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INTRODUCTION

The sum of accumulated evidence suggests that breast cancer metastases in mineralized bone are consequent upon establishment of tumor within marrow where, by production of osteoclastogenic factors, it promotes differentiation of marrow-residing osteoclast precursors into mature, skeleton-resorbing polykaryons. These cells, in turn, degrade bone, permitting extension of tumor into mineralized matrix.

In light of the above, we hypothesize that:

- 1) MOLECULES EXIST ON THE SURFACE OF BREAST CANCER CELLS WHICH MEDIATE THEIR ATTACHMENT TO COMPONENTS OF THE MARROW MICROENVIRONMENT;**
- 2) BREAST CANCER CELLS PRODUCE OSTEOCLASTOGENIC FACTORS WHICH CAN BE IDENTIFIED IN VITRO;**
- 3) INHIBITION OF OSTEOCLAST DIFFERENTIATION AND/OR FUNCTION DIMINISHES BREAST CANCER INDUCED BONE DESTRUCTION.**

To test these hypotheses we will:

- 1) CHARACTERIZE THE MOLECULES ON METASTATIC BREAST CANCER CELLS WHICH MEDIATE THEIR ATTACHMENT TO THE CELLULAR OR MATRICAL MARROW MICROENVIRONMENT;**
- 2) DEFINE THE MECHANISMS BY WHICH BREAST CANCER CELLS PROMOTE OSTEOCLASTOGENESIS IN VITRO;**
- 3) DETERMINE IF INHIBITION OF OSTEOCLAST DIFFERENTIATION AND/OR FUNCTION, IN VIVO, DIMINISHES BREAST CANCER-INDUCED BONE DESTRUCTION.**

STUDIES AND RESULTS

In the previous years reports we demonstrated that human breast cancer cell lines T47D and MCF-7 promote differentiation of osteoclasts when cocultured with bone marrow macrophage precursors (BMM). Osteoclast differentiation by these tumor lines was dependent on cell contact between the tumor cells and BMMs, and on the presence of vitamin D. In similar assays, a third cell line, the MDA-MB-231 (MDA-231) did not support osteoclastogenesis. This suggests that multiple mechanisms are used by different breast cancer cell lines to promote osteoclast differentiation. Because the T47D and MCF-7 cell lines were not very tumorigenic or metastatic in our *in vivo* bone metastasis model (intracardiac injection of malignant cells as described (1)), we have primarily concentrated on use of the MDA-231 cells for our *in vivo* metastasis assays. Following intracardiac injection, the MDA-231 cells metastasize to the skeleton and establish osteolytic lesions (Fig. 1). Thus, while MDA-231 cells fail to promote differentiation of osteoclasts *in vitro*, they are capable of recruiting the resorptive cells *in vivo*. In an effort to identify potential osteoclastogenic factors produced by the MDA-231 cells, marrow from tumor bearing bones were flushed with phosphate buffered saline (PBS), the cells pelleted and the supernate (marrow plasma) was assayed for presence of osteoclast inducing cytokines. It has been established that among various cytokines TNF- α , IL-1 and IL-6 are potent inducers of osteoclasts (2-7). Thus, the level of these cytokines in the serum plasma of tumor bearing femurs was compared to marrow plasma isolated from non-tumor bearing femurs. Using commercial ELIZA kits the presence of human (i.e. tumor cell derived), but not mouse (i.e. host derived) IL-6 was detected (Fig. 2). Analysis of the MDA-231 cells cultured *in vitro* showed that the cells constitutively secrete human IL-6 into the medium at up to 8 NG/10⁶ cells in 48 hrs (Fig. 3). In addition, the marrow plasma from tumor bearing bones express mouse, but not human, IL-1 (data not shown). TNF- α , either human or mouse, expression was undetectable (data not shown). Thus, the MDA-231 cells secrete the osteoclastogenic cytokine IL-6 and induce the production of IL-1 by the host, while TNF- α appears to not be involved in osteolysis in this system. It should be noted that the serum from the tumor bearing animals, similar to the normal control mice, expressed undetectable levels of TNF- α , IL-1 and IL-6, supporting the notion that these cytokines are produced and act locally. Experiments are planned to determine if inactivating antibodies to IL-6 and/or IL-1 will inhibit osteolysis induced by the MDA-231 cells.

