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13. ABSTRACT *(Maximum 200 words)*

Breast cancer often metastasizes to bone causing fractures and hypercalcemia due to osteolysis. This process likely involves recruitment by the cancer cells of osteoclasts from precursors. A coculture system was used to demonstrate that human T47D breast cancer cells promote differentiation of osteoclasts from precursors. This process was dependent on the concentration of cancer cells and the presence of $1,25(OH)_2D_3$. Clonal sublines of T47D were derived which lacked the ability to promote osteoclast differentiation. Using in vitro cancer cell attachment assays to human bone marrow stromal cells, two populations, adherent and non-adherent, of MDA-MB-231 cells, were isolated. When each population was tested in vivo for bone metastasis, the adherent cells showed a higher frequency of osteolytic lesions. It was established that MDA-MB-231 cells attach to human bone marrow stromal cells in part by α_v integrins. Cells were selected which expressed either high or undetectable levels of the $\alpha_v\beta_3$ integrin. When these cells were tested for bone metastasis in vivo, it was determined the cells that did not express $\alpha_v\beta_3$ caused more osteolytic lesions than cells expressing high levels of $\alpha_v\beta_3$. Analysis of bone tumors derived from either low $\alpha_v\beta_3$ expressing cells, or bone stroma adherent cells showed that both expressed low levels of the integrin. From these results we conclude that $\alpha_v\beta_3$ is not required for breast cancer metastasis to bone. Finally, our data demonstrate that cancer cells derived from a bone tumor metastasize to bone more frequently than the parental cells.

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INTRODUCTION

Skeletal metastasis is virtually ubiquitous among breast cancer patients dying of their disease (1). In fact, 80% of all metastatic lesions to bone are derived from either a breast or prostate primary (2). The fatal consequences of such metastasis include fracture and hypercalcemia, the latter occurring in one third of breast cancer patients with bone involvement (3). Thus, prevention of skeletal metastasis would greatly impact on the morbidity of breast cancer.

While the precise mechanisms by which breast cancer metastasizes to the skeleton and subsequently destroys bone are incompletely understood, osteoclasts probably play a critical role. It appears that breast cancer metastases in mineralized bone are consequent upon establishment of tumor within marrow. Once having achieved sufficient mass, the cancer promotes differentiation of marrow-residing osteoclast precursors into mature, skeleton-resorbing, polykaryons. These cells, in turn, degrade bone, permitting extension of breast cancer into mineralized matrix.

In light of the above, we hypothesized 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.**

Therefore, our specific aims were to:

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

We have initiated two strategies to define the role of attachment molecules, particularly integrins, in metastasis of human breast cancer cells to bone - a central tenet of our first specific aim. The first directed approach relied on selection of cancer cells that DID or DID NOT express a given cell adhesion molecule (CAM) on their surfaces, followed by measurement of the frequency of bone metastasis in vivo for each of the cell populations. The second approach relied on selection of cells that DID or DID NOT adhere to primary human bone marrow stromal cells. These cells were then assessed for bone metastasis potential in vivo, and the levels of various CAMs were compared, enabling us to draw correlations between expression of a particular CAM and bone metastasis. During the course of our experiments, described in detail below, we determined that T47D and MCF7 human breast cancer lines, mentioned in our original proposal, did not produce lytic bone lesions as consistently as the MDA-MB-231 line; therefore, all the in vivo experiments used the latter cell line.

