

Effects of Vitamin D-binding Protein-derived Macrophage-activating Factor on Human Breast Cancer Cells

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Abstract. *Background: Searching for additional therapeutic tools to fight breast cancer, we investigated the effects of vitamin D-binding protein-derived macrophage activating factor (DBP-MAF, also known as GcMAF) on a human breast cancer cell line (MCF-7). Materials and Methods: The effects of DBP-MAF on proliferation, morphology, vimentin expression and angiogenesis were studied by cell proliferation assay, phase-contrast microscopy, immunohistochemistry and western blotting, and chorioallantoic membrane (CAM) assay. Results: DBP-MAF inhibited human breast cancer cell proliferation and cancer cell-stimulated angiogenesis. MCF-7 cells treated with DBP-MAF predominantly grew in monolayer and appeared to be well adherent to each other and to the well surface. Exposure to DBP-MAF significantly reduced vimentin expression, indicating a reversal of the epithelial/mesenchymal transition, a hallmark of human breast cancer progression. Conclusion: These results are consistent with the hypothesis that the known anticancer efficacy of DBP-MAF can be ascribed to different biological properties of the molecule that include inhibition of tumour-induced angiogenesis and direct inhibition of cancer cell proliferation, migration and metastatic potential.*

Breast cancer is the most common type of cancer in women and one of the most common causes of cancer death. This leads to the continuous search for additional therapies that could be used as a complement to or alternative to surgery, radiation therapy and conventional chemotherapy. Among these novel approaches, those that target the immune system,

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often referred to as immunotherapy, and tumour-induced angiogenesis, appear promising.

Vitamin D-binding protein-derived macrophage-activating factor (DBP-MAF, also known as GcMAF) seems to be a good candidate for both immunotherapy and antiangiogenic therapy in that it has the ability to stimulate macrophages (1), and inhibit angiogenesis (2-5). The effects of DBP-MAF on 16 non-anaemic women who received weekly administration of DBP-MAF as treatment for metastatic breast cancer were recently described. Such a treatment yielded significant results, mainly attributed to activation of tumouricidal macrophages (6). It noteworthy, however, that the anti-angiogenic properties of DBP-MAF might also have contributed to these results, in particular considering that angiogenesis plays a key role in breast cancer progression (7). In fact, another recent study investigating the effects of DBP-MAF on tumour growth in a severe combined immunodeficiency (SCID) mouse xenograft model of human hepatocellular carcinoma (HCC) reported that the tumours in the treatment group had fewer microvessels than those in the untreated control group (8). In addition to these indirect anticancer properties, it was recently demonstrated that DBP-MAF directly inhibited proliferation, migration and metastatic potential of transformed cells, with particular reference to prostate cancer cells (9).

Therefore, based on these promising results, we decided to investigate the effects of a commercially available DBP-MAF preparation on a widely studied human breast cancer cell line MCF-7, focusing on the effects on proliferation, morphology, vimentin expression and cancer cell-induced angiogenesis. The MCF-7 cell line was chosen as an experimental model because of its characteristics that help to distinguish the effects of DBP-MAF from those of vitamin D (see the Discussion section).

Materials and Methods

Commercially available DBP-MAF and vitamin D-binding protein (VDBP), *i.e.* the precursor of DBP-MAF, were obtained from Immuno Biotechnology (Dover, Kent, UK). All other common reagents were from Sigma Aldrich (Milan, Italy).

Cell line. MCF-7 cells were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ in Eagle's minimum essential medium in Earle's Balanced salt solution, supplemented with 1 mM sodium pyruvate, 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Experiments with DBP-MAF were performed in medium containing 1% FBS after 24 h starvation in serum-free medium. In all experiments, control cells were treated with VDBP at the same concentration as that of DBP-MAF (0.4 ng/ml-40 ng/ml). We chose this procedure in order to rule out the possibility that the observed effects were due to non-specific protein interactions.

Cell proliferation. Assessment of cell proliferation was determined by Calbiochem Rapid Cell Proliferation Kit (Calbiochem, D.B.A., Milan, Italy) (10) and by direct cell counting. For this latter procedure, MCF-7 cells (2×10⁵ per well) were seeded in 24-well plates and the cell number assessed after 72 h using a haemocytometer. For both assessments, each condition was replicated in quadruplicate samples and each experiment was replicated three times. Differences between experimental values were evaluated by the Student's *t*-test.

Cell morphology. Cell morphology was studied by phase-contrast microscopy using an Optika inverted microscope (Model XDS-2; Optika Microscopes, Bergamo, Italy). This microscope had a positive-phase plate for phase-contrast imaging below a long working distance condenser lens, and an 8 Mp digital camera with LCD Screen (Optika Microscopes). The light source was a 6V/30W halogen pre-centered illuminator, with adjustable intensity. Phase-contrast imaging was performed on living cells without any fixation or treatment. A series of digital images of living cells were recorded for each experimental point and the most representative were chosen.