We have previously shown that MD-231 cells selected for preferential attachment to primary human bone marrow stromal cells, metastasize to bone up to six times more frequently than their marrow non-adherent counterparts. Analysis of the integrin expression pattern by these cells showed that the stroma-adherent, highly bone metastatic cells express lower levels of the $\alpha_v\beta_3$ integrin. This inverse correlation between $\alpha_v\beta_3$ expression

and bone metastasis was confirmed by isolation of $\alpha_v\beta_3$ high- and non-expressing variants of the parental MDA-231 line and demonstrating that the $\alpha_v\beta_3$ **non-expressors**, in fact, metastasize to bone as much as 10 times **more** frequently than the **high expressors**. To determine, if the negative correlation between $\alpha_v\beta_3$ expression and bone metastasis can be generalized to other breast tumor lines, we obtained $\alpha_v\beta_3$ high- and non-expressing variants of the MDA-435 line (generously provided by Dr. J. Smith of the Burnham Institute). In vivo metastasis data demonstrate that the low $\alpha_v\beta_3$ expressing MDA-435 cells metastasize to bone with a 78% frequency while the high integrin expressor metastasize to bone with a 25% frequency (Table 1). Thus, the inverse correlation between $\alpha_v\beta_3$ expression and bone metastasis may be a general characteristic of human breast cancer cells. It would be useful to determine if these results are extendable to primary human breast tumor cells.

Table 1. Low $\alpha_v\beta_3$ expression predisposes multiple breast cancer cell lines to preferentially metastasize to bone*

MDA-435	Number of	Number of mice	Number of weeks to
High $\alpha_v\beta_3$	8	2	3-4
Low $\alpha_v\beta_3$	9	7	3-4

*Data for the MDA-231 cells provided in last years progress report.

To determine if low $\alpha_v\beta_3$ expression is causal to increased bone metastasis, we re-expressed $\alpha_v\beta_3$, by transfection of the β_3 subunit, on the surface of the $\alpha_v\beta_3$ non-expressing lines MDA-231 C4 and C5. If a causal relation between expression level of the integrin and bone metastasis exists, then we would expect a decrease in rate of bone metastasis after re-expression of $\alpha_v\beta_3$. For controls, the cells were transfected with the empty expression vector. All transfected cells were selected for growth in the presence of the antibiotic G418. The β_3 transfected cells were subsequently sorted, using the $\alpha_v\beta_3$ complex specific antibody LM-609, to obtain a population that expresses the same level of $\alpha_v\beta_3$ as the original high $\alpha_v\beta_3$ expressing MDA-231 cells (Fig. 4). When the bone metastatic potential of the β_3 -transfected cells were tested in vivo, it was found that they metastasize to bone with the same frequency as their $\alpha_v\beta_3$ non-expressing parents (Table 2). This result suggests that while a strong negative correlation exists between $\alpha_v\beta_3$ expression and frequency of bone metastasis, low expression of this integrin alone is not causative to the observed increased frequency of osteolysis.

Table 2. Low $\alpha_v\beta_3$ expression correlates with, but is not causal to, increased frequency of osteolysis

MDA-231 subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Number of weeks to detection of bone metastases
MDA-231-C4 vector transfected	9	9	4-6
MDA-231-C4 β_3 transfected	9	6	4-6
MDA-231-C5 vector transfected	8	7	4-6
MDA-231-C5 β_3 transfected	9	9	4-6

In the previous experiments, we observed that mice injected with high $\alpha_v\beta_3$ expressing MDA-231 cells suffered from increased lung tumors as compared to animals injected with low $\alpha_v\beta_3$ expressing variant of MDA-231 cells (Fig. 5). This suggested that presence $\alpha_v\beta_3$ may be a determinant of lung targeting by circulating breast tumor cells. Because in the cardiac injection model the tumor cells are introduced in the thoracic cavity, the results of increased lung metastasis by high $\alpha_v\beta_3$ expressing cells was not conclusive as it was possible that the metastases arose from cells inadvertently introduced directly into the lungs. To exclude this possibility, the high and low $\alpha_v\beta_3$ expressing MDA-231 variants were injected into the tail vein as a model of hematogenous spread to the lungs. After three months, 100% of the mice (n=5) injected with the high $\alpha_v\beta_3$ expressing MDA-231 cells developed macroscopically visible lung tumors, while only 20% (n=5) of the mice injected with the $\alpha_v\beta_3$ non-expressing MDA-231 clone C5 developed lung metastases (Fig. 6). Histological analysis of the lungs from each group confirms the increased tumor burden in the lungs of mice injected with the high $\alpha_v\beta_3$ expressing MDA-231 cells (data not shown).