In our directed approach to assess the role of CAMs in bone metastasis, we used FACS sorting to select one population of MDA-MB-231 cells which expressed high levels of a given CAM and a second population which expressed undetectable levels of that CAM. Both cell types were then injected into left ventricles of nude mice and the number of animals from each group with lytic bone lesions, as determined by x-ray, was scored. This approach allows the evaluation of the role of single molecules in the metastatic process. We chose $\alpha_v\beta_3$ for our first experiments not only because of our previous experience with this integrin, but also because: 1) $\alpha_v\beta_3$ has been shown to be pivotal for metastasis of some melanomas, e.g. M21 and M3Dau (4,5). 2) This integrin was shown to be expressed on those breast cancer biopsies examined (6,7). 3) MDA-MB-231 cell attachment to primary human bone marrow stromal cells (described in more detail in the next section) was approximately 75% inhibitable by an anti-α_v antibody. By repeated FACS sorting with the $\alpha_v\beta_3$ complex specific antibody, LM609, we succeeded in isolating a population of MDA-MB-231 cells expressing 5-10 times more $\alpha_v\beta_3$ than the parental MDA-MB-231 cells as well as three populations in which $\alpha_v\beta_3$ was not detectable. We have injected 20 nude mice with the high $\alpha_v\beta_3$ expressing MDA-MB-231 variant and observed one bone tumors. This tumor was flushed from the bone marrow, cultured and its $\alpha_v\beta_3$ expression was unchanged. When low $\alpha_v\beta_3$ expressing MDA-MB-231 cells were injected into mice, 3 bone tumors out of 20 total injected animals were observed. When $\alpha_v\beta_3$ expression levels of two of these tumors were determined by FACS, one of the tumors maintained its original low expression while the second tumor expressed an incrementally higher level of the integrin. These results indicate that $\alpha_v\beta_3$ expression is not required for breast cancer metastasis to bone, and although the numbers are too low to be statistically significant (at this time) they indicate that $\alpha_v\beta_3$ may actually reduce the frequency of bone metastases.

The above experiments demonstrate the usefulness of the directed approach to determine the role of specific molecules in bone metastasis. Since our data with the α_v antibody described above indicates that α_v integrins are important for attachment of breast cancer cells to bone marrow stromal cells, we have recently initiated experiments to select for MDA-MB-231 cells that DO or DO NOT express any α_v integrin. Once cells have been selected, we will measure the rate of metastasis of each population to bone. We have determined that $\alpha_v\beta_3$ is not required for bone metastasis, but it will be of interest to determine which of the α_v integrins may mediate this process. Our immunoprecipitation data indicate that MDA-MB-231 cells express high levels of $\alpha_v\beta_5$, and we are selecting for subpopulations which do not express this integrin to determine the role of this integrin in metastasis. In the coming year, we plan to address the role of each of the α_v integrins in metastasis of human breast cancer cells to bone.

The second approach designed to identify CAMs which may play a role in breast cancer metastasis to bone, relied on selection of a subpopulation of MDA-MB-231 cells which preferentially adhered to human bone marrow stromal cells. Stromal cells were grown to confluence in microtiter plates, washed with PBS and used as substrate for MDA-MB-231 attachment. One MDA-MB-231 subpopulation was selected which ADHERED to the stromal cells within 5 minutes, while the second selected subpopulation DID NOT ADHERE even after 60 minutes. The selection process for each subpopulation was repeated 4 times to further enrich for adherent or non-adherent cancer cells. When nude mice were injected with each of these populations, 3/17 adherent and 1/14 non-adherent bone metastases were observed. Again our number of bone tumors vs. number of injected animals is too low to be statistically significant, but these initial results indicate that breast cancer cells that adhere preferentially to bone stroma in vitro also have a higher propensity to metastasize to bone in vivo. Currently, we have reisolated and cultured one primary bone tumor from the adherent and one from the non-adherent MDA-MB-231s. Each of these tumors was scanned by FACS for expression of $\alpha_v\beta_3$. Surprisingly the primary tumor derived from the 5 minute adherent cells expressed significantly lower levels of $\alpha_v\beta_3$ than either the non-adherent or the parental MDA-MB-231 cells. This observation independently confirms our conclusion from our directed approach, described above, that expression of $\alpha_v\beta_3$ is not necessary for metastasis of human breast cancer cells to bone. In fact high $\alpha_v\beta_3$ expression may lead to lower bone metastasis, but higher rate of metastasis to other organs such as lungs. We are currently investigating this possibility.