Papanicolaou staining was also performed. This staining results in very transparent cells, such that even thicker specimens with overlapping cells could be recorded. Briefly, cells were stained with Harris haematoxylin as nuclear stain. Orange G and EA-65 (Light Green, Bismarck Brown, and Eosin) were used for cytoplasmic staining (Sigma Aldrich, Milan, Italy). Slides were mounted with permanent mounting medium and observed under light microscopy (Nikon Instruments SpA, Milan, Italy).

Vimentin expression. Vimentin expression was studied by immunohistochemistry and by western blotting. For immunohistochemistry, a monoclonal primary antibody against human vimentin (Thermo Scientific, Fremont, CA, USA) diluted 1:100 was used; incubation was performed for 20 min at room temperature and it was followed by incubation with a goat anti-polyvalent horseradish peroxidase-conjugated antibody (Ultravision Plus Detection kit; Thermo Scientific). Cells were then observed under a light microscope (Nikon Instruments SpA).

Western blotting analysis was performed after homogenizing cells in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium dodecyl sulfate) supplemented with a protease inhibitor cocktail (Sigma Aldrich), and after centrifugation for 15 min at 4°C at 10000×g. The supernatant was collected and the protein concentration was measured using Bradford reagent (Sigma Aldrich). Aliquots

containing 20 µg of protein were diluted in 4× reducing Laemmli's sample buffer (250 mM Tris-HCl, pH 6.8, 20% glycerol, 8% Sodium Dodecyl Sulphate (SDS), 20% 2-mercaptoethanol, 0.008% bromophenol blue) and loaded onto 12% SDS-PolyAcrylamide Gel Electrophoresis (PAGE). Proteins were then transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked for 1 h at room temperature in 5% BSA-TPBS buffer (0.1% Tween-20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5), washed in TPBS, and incubated at 4°C overnight with anti-vimentin primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) (dilution 1:1000). Each experiment was repeated three times.

Densitometric analysis of vimentin was performed by Scion Image software (Scion Corporation, Frederick, MD, USA) for western blotting, β-actin signal was used as reference.

Angiogenic activity. Angiogenesis was studied by chorioallantoic membrane (CAM) assay (11). Briefly, fertilized chicken eggs were incubated at 37°C at constant humidity. On day 3, a square window was opened in the shell, and 2 to 3 ml of albumen were removed to allow detachment of the developing CAM from the shell. The window was sealed with a glass coverslip of the same dimension, and the eggs were returned to the incubator. On day 8, eggs were treated with 1 mm³ sterilized gelatin sponges (Gelfoam; UniPharma SA, Barbengo, Switzerland) placed on top of the growing CAM and loaded with: 5 µl PBS as negative control; 5 µl DBP-MAF (40 ng/ml); 5 µl MCF-7 suspension (6×10⁴ cells per sponge).

CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope equipped with a camera and image analyzer system (Nikon Instruments SpA). At day 12, the angiogenic response was evaluated by counting the number of vessels, centering the sponges within the focal plane of the CAM (circumfocal microvessel number, CFMN) (12). Mean±standard deviation (SD) were evaluated and the statistical significance of the differences between the counts was determined by Student's *t*-test.

Results

Effects of DBP-MAF on MCF-7 cell proliferation. MCF-7 human breast cancer cell proliferation was significantly inhibited by 72 h incubation with DBP-MAF in a dose-dependent manner. Figure 1A shows the results obtained by measuring proliferation with the colorimetric assay described in the Materials and Methods. DBP-MAF (at 0.4 ng/ml and 40 ng/ml) inhibited cell proliferation in a manner similar to that obtained with vitamin D (1,25(OH)-2D-3 at 100 nM and 1 µM), a known inhibitor of MCF-7 cell proliferation (13). Vitamin D (1 µM) and DBP-MAF (40 ng/ml) administered together led to almost total inhibition of cell proliferation in comparison to control cells. However, the results obtained with vitamin D at a concentration of 100 nM should be considered with care, since this concentration is well above the physiological level. Qualitatively similar results were obtained by counting the cell numbers after 72 h DBP-MAF treatment (Figure 1B). With this assay, significant inhibition of cell proliferation was also observed with 0.4 ng/ml DBP-

