demonstrated a direct relationship between neovascularization within the primary tumors and the incidence of the percentage distant micrometastases. The results showed that lung micrometastases developed when the percentage area of endothelial cells reached a threshold of 2%. The prevalence of metastases increased as this area within the primary tumor increased. Our findings are consistent with previous studies (14–17) that demonstrated a direct correlation between blood vessel density in primary tumors and their metastasis dissemination. Tumor microvessels often had fragmented basal membranes, which suggested that these vessels were structurally incomplete and leaky and, thus, that tumor cells could easily reach the circulation (14, 18).

Untreated mice had highly vascularized tumors, and microscopic lung metastases were observed in all of them. In contrast, CMDB7-treated mice exhibited a very low incidence of lung metastases. This low metastatic rate could reflect to the inhibited intratumoral angiogenesis because, at 50 mg/kg/week, CMDB7 very significantly blocked the growth of intratumoral vessels. CMDB7, when administered i.v. is also active and, moreover, is the most clinically applicable. CMDB7 as well as other polyanionic polysaccharides such as heparin could be active when given p.o. (19). Additional studies are necessary to confirm this hypothesis.

It has been reported that MDA-MB435 cells produce angiogenic factors, such as FGF-2 and vascular endothelial growth factor, that are detectable in their culture supernatants (20). Thus, CMDB7 could exert its antiangiogenic action by disrupting the autocrine and paracrine effects of growth factors released by the tumor cells.

We also demonstrated that CMDB7 had strong antiangiogenic and antimetastatic effects without inhibiting the primary tumor growth of xenografted MDA-MB435 tumors. This observation can be explained by the fact that MDA-MB435 mammary tumors grow slowly in vivo, and, thus, initial tumor growth may be independent of angiogenesis when the tumor size in ≤ 3 mm^3 (1). However, for rapidly growing s.c. tumors, such as xenografted MCF-7ras (8) and FGF-4-transformed human breast cells (IH9; Ref. 7), CMDB7 inhibited tumor growth in parallel with less tumor angiogenesis.

The antiangiogenic activity of CMDB7 was further supported by the results of experiments using the model of tumor-free angiogenesis FGF-2-enriched matrigel. Indeed, inclusion of CMDB7 in the matrigel inhibited FGF-2-induced angiogenesis in a dose-dependent manner. These findings are in agreement with our earlier studies, which showed that CMDB7 inhibited the in vitro migration and proliferation of endothelial cells without acting on cell viability (7). Thus, CMDB7 is cytostatic but not cytotoxic for endothelial cells. We reported previously (6) that CMDB7 inhibited HBL100 cell proliferation by interfering with the FGF-2 autocrine loop and that CMDB7 inhibited the paracrine mitogenic activity and receptor binding of fibroblast growth factor BB and platelet-derived growth factor BB (7, 8). Taken together, these new data confirmed that CMDB7, by blocking the activities of angiogenic growth factors as described in our previous reports, is a potent inhibitor of tumor angiogenesis and metastasis in vivo. We cannot exclude additional therapeutic effects of CMDB7 such as the inhibition of the number of tumor-associated macrophages infiltrating in vivo.

In conclusion, the data obtained with our model of human breast cancer highlighted: (a) the dependence of the incidence of distant metastasis development on the intratumoral angiogenesis of the primary tumors; and (b) the ability of CMDB7 to effectively inhibit angiogenesis in these xenografted tumors and prevent their distant metastasis to the lungs. Boehm et al. (21) demonstrated that a specific angiogenesis inhibitor (endostatin) did not induce drug resistance in three different types of transplantable murine tumors. Thus, CMDB7 and other antiangiogenic agents could be expected to be promising as novel pharmacologic agents in cancer therapy, especially when the patients develop acquired drug resistance to cytotoxic drugs. CMDB7 could prove beneficial in preventing the occurrence of secondary metastases and in inducing tumor dormancy through its cytostatic action on endothelial cells.

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References

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