CONCLUSIONS

In conclusion we have demonstrated expression level of the integrin $\alpha_v\beta_3$ correlates with organ-specific targeting of human breast tumor cells, such that low integrin expressing cells preferentially home to bone, while high $\alpha_v\beta_3$ expressing cells preferentially target to lungs. In addition, we have shown that the MDA-231 cells express the osteoclastogenic cytokine IL-6, and directly or indirectly promote expression of a second osteoclast promoting cytokine, IL-1 by the host. Expression of these cytokines likely contributes to the tumor induced osteolysis.

Based on these results, we will now focus on the following experiments.

A) We will establish if IL-1 or IL-6 have a significant role in tumor osteolysis. The approach will be to induce bone metastases in mice, and determine if inactivating antibodies to either cytokine will reduce the extent of osteolysis when compared to none immune IgG treated animals. These antibodies will be administered i.p. under two regiments: i) immediately following inoculation with the tumor cells to determine if inhibition of either cytokine will prevent osteolysis. ii) In the second regiment, the antibodies will be administered i.p. following the initial detection osteolytic metastases to determine if inhibition of either IL-1 or IL-6 will reverse the tumor induced bone destruction. In the event that antibodies to both cytokines show partial activity, we will administer the antibodies together to determine if the combination therapy is more effective in preventing or reversing the osteolysis.

B) In vivo metastasis data using two breast cancer cell lines demonstrate a negative correlation between $\alpha_v\beta_3$ expression and frequency of bone metastasis. It is important to determine if primary breast tumor cells also behave in the mouse model similarly to the MDA-231 and MDA-435 lines. For this purpose, we will initially measure the expression level of $\alpha_v\beta_3$ by primary tumor cells using FACS analysis to quantitate the levels of this integrin. Next the in vivo metastasis potential of low and high $\alpha_v\beta_3$ expressing primary tumor cells will be assessed in our mouse model. In the event that tumor cells isolated from a single patient contains a mixed population of low and high $\alpha_v\beta_3$ expressing cells, then we will first derive low and high integrin expressing sublines (similar to our approach with the MDA-231 cells) prior to the in vivo metastasis assays. These experiments will determine the potential diagnostic value of $\alpha_v\beta_3$ expression in patient at risk of breast cancer metastasis to bone.

C) In the previous year's report, we demonstrated isolation of highly bone metastatic derivatives of MDA-231 cells generated by repeated in vivo passage of the tumor cells. We are in the process of raising monoclonal antibodies which specifically bind to the surface

of the passaged, highly bone metastatic tumor cells, but not to the parental, low osteolytic MDA-231 cells. The ability of these antibodies in preventing osteolysis will be tested using the scheme outlined above for antibodies to IL-1 and IL-6. Identification of such inhibitory antibodies will then allow the characterization of the antigen which presumably plays a significant role in bone metastasis.

METHODS

Reagents. Sources of antibodies used in this study were: LM609 anti- $\alpha_v\beta_3$ (8,9) generously provided by Dr. Cheresch (Scripps Research Institute); P1E6 anti- α_2 , P1B5 anti- α_3 , P4C2 anti- α_4 , P1D6 anti- α_5 and anti- $\alpha_v\beta_5$ P1F6 (all purchased from Gibco/BRL); anti- α_6 GoH3, anti- β_1 MAR4 (both purchased from Pharmingen); and anti- α_v L230 (American Type Culture Collection).

Cell Culture. MDA-231 cells were obtained from the American Type Culture Collection, and cultured in α -MEM containing 10% fetal bovine serum (α -10-MEM; Gibco/BRL). The cells were passaged by trypsinization twice weekly.

