Inhibition of *In Vivo* Tumour Growth by the Blocking of Host $\alpha_v\beta_3$ and $\alpha II_b\beta_3$ Integrins

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Abstract. *Background: The $\alpha_v\beta_3$ integrin in the endothelial cell membrane is important for the growth and migration of capillaries into tumour tissue and is also a survival factor for these cells. The $\alpha II_b\beta_3$ (GPIIb/IIIa) integrin is responsible for platelet activation and, with concomitant release of different stored proangiogenic factors, and tumour cell-platelet interactions. Materials and Methods: An immunodeficient nude rat model was used to study tumour growth in tibial bone, with tumour cells negative for the target $\alpha_v\beta_3$ and $\alpha II_b\beta_3$ integrins. Results: Daily intraperitoneal injections of m7E3 F(ab')$_2$ antibody fragment, blocking human and rat $\alpha_v\beta_3$ and $\alpha II_b\beta_3$ integrins, reduced the measured size of the tumours growing in the tibial bone by 35% (p=0.012), and also the microvessel density in these tumours. The concentration of the important proangiogenic factor bFGF was significantly reduced by 41% in the treated tumours. The treatment slightly increased the time to the appearance of the tumour from 22.2 to 24.9 days, indicating a small but significant effect on the early stages of tumour growth and invasion through the bone tissue. Conclusion: Integrin-targeted treatment reduced tumour growth, solely targeting the host angiogenesis. This treatment strategy should be further exploited for use in combination with conventional treatment strategies, or the combined targeting of alternative antiangiogenic pathways.*

The formation of new blood vessels in tumour tissue, neoangiogenesis, is of vital importance for the survival and growth of the tumour and thus, the progression of the malignant disease. Inhibition of this process in order to starve the tumour cells has been suggested as a therapeutic strategy. Promising activity has been observed in animal models with different antiangiogenic molecules and, based on this, a number of clinical trials with different compounds have been performed (1-4).

Monoclonal antibodies have successfully been used for targeting molecules of importance for tumour progression and survival, including factors such as vascular endothelial growth factor (VEGF)-A, which is involved in the formation of new vasculature in tumours (5). An antibody to VEGF-A has been used in breast cancer and failed to increase the time to tumour progression in heavily pretreated patients with metastatic disease (3), but did increase the time to progression when used for first-line therapy in combination with chemotherapy in these patients (6). However, this antiangiogenic treatment in combination with chemotherapy is a part of routine clinical practice *e.g.* in patients with metastatic colorectal carcinoma (7). Thus, the tumour type, disease stage and concomitant treatment may be important factors that significantly impact the efficacy of this treatment.

Treatment with integrin antagonists targeting the $\alpha_v\beta_3$ integrin has demonstrated a reduction in angiogenesis and tumour growth in melanoma (8-10). It is well known that the $\alpha_v\beta_3$ integrin receptor may act as a survival factor for melanoma cells and treatment targeted to this integrin may reduce the surviving fraction of the melanoma cells (11). Such a relationship has not been shown for other tumour types, such as for example carcinomas (12), although the expression of the $\alpha_v\beta_3$ molecule on the cell surface may modify the phenotype of malignant breast cancer cells in experimental systems (13). Endothelial cells express the $\alpha_v\beta_3$ integrin, independently of the tumour stimulating the angiogenic process. This molecule may therefore be used for targeted inhibition of tumour angiogenesis, as shown in a previous study (9). Treatment with a monoclonal antibody toward the $\alpha_v\beta_3$ integrin was able to inhibit growth of tissue from human breast cancer biopsies when transplanted in a human skin model (14) and has also been shown to inhibit the angiogenesis in a skin transplant model of human melanoma (15).

As described above, the toxicity profile of antibodies makes them suitable for combination with other treatment

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Key Words: Angiogenesis, animal model, metastasis, experimental therapy, integrin.

regimens. However, metastatic deposits to bone tissue represent a significant clinical challenge for many tumour types. Another study has indicated that treatment targeting the α_v integrin reduced the formation of capillaries and the destruction of bone implants (16). However, not all tumor cells are positive for the α_v integrin. It is therefore of considerable interest to investigate the possibility for using such a therapeutic strategy in an animal model based on a target ($\alpha_v\beta_3$ and aIIbB3 integrin) negative tumour cell line (a metastatic variant of the HeLa cell line) able to grow into and metastasize to bone (17).