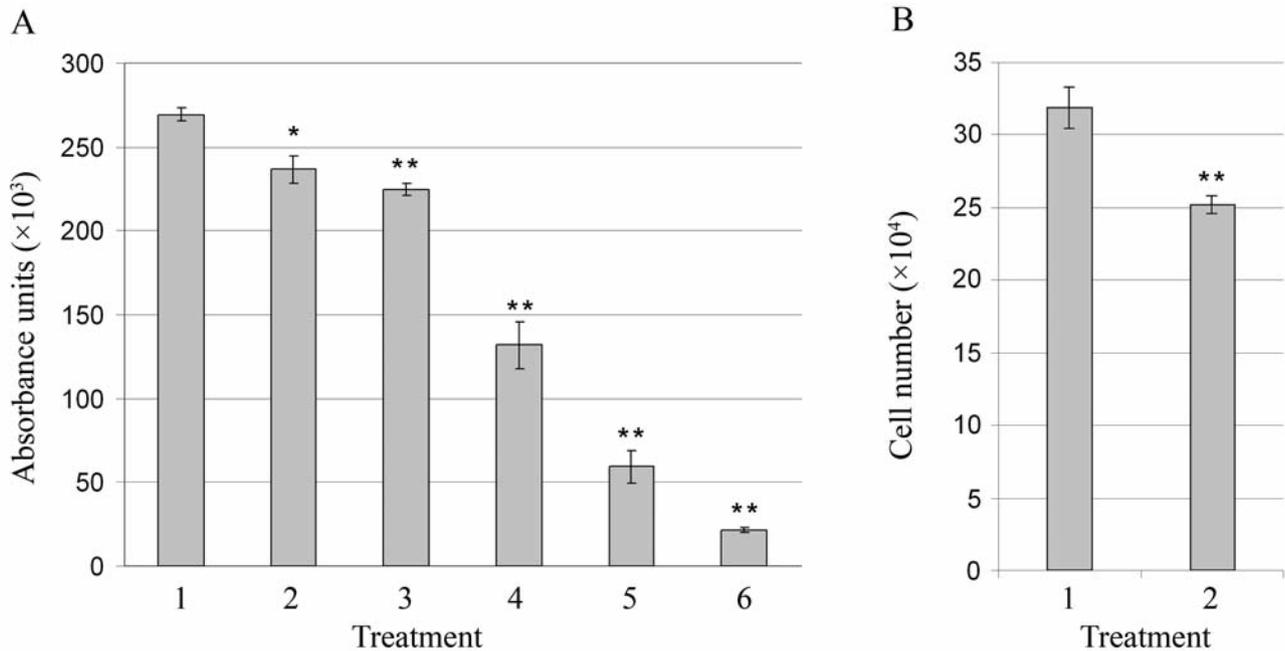


Figure 1. Effects of 72 h treatment with Vitamin D-binding Protein-derived Macrophage-activating Factor (DBP-MAF) on MCF-7 cell proliferation. A: Assessment of cell proliferation by methyl-thiazol-tetrazolium (MTT)-based assay. Treatment columns 1: control (VDBP, 40 ng/ml); 2: DBP-MAF, 0.4 ng/ml.; 3: vitamin D (1,25(OH)-2D-3), 100 nM; 4: DBP-MAF, 40 ng/ml; 5: 1,25(OH)-2D-3, 1 μ M; 6: 1,25(OH)-2D-3, 1 μ M + DBP-MAF, 40 ng/ml. Values represent means \pm SEM (n=12). *p<0.05 vs. Control; **p<0.01 vs. control. B: Assessment of cell proliferation by cell counting. MCF-7 cells (2×10^5 cells per well) were seeded in 24-well plates and cell number after 72 h assessed using a haematocytometer. Treatment columns 1: control; 2: DBP-MAF 0.4 ng/ml. Values represent means \pm SEM (n=12). **p<0.01 vs. control.

MAF, a concentration similar to that required to stimulate human peripheral blood mononuclear cells (5). Taken together, these results demonstrate that DBP-MAF directly inhibited MCF-7 cell proliferation in a manner analogous to that observed by Gregory *et al.* for LnCaP prostate cancer cells (9).

Effects of DBP-MAF on MCF-7 cell morphology. Cell morphology was first studied in living MCF-7 cells observed under phase-contrast light microscopy. In other words, in these experiments cells had not undergone any preparation for microscopy, *i.e.* washing, fixation or staining. Figure 2A shows control cells after 72 h incubation. As expected, cells did not show contact inhibition and formed clusters (indicated by arrows in the figure). Cell morphology and size were nonhomogeneous. Figure 2B shows MCF-7 cells treated with 40 ng/ml DBP-MAF for 72 h. No major clusters were observed and the culture appeared as a monolayer of cells, regularly polygonal and uniform in morphology and size. The DBP-MAF concentration required to induce the morphological changes depicted in Figure 2B was higher than that required to inhibit cell proliferation and identical to that required to inhibit angiogenesis [see following paragraph and (5)]. It is worth noting, however, that minor, although evident,

morphological changes were also observed at a lower concentration (0.4 ng/ml DBP-MAF). Figure 2C shows phase-contrast microscopy of control MCF-7 cells, whereas Figure 2D shows cells treated with 0.4 ng/ml DBP-MAF for 72 h. MCF-7 cells treated with DBP-MAF also appeared less nonhomogeneous in morphology and size compared with control cells. Morphological changes were also apparent after 24 h treatment by phase-contrast microscopy (Figure 3). In this case, a higher concentration of DBP-MAF was required to induce observable significant changes. Figure 3A shows control MCF-7 cells, whereas Figure 3B shows cells treated with 40 ng/ml DBP-MAF for 24 h. The differences in homogeneity, morphology and size are evident.