Studies with melanomas have previously demonstrated that it is possible to isolate more metastatic cells by serial passage in vivo from less metastatic parental cells (4). Based on this model, we isolated cells from metastatic bone tumors of MDA-MB-231 expressing high or low levels of $\alpha_v\beta_3$, and adherent or non-adherent to marrow stromal cell monolayers. To date we have tested the metastatic potential of low $\alpha_v\beta_3$

expressing cells as well as bone marrow stroma adherent tumor cells. In both cases, we find that incidents of bone metastases increased from 1-2 per 10 injected mice for the parental cells, to 8-10 bone tumors per 10 animals injected. It is noteworthy that the animals injected with cells derived from primary bone tumors, developed lytic bone lesion in shorter time (4 weeks or less) as compared to animals injected with the parental cells (6-8 weeks). Additionally, the animals injected with the primary tumor cells (as opposed to those injected with the parental cells) did not appear cachectic at the time of detection of the initial lytic bone lesions. These results demonstrate that it is in fact possible to isolate more aggressive bone metastatic tumors by serial passage in vivo. Comparisons of CAM expression in the parental, primary and secondary tumors will allow us to draw correlations between expression of a given CAM by a cell and the bone metastasis potential of that cell. Once such a correlation has been shown, we can select subpopulations of cells that do not express this CAM, inject them into mice and determine their metastatic potential. The usefulness of this approach is demonstrated by our experiments using cells selected for high and low expression of $\alpha_v\beta_3$ summarized above.

We find that cells derived from bone tumors have significantly higher incidence of bone metastasis (80-100%) than their respective parental cells (10-20%). Using these populations with low and high bone metastasis, a strategy for identification of CAMs involved in metastasis was outlined above. Bone metastasis may also result from expression of a novel CAM, or inappropriate expression of a secreted or cytoplasmic protein. These possibilities will be addressed by either differential cDNA cloning, or by differential display PCR which facilitate the identification of molecules that are expressed at different levels in the low and high metastatic cells. Other cloning strategies, such as subtractive cDNA cloning or expression cloning, are also possible and will be considered if we fail with above methods.

The second specific aim of our proposal focused on delineating the mechanisms by which breast cancer cells promote osteoclast differentiation in vitro. Our initial results presented in the original proposal led us to believe that the cancer cells secreted a factor(s) which recruited osteoclasts from precursors. Further experiments in our in vitro osteoclastogenesis system shows that to not be the case. We have confirmed that co-culture of T47D cells with murine bone marrow macrophages (BMMs) does indeed result in osteoclast differentiation (Figs. 1A & 1B - see Appendix). Furthermore, generation of osteoclasts is dependent on presence of $1,25(OH)_2D_3$ in the co-cultures (Fig. 2 - see Appendix). However, when T47Ds and macrophages were separated in transwell dishes, or by a layer of agar between the two cell types, we failed to generate any osteoclasts (Fig. 3 shows the transwell data - see Appendix). Thus it appears that the cancer cells recruit osteoclasts by a cell contact mediated event rather than by a secreted factor.

A different strategy was used to identify putative molecule(s) expressed by T47Ds which recruit osteoclasts from precursors. In this case, the T47Ds were randomly cloned, and seven clonal sublines of T47Ds (T47D clone 1-7) were obtained. Each of the clonal sublines was assayed in the osteoclastogenic co-culture system. The results demonstrated that four of the clones were at least as efficient as the parental T47D in promoting osteoclast differentiation. More interestingly, three of the clones failed to promote osteoclast differentiation (Fig. 4 - see Appendix). Our original plan was to use a differential cloning technique to identify differences between the two types of T47D clones. However, the clones which initially did not promote osteoclasts had an unstable phenotype, and with passage in vitro they regained the ability to promote osteoclast differentiation. We are now attempting to isolate a non-osteoclastogenic clone of T47D with a stable phenotype.

In the third specific aim of our proposal, we ask whether inhibition of osteoclast differentiation or function diminishes breast-cancer induced osteolysis. During the past year we have generated the critical tools to address this question. Specifically, we have generated subpopulations of MDA-MB-231 cells which metastasize to bone in 80-100% of the recipients, and do so in a relatively short period of time. With this system in hand, we will assess the effect of administering the osteoclast proton pump inhibitor TI-EDTA to animals injected with highly metastatic MDA-MB-231 cells. Since our in vitro cell attachment data indicates that α_v integrins are important for bone metastasis, we will also test the effects of infusing recipient mice with an antibody to human α_v that prevents any α_v integrin from binding to extracellular matrix.