Materials and Methods

Antibodies. The m7E3 F(ab')$_2$ fragment used for treatment of the animals and immunostaining, recognising the $\alpha_v\beta_3$ and the $\alpha II_b\beta_3$ (GPIIb/IIIa) integrin, was supplied by Centocor, Inc (Malvern, PA, USA). The anti-$\alpha_v\beta_3$-integrin antibody used was from clone P1F6 (originally obtained from Becton Dickinson Cellular Imaging Systems, San Jose, CA). The $\alpha_v\beta_5$ antibody was also obtained from Becton Dickinson, CA.

Cells. The metastatic variant of the HeLa cells used in this study has previously been characterised as described in (17). The cells were originally described as originating from breast tissue (18), but cytogenetic analyses have confirmed the cells to be a variant of the HeLa cell line, as described elsewhere (19). The establishment and characterisation of the MA-11 breast cancer cell line has previously been described (20). Both cell lines were grown in RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and glutamax (Life Technologies, Paisley, Scotland) and kept in a standard tissue culture incubator at 37˚C. Confluent cells were detached with 0.01 M EDTA. For the injection into the animals, cells in exponential growth phase were used.

Flow cytometric analysis. For a detailed description of this method see (17). The F'ab fragment m7E3 used for flow cytometric analysis was identical to the F'ab fragment m7E3 used for treatment of the animals. The tumour cells were detached and resuspended in phosphate buffered saline (PBS) with 1% human serum albumin (HSA). The antibodies were then added at a concentration of 20 μg/ml and cells were incubated with gentle rotation for 40 min at 4˚C. The cells were then washed twice, before the addition of a 1:20 dilution of fluorescein isothiocyanate (FITC) conjugated secondary antibody (F0313, DAKO, Glostrup, Denmark). After 40 minutes at 4˚C, the cells were washed and analysed on a Becton Dickinson FACStar Plus equipped with an argon laser.

Animals. All procedures and experiments involving animals were approved by The National Animal Research Authority and carried out according to the European Convention for the Protection of Vertebrates used for Scientific Purposes. Nude rats (Han: rnu/rnu Rowett) were bred in our nude rodent facility. Animals were kept in a specific pathogen-free environment, in positive pressure rooms with filtered and humidified air. Four- to five-week old animals of both sexes were used. Rats injected with cells in the left cardiac ventricle or the tibial bone were anesthetized with a subcutaneous injection of a mixture containing 0.1 mg/kg fentanyl, 5 mg/kg fluanison and 2.5 mg/kg midazolam. The treatment with intraperitoneal injection of the F'ab fragment was performed in animals anesthetized briefly with halothane and N_2O, mixed with O_2.

Tumour cell inoculation – experimental endpoints. Primary tumor: Percutaneous intraosseal (intratibial) injection was performed by drilling a 19-gauge needle into the marrow cavity immediately proximal to the *tuberositas tibia* (21) and injecting a 0.1 ml cell suspension containing 1×10^6 HeLa cells into the distal end of the tibial bone marrow cavity. Tumour formation was detected by palpation of the tibial bone, and the time from inoculation of the tumour cells to the initial palpatory signs of tumour growth were recorded as the tumour free-survival. The animals were sacrificed when progressive growth of the tumour was recorded and well before the rats became distressed by the leg tumour.

Tumor metastasis: Intracardial (LV) injection of anaesthetized animals was performed as described elsewhere (22). Briefly, a microinfusion set containing a 27-gauge needle connected to an infusion tube was used. The needle was inserted through the third intercostal space into the left ventricle of the heart after a midline skin incision. When, upon aspiration, light red pulsating blood was observed in the tube, 0.2 ml of cell suspension containing 10^6 HeLa cells was injected into the ventricle, the needle withdrawn and the skin closed with staples. The animals were then inspected daily with respect to symptoms of tumour-related disease and general condition. The animals were sacrificed when symptoms of metastatic disease appeared. As described elsewhere, disease related to growth of metastases caused paresis and neurological symptoms, cachexia and weight loss respectively (17). The period from inoculation of the tumour cells to the appearance of symptoms of metastasis was recorded as the tumour free-survival time.