We then examined MCF-7 cells using classical Papanicolaou stain (Figure 4). In this case, cell cultures underwent washing, fixation and staining. Figure 4A shows control MCF-7 cells at 72 h incubation. Cells grew one on top of the other, forming typical clusters as indicated by arrows. Cell size, morphology and staining were nonhomogeneous. Large empty spaces between clusters suggest poor adherence to the well surface. This particular feature was not apparent under phase-contrast microscopy since it was evident only after washing the cultures during the staining procedure. Figure 4B shows cells treated with 40

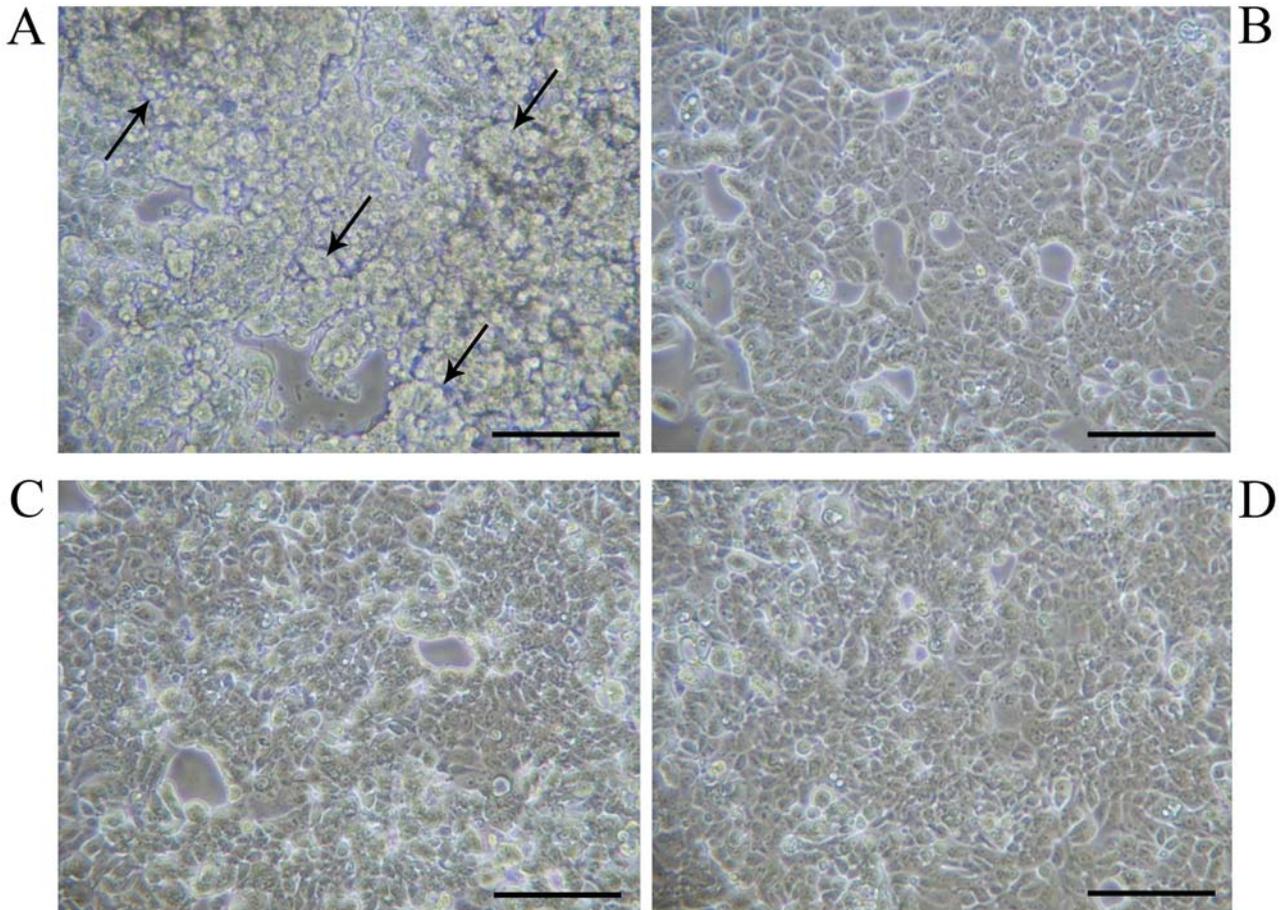


Figure 2. Effects of 72 h treatment with Vitamin D-binding Protein-derived Macrophage-activating Factor (DBP-MAF) on MCF-7 cell morphology under phase-contrast light microscopy. MCF-7 cells treated for 72 h with 40 ng/ml Vitamin D-binding Protein (VDBP) (control) (A; arrows indicate cell clusters), 40 ng/ml DBP-MAF (B), 0.4 ng/ml VDBP (C), and with 0.4 ng/ml DBP-MAF (D). Scale bar=50 μ m.