In vivo metastasis assays. For skeletal metastasis, subconfluent MDA-231 cells were harvested non-enzymatically with cell dissociation medium (Sigma), washed, and the concentration adjusted to 10^6 cells/ml in PBS. These cells were kept at 4°C until injected into mice. Athymic nude mice were anesthetized with 65 mg/kg body weight of pentobarbital administered intraperitoneal. Each animal was injected with 10^5 cells (0.1 ml) in the left ventricle, by insertion of syringe needle between the second and third rib, and 2-3 mm to the left of the sternum (1,10). Entry of oxygenated (bright red) blood in the needle assured injection in the left ventricle (1). Progression of osteolytic metastases was monitored by x-ray of the animals using a CGR Sonograph 550T Dedicated Mammography Unit with Kodak MinR film at weekly intervals.

For lung metastasis, the MDA-231 cells were prepared as above, and 10^5 cells in 0.1 ml of PBS were injected into the lateral tail vein. The animals were sacrificed after three months, and the lungs examined for the presence of metastatic tumors.

Isolation of high $\alpha_v\beta_3$ -expressing population and $\alpha_v\beta_3$ non-expressing C4 and C5 clonal variants of MDA-231. Subconfluent MDA-231 cells were lifted from culture plates with cell dissociation medium, washed into serum-free α -MEM, concentrated to 10^7 cells/ml, incubated on ice with 10 μ g/ml of anti- $\alpha_v\beta_3$ heterodimer-specific antibody LM-609 (8,9) for 60 minutes. The primary antibody was washed, and the cells incubated with a fluorescein labeled anti-mouse secondary (Jackson Immunochemicals). After washing excess secondary antibody, the cells were sorted using a FACSCAN (Becton-Dickinson). The

highest and the lowest 0.5% fluorescent labeled cells were selected and grown. This procedure was repeated four times to isolate the high $\alpha_v\beta_3$ expressing subpopulation of MDA-231 cells. Since this approach failed to yield a low $\alpha_v\beta_3$ expressing population, the FACS was repeated except that the low $\alpha_v\beta_3$ expressing cells were cloned by limiting dilution to obtain clonal lines. The $\alpha_v\beta_3$ expression of each line was measured by FACS and only clones, MDA-231 C4 and C5, expressing undetectable expression of the integrin were further propagated.

Expression of β_3 cDNA. The full-length human β_3 cDNA cloned in the pRc/RSV expression vector (Invitrogen) was provided by Drs. S. Blystone and E. Brown (11). Five μ g of this plasmid or pRc/RSV vector, as control, in 200 μ l of Opti-MEM (Gibco/BRL) was mixed with 20 μ g of Lipofectamine in 200 μ l of Opti-MEM. After 30 minute incubation, the lipid/DNA mixtures were added to MDA-231 clones C4 and C5. After six hrs the medium was changed, and after 48 hrs the cells were passaged at low density into α -10-MEM containing 650 μ g/ml G418. Antibiotic resistant clones became identifiable after 10 days. These cells were stained with LM609 and the highest 0.5% $\alpha_v\beta_3$ expressors isolated by FACS. The FACS sorting was repeated three times to isolate populations of transfected MDA-231 C4 and C5 cells over-expressing $\alpha_v\beta_3$ at a similar level to that expressed by the high $\alpha_v\beta_3$ expressing population. The populations of MDA-231 clones C4 and C5 transfected with the control pRc/RSV vector did not express $\alpha_v\beta_3$ as demonstrated by FACS analysis using LM609.

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Tondravi, M., Quiroz, M., Scott, P., and Teitelbaum, S.L. (1997) High $\alpha_v\beta_3$ expression inversely correlates with metastasis of human breast cancer cells to bone. *J Bone Miner Res* 12:S397.