Treatment of the animals. The HeLa cells were inoculated into the tibial bone marrow cavity, the animals were treated with daily intraperitoneal injection of m7E3 F(ab')$_2$ fragment at a dose of 6 mg/kg/day, with the start of treatment on the day of the tumour inoculation. The dose level was selected based upon pilot experiments using the F(ab')$_2$ fragment (data not shown). Two separate experiments were performed and the results were pooled for further analysis. Control animals were anaesthetized in the same manner and injected with the diluent used for the F'ab fragment (PBS).

Histopathological examination. The tumour growth in animals injected in the tibial bone marrow cavity was closely monitored and the study was terminated when the animals in the control group had to be sacrificed due to the size of the tumour. The animals injected intracardially with tumour cells were sacrificed when clear symptoms of progressive disease appeared (pareses, weight loss or distress). The tissues from rats injected intratibially and intracardially with tumour cells were removed, fixed in 4% formalin buffered in PBS and prepared for histopathological examination by dehydration in gradients of ethanol and embedding in paraffin. Sections, 5 μm-thick, were cut, stained with hematoxylin and eosin and examined microscopically for the presence of metastases in the different tissues.

Analysis of neoangiogenesis and angiogenesis promoting factors. Tumour tissue from the animals injected with HeLa cells in the tibial bone marrow cavity was cut into three parts and snap frozen in

liquid nitrogen, slowly frozen embedded in TissueTek (Sakamura, Zoeteroude, the Netherlands) and immersed in 4% buffered formalin. Frozen sections were cut at a thickness of 5 μm and stained with an antibody recognising the CD31 molecule, an endothelial cell marker (cat. # 550300, BD Pharmingen, San Diego, CA, USA) or a polyclonal rabbit anti-human von Willebrand factor antibody (cat. # A0082, Dako, Glostrup, Denmark) according to the description provided by the manufacturer. The EnVision system (Dako) was used for secondary labelling, counterstained with hematoxylin and eosin and sealed with cover-slips. Three high-power fields (x400 magnification) were counted and the mean number of capillaries calculated for each treatment group, without knowledge of the treatment given.

The frozen tissue specimens were placed in ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 100 mM NaF, 10% glycerol and 0.1% NP-40, with 1 mM phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate and 10 μg/ml of leupeptin, pepstatin and aprotinin. The tissue specimens were then ground and incubated on ice for 60 minutes and thereafter sonicated. The samples were then centrifuged for 15 min at 15,000 rpm, before the sedimented cell remnants were discarded and the protein lysates frozen at –70˚C.

The total protein concentration in the samples was estimated using the BCA protein assay kit (Pierce, Rockford, IL, USA). The concentration of the bFGF in the protein lysates was determined by the use of human bFGF ELISA kit (BioSource, Camarillo, CA, USA), and the VEGF-A content was measured using the Human VEGF Quantikine ELISA kit (R&D systems, Abingdon, UK), both according to the manufacturers' guidelines. Standard curves were generated with the components delivered with the kit.

Statistical evaluation. The results of the two experiments in the intratibial growth model were pooled and analysed for tumour-free survival using the Log-rank test. The significance levels of the differences in the treated and control groups with respect to the measured tumour size, tumour weight, capillary density, bFGF and VEGF-A concentration were determined using the Mann-Whitney *U*-test.

Results

Flow cytometric analysis of integrin expression. The HeLa cells and the human breast cancer MA-11 cells were stained with the m7E3 F(ab')$_2$ fragment and an antibody recognising the $\alpha_v\beta_5$ integrin. The HeLa cells did not stain for the m7E3 F(ab')$_2$ fragment, but were positive for the $\alpha_v\beta_5$ integrin, used as a control antibody (Figure 1A). The MA-11 cells stained positively using both antibodies (Figure 1B). The $\alpha_v\beta_3$ integrin-deficient HeLa cells were chosen for this study, as this would ensure that the effects obtained in the planned experiments were dependent solely on the host integrin expression.