ng/ml DBP-MAF for 72 h. Cells were smaller, regularly polygonal and uniform in size and morphology, and the culture grew as a monolayer. Cells appeared to be well adherent to each other and to the well surface, as suggested by the absence of large empty spaces.

Effects of DBP-MAF on vimentin expression in MCF-7 cells.

Although the MCF-7 line is of epithelial origin and retains several characteristics of differentiated mammary epithelium, it expresses vimentin, the archetypal mesenchymal marker that is also over expressed in breast carcinomas undergoing epithelial/mesenchymal transition and exhibiting increased cell migration and invasion. In fact, vimentin expression is considered a hallmark of human breast cancer progression and, during progression toward a more malignant phenotype, the cell intermediate filament status changes from a keratin-rich to a vimentin-rich network (14).

Our hypothesis that DBP-MAF-induced morphological changes could be interpreted as if DBP-MAF reverted cancer cell malignant phenotype was corroborated by the study of vimentin expression. Figure 5 demonstrates that exposure to 40 ng/ml DBP-MAF for 72 h significantly reduced vimentin expression in MCF-7 cells. Figure 5A (immunohistochemistry) shows control MCF-7 cells; significant brown staining was observed, indicating strong vimentin expression. In this case, large empty spaces after repeated washing also suggest poor adherence to the well surface. Figure 5B (immunohistochemistry) shows MCF-7 cells treated with 40 ng/ml DBP-MAF for 72 h. Brown staining was less pronounced and cells also appeared more uniform in morphology and size, and more adherent to the substrate. Densitometric analysis (Figure 5C) confirmed these observations. Western blot analysis (Figure 5D), yielded results consistent with those obtained by immunohistochemistry.

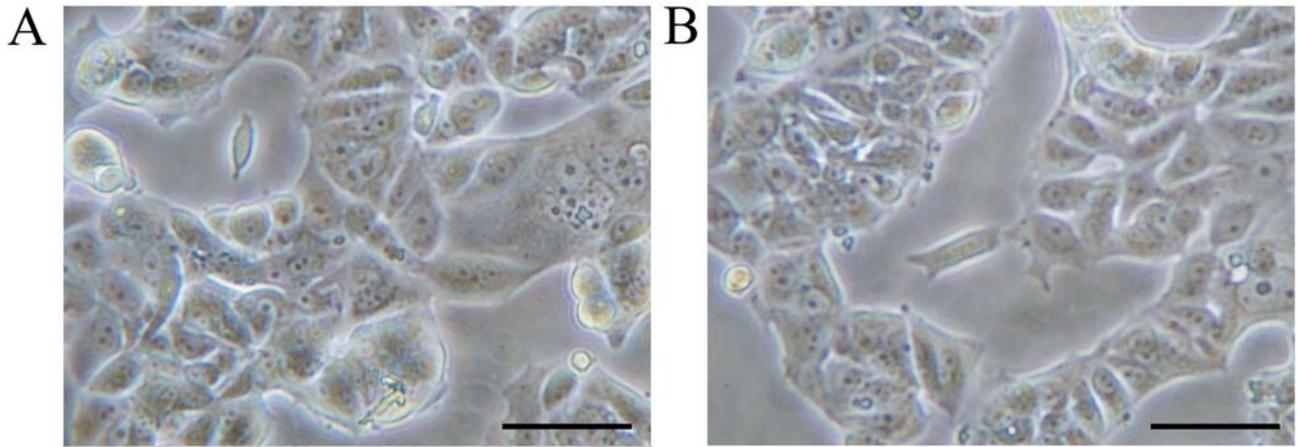


Figure 3. Effects of 24 h treatment with Vitamin D-binding Protein-derived Macrophage-activating Factor (DBP-MAF) on MCF-7 cell morphology under phase-contrast light microscopy. MCF-7 cells treated for 24 h with 40 ng/ml VDBP Vitamin D-binding Protein (VDBP) (control) (A) and with 40 ng/ml DBP-MAF (B). Scale bar=25 μ m.