FIGURES

- Fig. 1 Human breast cancer cells metastasize to bone and cause osteolytic lesions. X-ray of a mouse bearing two osteolytic lesions (arrows, left panel). Histological section of a metastatic bone tumor stained for tartrate-resistant acid phosphatase showing abundant osteoclasts recruited by the malignant cells (right panel).
- Fig. 2 Human breast cancer cells express osteoclastogenic cytokine IL-6 in vivo. Marrow from femurs of a normal mouse (no tumor) or from three animals with osteolytic lesions, resulting from metastasis of MDA-231 cells, were flushed with PBS, the cells pelleted and the supernates assayed for presence of human IL-6 using a commercial ELISA kit. The marrow plasma from each of the animals harboring metastases expresses significant levels of IL-6 while this cytokine is undetectable in the control.
- Fig. 3 MDA-231 human breast tumor cells express IL-6 in vitro. The parental MDA-231 or indicated sublines of it were grown to confluence, transferred to serum-free medium for 48 hrs., and level of secreted IL-6 measured using a commercial ELISA kit. As indicated, the parental MDA-231 and all of its derivatives produce significant quantities of IL-6.
- Fig. 4 Over expression of $\alpha_v\beta_3$ in $\alpha_v\beta_3$ non-expressing MDA-231 clonal line C5. The top panel shows the low level of $\alpha_v\beta_3$ expressed by the MDA-231 C5 subline. The β_3 transfected MDA-231 C5 cells (bottom) express the same level of the integrin, detected by LM609 staining, as the high $\alpha_v\beta_3$ expressing population previously selected by FACS (middle).
- Fig. 5 Low $\alpha_v\beta_3$ expressing MDA-231 cells metastasize less frequently to the lungs than high $\alpha_v\beta_3$ expressing MDA-231 cells. Representative sections through the lungs of mice injected intracardiac with: i) high $\alpha_v\beta_3$ expressing MDA-231 cells (Panel A), ii) $\alpha_v\beta_3$ non-expressing MDA-231 clones C4 (Panel B) and C5 (Panel C). All sections were stained with hematoxylin and eosin, and photographed at 40X magnification to show a large area of the section. All of the tumors in each section are indicated with arrow heads. The numbered tumors in each panel were also photographed at 200X magnification and are shown as insets (to the left of each panel). Note that the high $\alpha_v\beta_3$ expressing cells produced greater number as well as larger tumors than clonal lines C4 and C5 which do not express $\alpha_v\beta_3$.

Fig. 6 Injection of MDA-231 cells into the tail vein confirms that high $\alpha_v\beta_3$ expressing MDA-231 cells preferentially metastasize to the lungs. Top panels show a photograph of the right and left lobes of lungs from a mouse injected with the high $\alpha_v\beta_3$ expressing subline of MDA-231. The tumors are indicated by the arrow heads. The bottom panels show the absence of lung tumors in a mouse injected with the low $\alpha_v\beta_3$ expressing MDA-231 C5 subline.