Intraperitoneal anti-integrin treatment of intratibial tumours. Treatment with the m7E3 F(ab')$_2$ fragment increased the tumour-free survival by 12%, from 22.2 days to 24.9 days (log rank, $p \leq 0.0336$; n=13 in control and n=13 treatment group; Figure 2). The effect of the treatment was more pronounced when comparing the tumour/tibia volume in treated and untreated animals at the end of the experiment. Treatment with the m7E3 F(ab')$_2$ fragment reduced the tibia/tumour volume by 35% ($p \leq 0.012$; Mann-Whitney *U*-test). Furthermore, the average tumour weight was reduced in the animals treated with the F(ab')$_2$ fragment to 1.05 g, compared with 1.85 g in the control animals. However, this difference did not reach statistical significance ($p \leq 0.0769$; Mann-Whitney *U*-test).

Treatment with the m7E3 F(ab')$_2$ fragment on five out of seven days in the week had no influence on the tumour-free survival or the growth of the intratibial tumour.

Intraperitoneal anti-integrin treatment of tumour cell metastasis. Histopathological examination revealed metastases to the lungs in two of the animals inoculated with HeLa cells in the tibial bone marrow cavity. A separate experiment was therefore performed injecting the HeLa cells into the left cardiac ventricle of the immunodeficient animals. Treatment with the m7E3 F(ab')$_2$ fragment was started the same day as the inoculation of the tumour cells, but did not affect the survival of the animals. The mean symptom-free survival time in the control group (n=9) was 24 days, no different from the 23.6 days in animals receiving the m7E3 F(ab')$_2$ fragment (n=9). One animal in the control group and two animals in the treatment group were sacrificed without symptoms, but all of the animals were shown to have tumours metastasized to the brain and/or the tibial bone at histopathological examination. There was no difference in the frequency of metastases to the different organs between the treated and the untreated animals (Table I).

Analysis of neoangiogenesis and angiogenesis promoting factors in m7E3 F(ab')$_2$ treated animals. The microvessel density in the tumours from one of the experiments was determined by counting the number of capillaries on anti-CD31– or anti-human von Willebrand factor-stained frozen-sectioned tumour specimens (Figure 3). The density was reduced from an average of 26.8 in the control specimens to 20.9 in the m7E3 F(ab')$_2$ fragment-treated specimens per field of view (×400 magnification; $p \leq 0.0143$, Mann-Whitney U-test; Figure 3C). Such a change in microvessel density may be mediated by several factors of importance for neoangiogenesis in tumours. Two important factors for vessel formation and growth in tumours are bFGF and VEGF. The expression of these factors was determined by ELISA on protein lysates made from tumour tissue snap-frozen in liquid nitrogen. bFGF content in the tumour specimens was reduced from 3.2 to 1.9 pg bFGF per μg total protein in the PBS-treated group compared with the m7E3 F(ab')$_2$ treated animals ($p \leq 0.0109$, Mann-Whitney *U*-test; Figure 3D). The average VEGF concentration in the tumour specimens of the m7E3 F(ab')$_2$ fragment treated animals was 0.28 pg/μg total