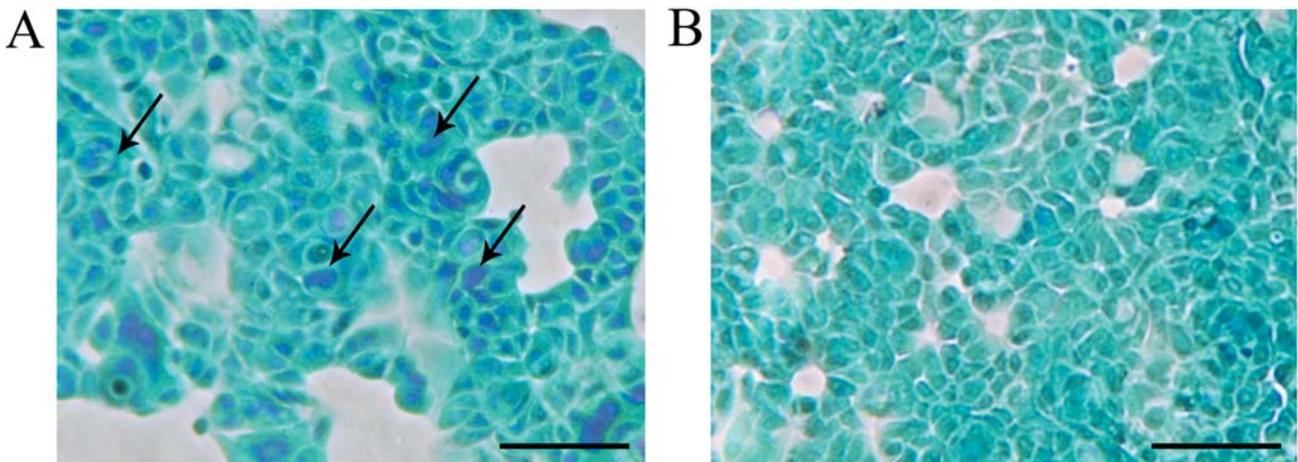


Figure 4. Effects of 72 h treatment with Vitamin D-binding Protein-derived Macrophage-activating Factor (DBP-MAF) on MCF-7 cell morphology after Papanicolaou stain under light microscopy. MCF-7 cells treated for 72 h with 40 ng/ml Vitamin D-binding Protein (VDBP) (control) (A; arrows indicate cell clusters), and with 40 ng/ml DBP-MAF (B). Scale bar=25 μ m.

Effects of DBP-MAF on breast cancer cell-stimulated angiogenesis. It is well reported that DBP-MAF inhibits angiogenesis, and inhibition of angiogenesis is considered a key feature of its anticancer properties (2, 3, 8, 15). It is worth noting, however, that until recently, DBP-MAF was not commercially available and each laboratory had to extract and purify DBP-MAF using complex procedures that varied from laboratory to laboratory (8, 9). An example of the difficulties in preparing DBP-MAF is reported in (16). Therefore, the effects of commercially available DBP-MAF on cancer cell-stimulated CAM assay, a reliable and inexpensive method to study angiogenesis, have not yet been tested. In order to fill this gap, we tested its effects both under basal conditions and when administered together with MCF-7 cells. DBP-MAF did

not alter basal angiogenesis (Table I) or chick embryo development (not shown). As expected, MCF-7 cells, directly implanted in the CAM, strongly stimulated angiogenesis (Table I), as previously described (11). In fact, it is well known that MCF-7 cells produce pro-angiogenic factors (17), and stimulation of angiogenesis by MCF-7 cells was comparable to that induced by prostaglandin E₁, a known inducer of proangiogenic factor production (5). DBP-MAF (40 ng/ml) significantly inhibited MCF-7-stimulated angiogenesis (Table I). It is worth noting that the DBP-MAF concentration required to achieve full inhibition of stimulated angiogenesis was 40 ng/ml, *i.e.* a concentration 100-fold higher than that required to inhibit MCF-7 cell proliferation under our experimental conditions. This DBP-MAF concentration, however, was