Figure 1

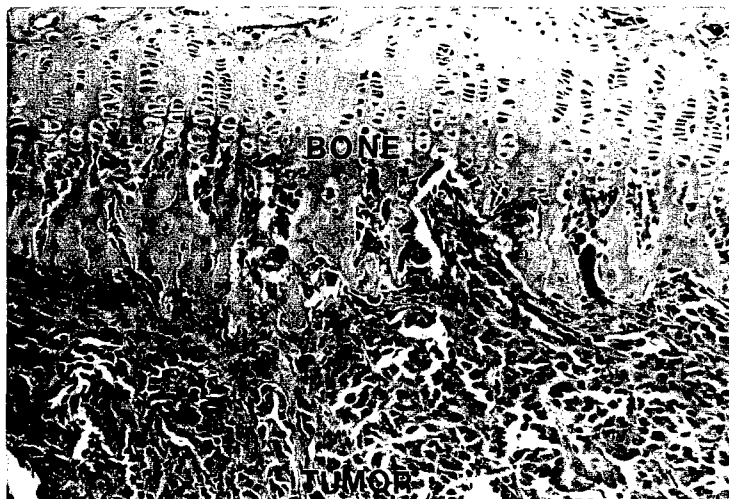


Figure 2

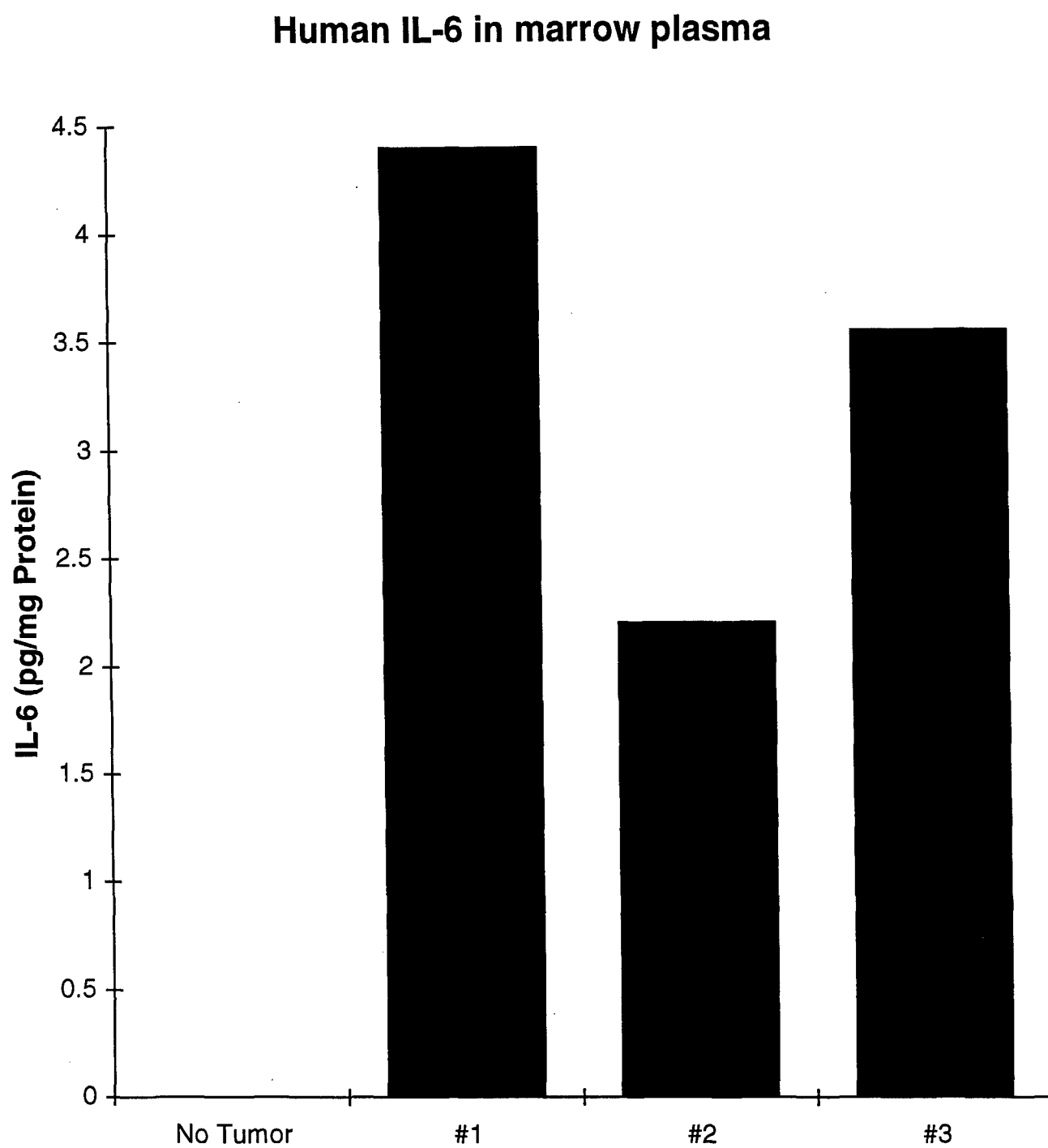


Figure 3