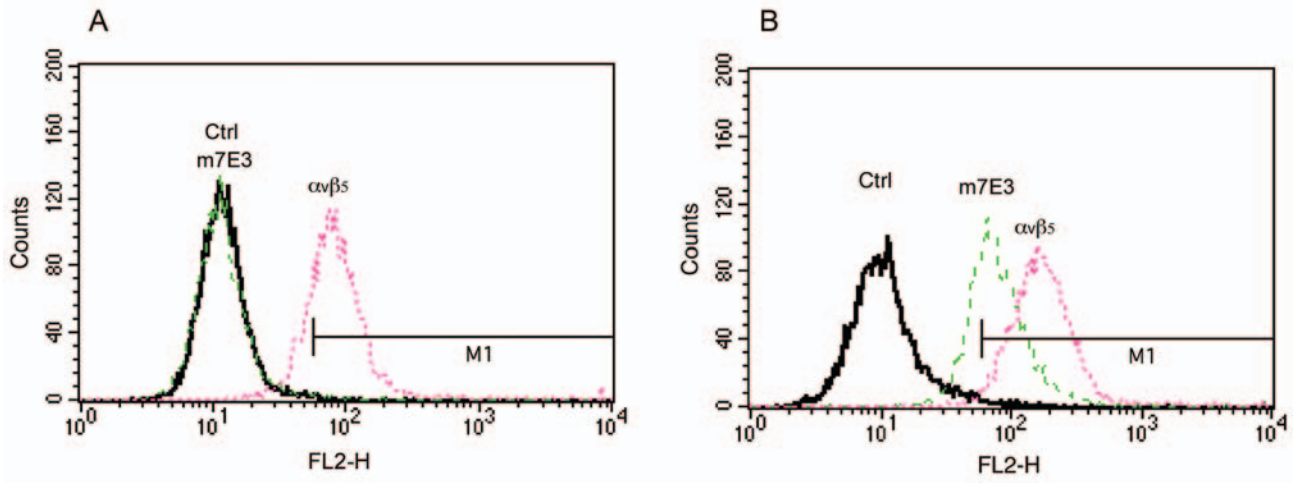

Figure 1. *Flow cytometric analysis of integrin expression in HeLa (A) and MA-11 cells (B). The HeLa cells were negative for the m7E3 F(ab')$_2$ fragment, but largely stained positively for the $\alpha_v\beta_5$ integrin (81.7% of the cells were positive with the indicated threshold). The MA-11 cells stained positively both with the antibody recognizing the αvβ5 integrin and the m7E3 F(ab')$_2$ fragment binding the $\alpha_v\beta_3$ integrin (98.8% and 73.0% of the cells were positive with the respective staining, with the threshold set as indicated).*

protein compared with 0.64 pg/μg total protein in specimens from the PBS control-treated animals ($p \leq 0.1279$; Mann-Whitney U-test; Figure 3E).

Discussion

Neoangiogenesis, the formation of new capillaries, is a key event for the sustained growth of a tumour. Inhibition of this process may represent an attractive possibility for targeted anticancer treatment used as an adjuvant to standard treatment regimens. Conventional anticancer therapies are focused on the inhibition of DNA replication. However, genetic instability in the cancer cells often leads to treatment resistance, either due to inherent resistance mechanisms or as a consequence of a loss of the targeting epitope. As described by others (23), inhibition of neoangiogenesis by targeting endothelial cells circumvents this problem, as these cells are under normal growth control and are less likely to develop resistance to the treatment given. In this study, the treatment effect of the m7E3 F(ab')$_2$ was independent of the antigen expression of the tumour cells, as the tumour cells did not express the targeting antigen. This emphasises the versatility of such a treatment regimen. It is well known that targeting $\alpha_v\beta_3$ or $\alpha_v\beta_5$ on tumour cells may reduce the growth of tumour cells positive for this epitope (10). Thus, the 35% reduction of the tumour volume relative to the control treated tumours achieved in our study, with a highly aggressive tumour negative for the targeting epitope, may be regarded as a minimum of what may be obtained in the *in vivo* situation.

The increase in tumour-free survival (time delay before the tumour could be detected) was modest in this animal model. The growth of the malignant cells may initially be

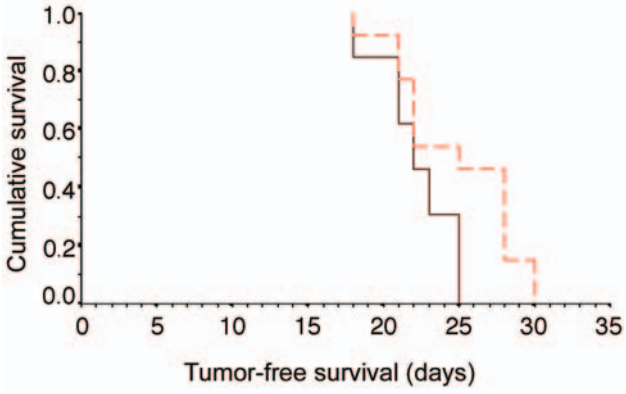

Figure 2. *Survival curves of the animals treated with daily intraperitoneal injections of the m7E3 F(ab')$_2$ fragment (dotted line; n=13) and the control animals (solid line; n=13). This illustrates the tumour-free survival of the animals, i.e. the time from the injection of the tumour cells to the appearance of a tumour in the inoculated tibia.*

angiogenesis independent. The treatment effect became larger with the growth of the bulky tumour, possibly due to the increased need for neoangiogenesis to sustain the growth. The capillary density in the tumour was reduced by the treatment, indicating a direct effect of the anti-integrin treatment on the survival, growth and migration of the endothelial cells. However, it cannot be ruled out that this treatment indirectly inhibited factors other than the migration of the endothelial cells, as indicated by a reduction in the bFGF content of the tumour tissue.