CONCLUSIONS

We have demonstrated that when breast cancer cells are selected for attachment to bone marrow stroma in vitro they metastasize to bone more frequently in vivo. The cancer-cell bone marrow stroma interaction is at least partially mediated by the integrin family of cell adhesion molecules. This conclusion is supported by the fact that attachment of cancer cells is inhibited by RGD but NOT RGE containing peptides, and is also inhibited by an anti-α_v antibody. The importance of one α_v integrin, namely $\alpha_v\beta_3$, in metastasis of breast cancer to bone was evaluated directly by selecting by FACS for subpopulations of cancer cells which did or did not express the integrin. The results demonstrated that $\alpha_v\beta_3$ is not necessary for bone metastasis, and that its expression may actually lower the frequency of bone metastases. This conclusion was independently confirmed by analyzing the expression level of $\alpha_v\beta_3$ on bone metastatic cells derived from cells selected for preferential attachment to bone marrow stroma.

We have also demonstrated that cancer cells derived from bone tumors are more metastatic to bone than their parental cells. By isolating cells that have low or

high bone metastasis frequency, we are in the position to characterize the changes that lead to more aggressive bone metastasis. Specifically we will analyze changes in expression of CAMs by FACS analysis, and changes of expression of other molecules by differential display PCR. In either case, once a candidate molecule involved in metastasis has been identified, we will select for cell lines that do not express the given molecule (using methods outlined above) and determine whether the metastatic behavior of the cell changes.

We have completed our initial studies of T47D induced osteoclast differentiation in vitro. Our results indicate that this process requires the presence of $1,25(OH)_2D_3$ and cell contact between the cancer cells and the osteoclast precursors. We have derived subclones of T47D which have lost their ability to promote osteoclast differentiation in vitro. If we are able to generate such T47D subclone that demonstrates a stable phenotype, then we will be in the position to use differential display PCR to determine differences in gene expression between that clone and one that does promote osteoclast differentiation in vitro.

Having generated breast cancer cells which metastasize to bone with high frequency, we are in the position to initiate experiments aimed at determining whether inhibition of osteoclast differentiation or function in vivo will diminish the frequency of metastasis induced osteolytic lesions.

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American Society for Bone and Mineral Research Meeting - September 9-13, 1995 Baltimore, Maryland

Tondravi, M.M., Quiroz, M., Ross, F.P., and Teitelbaum, S.L.: The human breast cancer cell line T47D supports osteoclast differnetiation in vitro. Journal of Bone and Mineral Research (in press).

Tondravi, M.M., Quiroz, M., and Teitelbaum, S.L.: Role of α_v-integrins in attachment and metastasis of human breast cancer cells to bone. Journal of Bone and Mineral Research (in press).

Keystone Symposia on Molecular & Cellular Biology/Cancer Cell Invasion and Motility - February 5-11, 1995, Tamarron, Colorado

M. Mehrdad Tondravi, Marisol Quiroz, F. Patrick Ross and Steven L. Teitelbaum: Human breast cancer cell lines support osteoclast differentiation in vitro. J Cellular Biochem Suppl. 19B:28, 1995.

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THE HUMAN BREAST CANCER CELL LINE T47D SUPPORTS OSTEOCLAST DIFFERENTIATION IN VITRO. M.M. Tondravi, M. Quiroz*, F.P. Ross and S.L. Teitelbaum, Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.