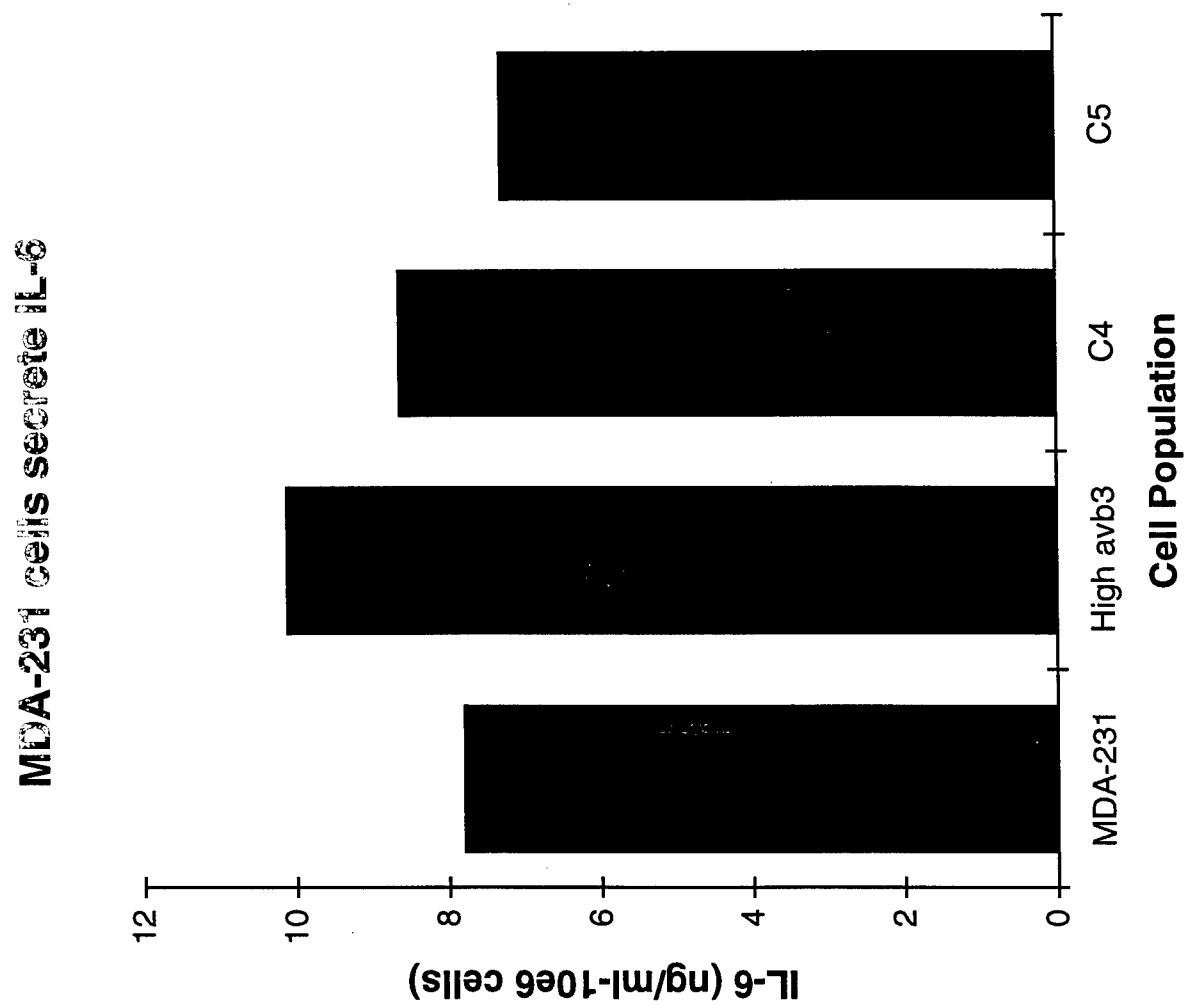


Figure 4

Over expression of $\alpha_v\beta_3$ integrin in $\alpha_v\beta_3$ non-expressing MDA-231 C5 cells.