Treatment interfering with the $\alpha_v\beta_3$ integrin function is known to reduce bFGF-mediated angiogenesis (24, 25). Blocking the $\alpha_v\beta_3$ integrin on endothelial calls may therefore

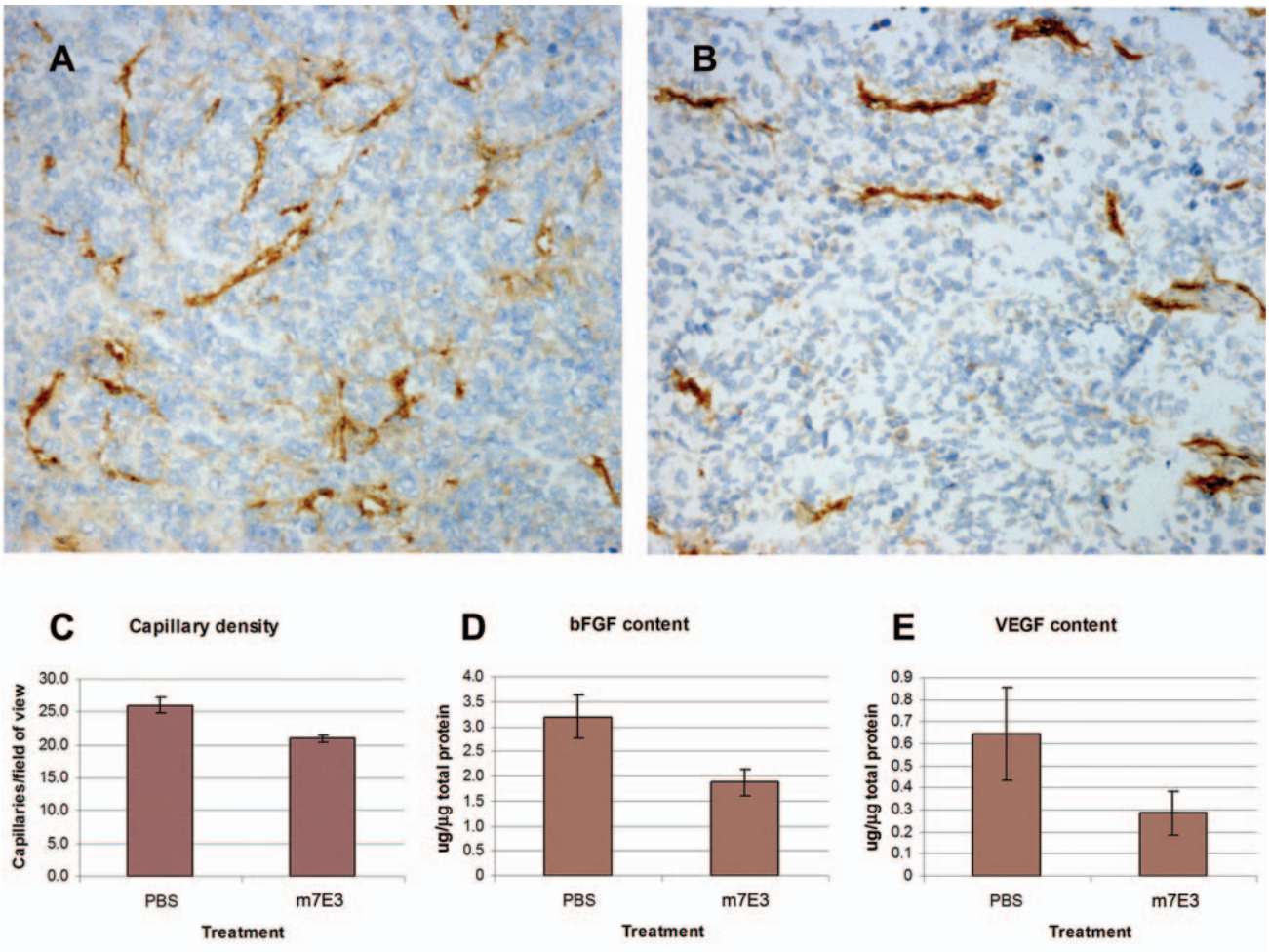

Figure 3. *The effect of treatment with the m7E3 F(ab')$_2$ fragment on angiogenesis. Photomicrographs (×10) of tumor samples from control (A) and treated (B) animals, demonstrating a reduced number of blood vessels in the treated samples. C, Blood vessel counts in tumor samples from treated and untreated animals. D, The bFGF content was significantly reduced in the tumor samples from treated animals. E, The VEGF-A content was lower in the tumor samples from treated animals. The difference was not significant.*

interfere with bFGF-mediated signalling and thereby contribute to the inhibition of neoangiogenesis. This may in fact be an interruption of a vicious circle, blocking the stimulation of the endothelial cells by bFGF, decreasing the capillary proliferation, with a reduction of the blood supply reducing the viability of the tumour cells, thus leading to a reduction in the production of angiogenic factors. An inhibition of the angiogenic effects of VEGF cannot be ruled out, as the $\alpha_v\beta_3$ integrin was found to be important for the activation of the VEGFR-2 receptor (26). This receptor is responsible for the effects of VEGF-A, one of the key stimulators in the process of neoangiogenesis (27). Both of these factors may be induced by a lack of blood supply and hypoxia. However, this did not happen in our study, as both the content of bFGF and VEGF in the tumours were reduced, although only the former reached statistical significance.

To what extent binding of the m7E3 F(ab')$_2$ to the platelet integrin receptor GPIIb/IIIa mediated the effects seen on the tumours is not known. In a previous study, antibodies to this receptor were found to inhibit the sprouting of endothelial cells stimulated by platelet-rich plasma. However, such an effect was also observed by blocking the $\alpha_v\beta_3$ integrin alone (28). However, it cannot be ruled out that a reduction in platelet-derived growth factors, mediated by inactivation of the platelets by binding of the GPIIb/IIIa, may have contributed to the treatment effect in the present study.