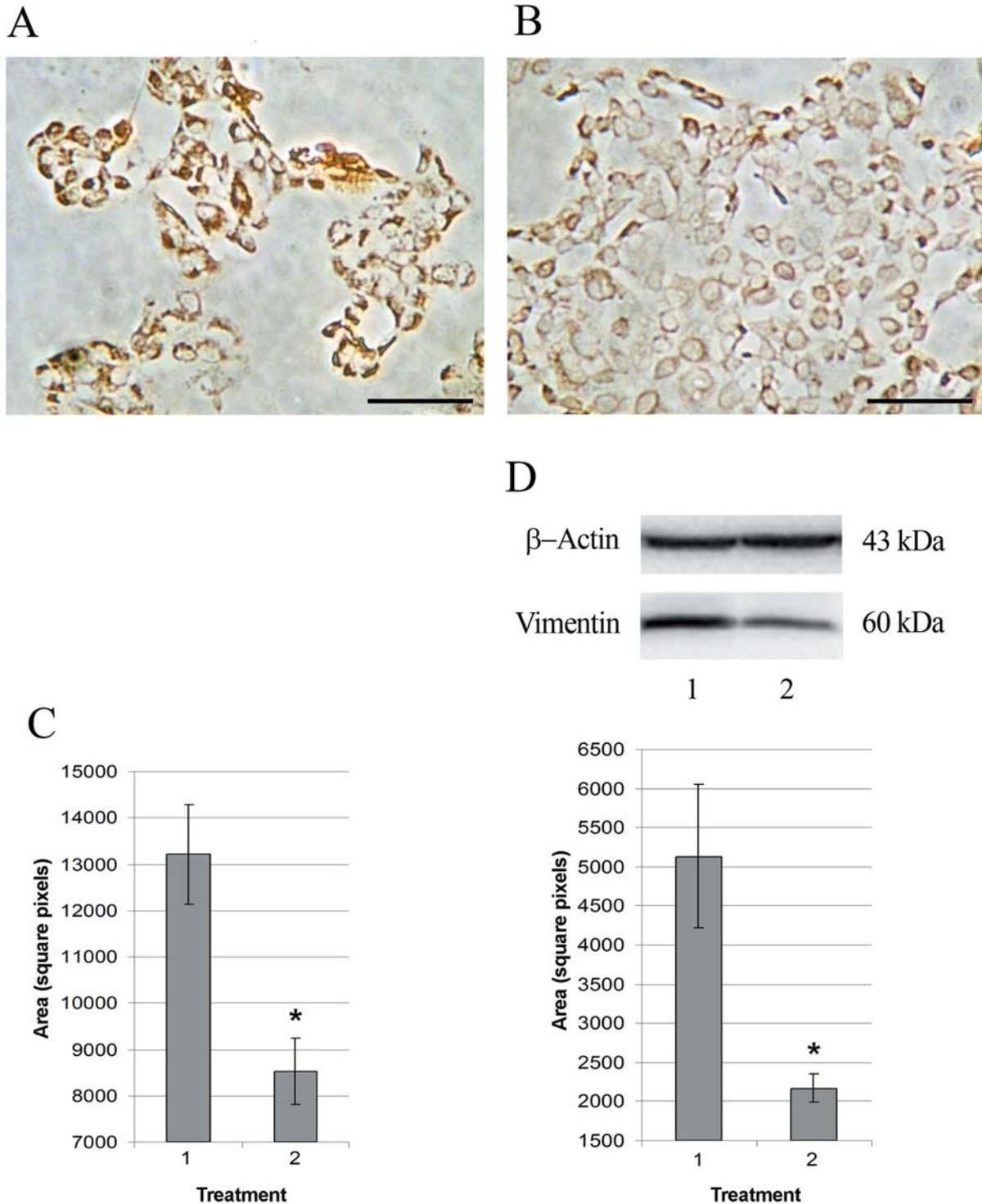


Figure 5. Effects of 72 h treatment with Vitamin D-binding Protein-derived Macrophage-activating Factor (DBP-MAF) on vimentin expression in MCF-7 cells. Immunohistochemistry of MCF-7 cells treated for 72 h with 40 ng/ml Vitamin D-binding Protein (VDBP) (control) (A) and with 40 ng/ml DBP-MAF (B). Scale bar=25 μ m. Densitometric analysis of vimentin expression determined by immunohistochemistry (C). Brown stained areas of panels A (column 1, control), and B (column 2, DBP-MAF) were measured by Scion Image software and expressed as square pixels. Western blot analysis and densitometric evaluation of vimentin expression (D). Band 1 and column 1 (densitometric analysis of the actual band) refer to MCF-7 cells treated for 72 h with 40 ng/ml VDBP. Band 2 and column 2 refer to MCF-7 cells treated for 72 h with 40 ng/ml DBP-MAF. Areas of bands 1 (control), and 2 (DBP-MAF) were measured by Scion Image software and expressed as square pixels. Values in densitometric analyses indicate means \pm SEM (n=3). *p<0.01 vs. control. B-Actin signal was used for reference.