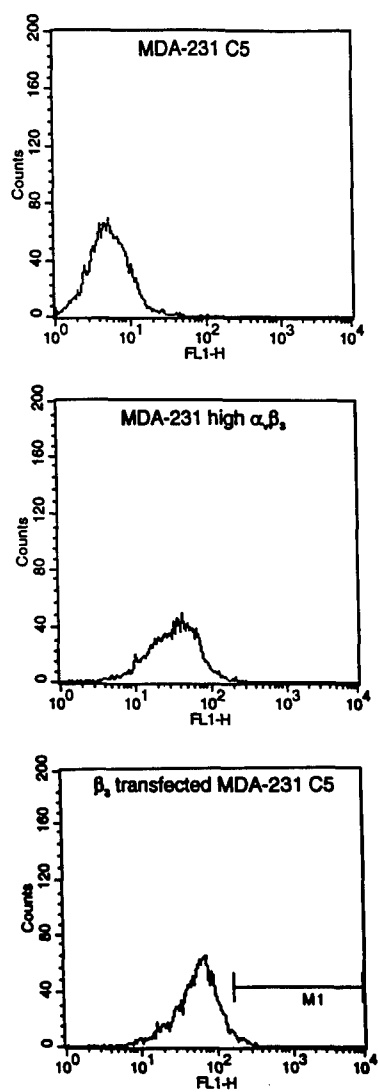


Figure 5

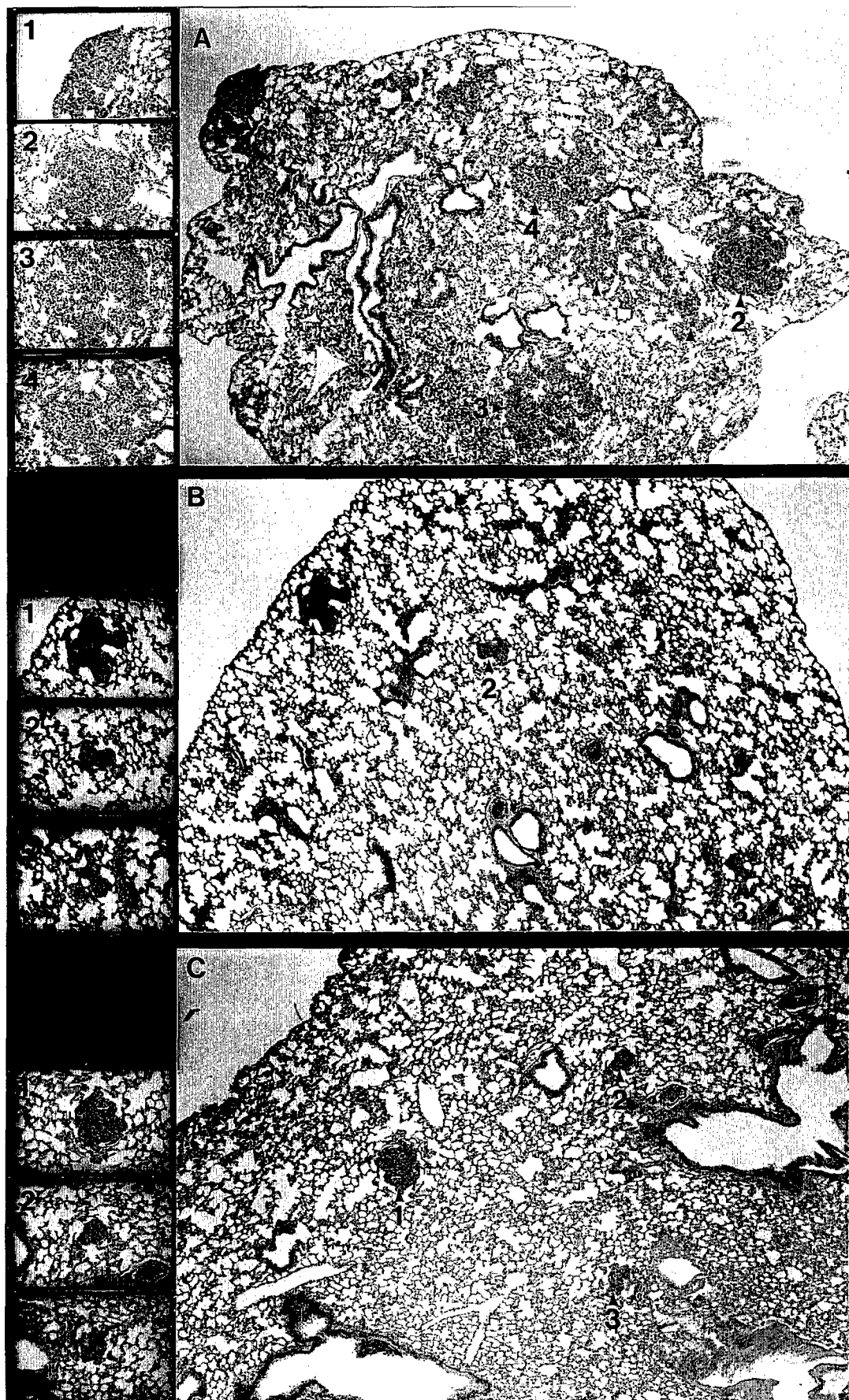


Figure 6