Phase I studies targeting the $\alpha_v\beta_3$ integrin for the inhibition of angiogenesis have recently been published (29-33). Both monoclonal antibodies and small molecules inhibiting the integrin function have been administered at dose levels known to saturate the integrin molecules *in vitro*,

Table I. *The distribution and frequency of metastases in m7E3 F(ab')$_2$-treated and control immunodeficient rats inoculated with HeLa cells in the left cardiac ventricle. All the animals were found to have metastases at the end of the experiment by histopathological examination.*

Treatment	Metastatic sites Number of animals with metastases (% of animals)					
	Tibial bone	Spine	Lung	Liver	Brain	Adrenals
m7E3 F(ab')2	6/9 (66%)	6/9 (66%)	2/9 (22%)	0	8/9 (89%)	6/9 (66%)
Control	8/9 (89%)	7/9 (78%)	1/9 (11%)	0	6/9 (66%)	8/9 (89%)

without serious side-effects. Such treatment has resulted in few objective responses, although disease stabilisation has been reported in some of the patients for extended time periods (31, 33). Due to the limited toxicity seen with these compounds, they may be suitable for combination with other therapeutic strategies. Prospects for such therapy and hints of synergistic activity have been observed in the combined treatment of breast cancer with radioimmunotherapy, where the integrin-targeting alone was ineffective (34).

In conclusion, we have demonstrated that blocking the $\alpha_v\beta_3$ and the GPIIb/IIIa integrins *in vivo* reduce the tumour growth in bone, by inhibition of neoangiogenesis as confirmed by the reduced number of capillaries in the treated tumours. The possibility of translating such a treatment strategy for the clinical treatment of solid tumour metastasis, preferably combined with conventional treatment regimens or other antiangiogenic treatment strategies, should be further explored.

Acknowledgements

The authors acknowledge the technical assistance of the Research Laboratory, Department of Pathology, The Norwegian Radium Hospital for processing and immunocytochemical staining of the tissue specimens. Øystein Fodstad was supported by the Norwegian Cancer Society. As part of a research project collaboration, funding for materials in this study was contributed by Centocor, Inc.

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Received September 25, 2008
Revised December 10, 2008
Accepted December 16, 2008