Table I. *Quantitative evaluation of angiogenesis on chorioallantoic membrane (CAM) assay. Effects of Vitamin D-binding protein-derived macrophage-activating factor (DBP-MAF) on MCF-7-stimulated angiogenesis.*

Treatment	Circumfocal microvessel number
PBS	15.3±1.4
DBP-MAF (40 ng/ml)	16.4±1.8
MCF-7	28.5±1.3
MCF-7 + DBP-MAF (40 ng/ml)	16.7±1.0*

Data are the average number of blood vessels derived from scoring small (≤ 1 mm diam.), large (> 1 mm diam.), and tortuous microvessels surrounding the sponge. Data are reported as means \pm SEM (n=18). * $p < 0.02$ vs. MCF-7 cells alone.

identical to that required to observe the major morphological changes reported in Figures 2-4, as well as reduced vimentin expression (Figure 5). This observation is consistent with previous results demonstrating that the concentrations of DBP-MAF required to inhibit angiogenesis in CAM assay or in other assays were higher than those required to activate macrophages or inhibit cancer cell proliferation (3, 4, 9). In addition, these results, consistent with those reported in (2, 3, 8, 15), seem to indicate that inhibition of angiogenesis by DBP-MAF is independent of the pro-angiogenic factor(s) used to stimulate angiogenesis in the different experimental models tested.

Discussion

The biological effects of DBP-MAF were first described in 1994 (18) and shortly afterward its anti/tumour properties in BALB/c mice bearing Ehrlich ascites tumour were described (19). Further studies on the effects of DBP-MAF on cancer patients reported successful immunotherapy of prostate cancer (20), and metastatic breast (6) and colon (21) cancer. Accumulating evidence indicates that the anticancer efficacy of DBP-MAF can be ascribed at least to three biological properties of the molecule: i) activation of tumoricidal macrophages; ii) inhibition of tumour-induced angiogenesis; and iii) direct inhibition of cancer cell proliferation, migration and metastatic potential. The direct inhibition of cancer cell proliferation, however, before this study has been described only for prostate cancer cells (9).

Not surprisingly, the anticancer effects of DBP-MAF attracted great attention and a quick search of the Internet reveals that several cancer clinics in different countries are currently proposing DBP-MAF as treatment for cancer patients (<http://www.klinik-st-georg.de/dr-douwes-informiert/wahre-hilfe-bei-krebs/>, <http://immunemedicine.com/available-therapies/gcmf/>, http://www.gcmf.eu/info/index.php?option=com_content&view=article&id=122&Itemid=56 web page accessed on June 30th 2011). The interest for the therapeutic

potential of DBP-MAF is further demonstrated by the applications for patents related to DBP-MAF use (for the latest patent application see number 20110123591, publication date: 05/26/2011, by Knezevich and Silvetz, available at: <http://www.faqs.org/patents/app/20110123591>, web page accessed on June 30th 2011).

However, one major hindrance to basic and clinical research on DBP-MAF, is that, so far, most laboratories have produced their own DBP-MAF with different procedures, all based on sequential deglycosylation of its precursor, *i.e.* VDBP. As recently demonstrated by Ravnsborg *et al.* (16), the sequence of deglycosylation is critical and this might explain why different laboratories reported different specific activities for DBP-MAF as far as biological effects were concerned (3-5, 9). With the aim of allowing reproducibility of our results, in this study, we used a commercially available source of DBP-MAF to assess its effects on cultured human breast cancer cells. Our results are in agreement with the observation of Gregory *et al.* (9) who demonstrated a direct inhibitory effect on prostate cancer cells, and lend credit to the hypothesis that DBP-MAF has a non type-specific anticancer activity. This hypothesis is further reinforced by preliminary data indicating a direct inhibitory effect of DBP-MAF on a human acute monocytic leukemia cell line (Pacini *et al.*, manuscript in preparation). Our results also demonstrate for the first time that DBP-MAF induced changes of several morphological features of breast cancer cells, a phenomenon that we interpreted as reversal of the malignant phenotype. This phenomenon was consistent with the observation that DBP-MAF reduced the expression of vimentin, a hallmark of malignant progression (14), since trans-differentiation from an epithelial to a mesenchymal phenotype is responsible for increased aggressiveness and metastatic propensity (22).

We hypothesize that the observed effects of DBP-MAF on MCF-7 cells may be ascribed to stimulation of a cell surface receptor, with triggering of a cascade of intracellular signalling, possibly involving cAMP production, as we observed in human mononuclear cells (5). In fact, MCF-7 cells, unlike other human breast cancer cell lines, do not internalize DBP (23) and therefore it is not likely that they internalize DBP-MAF. It is worth noting that among different human breast cancer cell lines, MCF-7 cells were chosen as an experimental model in this study because of this feature, which helps distinguish the effects of DBP-MAF from those of vitamin D possibly bound to DBP-MAF. In fact, MCF-7 cells are cultured in medium containing FBS, and FBS contains vitamin D (24) as well as DBP (23).

In conclusion, our results support and reinforce the hypothesis that DBP-MAF is a molecule endowed with multiple biological activities relating to its anticancer properties. It can be hypothesized that future directions of research in this field will involve the study of the effects of

DBP-MAF in wound-healing, migration and cell death assays, and in xenograft animal models in order to further characterize the multifaceted properties of DBP-MAF.

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