Breast cancer often metastasizes to bone. These bone metastases cause osteolysis resulting in bone fractures and hypercalcemia. Although the precise mechanisms of breast cancer metastasis to bone is not understood, osteoclasts probably play a critical role. Several lines of evidence indicate that the cancer cells do not resorb bone per se, and have led us to the hypothesis that once a tumor has been established in the bone marrow environment, the cancer cells promote the differentiation of marrow-residing osteoclast precursors into mature, bone-resorbing, polykaryons. As a test of this hypothesis, we have established a cell culture system whereby breast cancer cells promote osteoclast differentiation in vitro. The osteoclast is a member of the monocyte/macrophage lineage. In mammals, differentiation of macrophage precursors into osteoclasts requires the presence of accessory cells which include stromal cells or osteoblasts. We have established that the T47D human breast cancer cell line can assume the role of the accessory cells in osteoclastogenesis. We next asked whether this cancer cell line induces osteoclast differentiation through a secretory factor by culturing the osteoclast precursors in the bottom of transwell dishes and the cancer cells on the separating filter. The results indicate that a soluble factor is not involved and that cell contact is required for osteoclast differentiation in this system. In addition, we have generated clonal sublines of the T47D breast cancer cells and have identified clones of T47Ds which do not support osteoclastogenesis in vitro while other sublines do. These two types of clonal sublines of T47D cells will be useful in identifying molecules preferentially expressed by the osteoclast promoting clones.

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ROLE OF α_v-INTEGRINS IN ATTACHMENT AND METASTASIS OF HUMAN BREAST CANCER CELLS TO BONE. M.M. Tondravi, M. Quiroz*, and S.L. Teitelbaum (Intr. by L.R. Chase), Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.

Breast cancer is one of the few neoplasms which preferentially metastasizes to bone, and is often associated with significant morbidity due to osteolysis. Cellular adhesion molecules play key roles in dissemination and selective targeting of metastatic tumor cells. We have initiated experiments to test the role of integrins in the targeting of human breast cancer cells to bone marrow using *in vitro* cell attachment and *in vivo* metastasis. The *in vivo* studies utilize the nude mouse model whereby intraventricular injection of the tumor cells leads to bone metastasis and subsequent osteolysis. Initially a cell attachment assay was developed in which primary human bone marrow stromal cells (HBMSC) were grown as a monolayer, and their extracellular matrix used as the substrate for attachment of the human breast cancer cells MDA-MB-231 cells. Binding of MDA-MB-231 cells to the HBMSC matrix is saturable. The attachment is approximately 75% inhibited by the peptide GRGDSP but not by GRGESP, indicating a role for RGD-dependent integrins in this attachment process. Attachment of MDA-MB-231 cells to HBMSCs is also inhibited by approximately 70-80% with an anti-α_v antibody. This attachment is partially inhibited (30%) by an anti-$\alpha_v\beta_3$ and to a lesser extent (10-30%) by an anti-$\alpha_v\beta_5$ antibody. These results indicate the involvement of other α_v integrins possibly $\alpha_v\beta_1$. In addition, we have used this attachment assay to select for a population of MDA-MB-231 cells which adhere within 5 minutes and a second population which do not attach within 60 minutes. We find that a higher concentration of the anti-α_v antibody is required to inhibit the attachment of the rapidly adhering cells to HBMSCs. Previously, a histological correlation was shown in human breast cancers between expression level of the integrin $\alpha_v\beta_3$ and metastasis to bone. In order to test whether expression of $\alpha_v\beta_3$ is causal to bone metastasis, we have derived sublines of MDA-MB-231 cells which either express high or very low levels of this integrin. The metastatic potential of these MDA-MB-231 derivatives are currently being tested by intraventricular injection of the cells in nude mice.

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B1-412 A SMALL MOLECULAR WEIGHT G-PROTEIN, *rho p21*, ACTIVATES PROTEIN KINASES AND INDUCES PROTEIN TYROSINE PHOSPHORYLATION. Shuh Narumiya, Naokazu Kumagai, and Narito Morii, Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan

rho p21 is a *ras p21*-related small molecular weight GTP-binding protein. This protein is specifically ADP-ribosylated by C3 exoenzyme produced by *Clostridium botulinum*, and inactivated. Studies using this enzyme and several *rho* mutants as probes have revealed that *rho p21* is activated in response to extracellular stimuli and induces activation of integrin molecules and stress fiber formation to stimulate the cell-substrate adhesion. *rho p21* is also suggested to regulate the contractile ring formation during cytokinesis. In order to elucidate the molecular mechanism of these actions, we used botulinum C3 exoenzyme and examined its effects on lysophosphatidic acid (LPA)-induced activation of protein kinases and tyrosine phosphorylation. Subconfluent cultures of Swiss 3T3 cells and rat 3Y1 cells were subjected to serum starvation and incubated with or without C3 exoenzyme for 48 h. The cells were then washed and stimulated by LPA. After incubation, the cells were lysed with the modified RIPA buffer and the lysates were used for the analyses. The immunoblot and immunoprecipitation experiments showed that LPA stimulation induced tyrosine phosphorylation of a number of cellular proteins including p43 ERK-2, p64, p72 paxillin, p88 and a group of proteins of 110-130 kDa including p125 FAK. The treatment with C3 exoenzyme induced the *in situ* ADP-ribosylation of *rho p21* in the cells and significantly suppressed the LPA-stimulated phosphorylation of ERK-2, paxillin and FAK. In order to examine protein kinases involved in this process, the renaturation kinase assay was performed in the cell lysates. This analysis revealed that LPA activated protein kinases of 43, 60, 64, 85 and 145 kDa. Among them, activation of 60/64 kinases was significantly suppressed by the prior C3 exoenzyme treatment. These results suggest that *rho p21* activates a kinase cascade and induces tyrosine phosphorylation of cellular proteins in LPA signalling. Beacuse LPA induces the integrin-mediated cell adhesion via *rho p21*, the above mentioned activation of a kinase cascade may underlie the stimulus-evoked adhesion of cells to substratum.

B1-413 INFLUENCE OF ANTIESTROGENS AND GROWTH FACTORS ON THE INVASIVENESS OF BREAST CANCER CELLS IN CULTURE, J. Thomas Pento, Talitha T. Rajah, S.M. Abbas Abidi and Gina M. Hurt, Department of Pharmacology and Toxicology, University of Oklahoma, Health Sciences Center, Oklahoma City, OK 73190

The influence of antiestrogens [tamoxifen (TAM), ICI-182,780 (ICI) and Analog II (1,1dichloro-Cis-2,3-diphenylcyclopropane)(AII)] on invasiveness and the chemotactic potential of growth factors [EGF, IGF-1 and TGF-β] was examined in MCF-7 (ER+) and MDA-MB-231 (ER-) human breast cancer cells. In addition, cell morphology was examined using scanning electron microscopy (SEM) and type IV collagenase release was assayed. In the invasion assay, cells were treated with antiestrogen (10^{-10}-10^{-6}M) for 4 days and seeded ($3x10^5$cells/well) onto the upper surface of Matrigel coated (100 μg/sq cm) nucleopore membranes (Costar Transwells; 12 μm pore) and incubated for 24 hrs in the presence of antiestrogen. In the chemotaxis assay, cells were treated with antiestrogen (10^{-6}M) for 4 days and seeded ($3x10^5$ cells/well) onto the upper surface of collagen coated (1.5 μg/sq cm) nucleopore membranes (8 μm pore) and allowed to migrate for 6-12 hrs with growth factors (10^{-8}M) added to the lower chamber. Cells on the membrane surface were then fixed, stained with Diff Quik and invasiveness or chemotaxis determined by counting cells in 10-20 fields on the lower surface. In addition, collagenase activity in media was determined. The results indicate that AII and TAM (10^{-6}M) caused a 20 % reduction in the invasiveness of MDA cells. It was further determined that collagenase release from MDA cells, which was greater than from MCF cells, was reduced by AII and ICI (10^{-6}M) treatment. It was observed by SEM that MDA cells developed cell surface microvilli and cellular extensions "invadopodia" that reached into membrane pores. TGF-ß was found to be more active than EGF or IGF-1 in stimulating tumor cell chemotaxis. In conclusion, our results indicate that AII and TAM reduced tumor cell invasiveness and that ICI produced the greatest inhibition of collagenase release in vitro. The antiestrogens produced minor changes in growth factor-mediated tumor cell movement. (This project was supported in part by OCAST grant HR2-009 and NIH/NCI grant CA 62117.)

B1-414 MOTILITY OF HEPATOCYTES AND HEPATOCELLULAR CARCINOMA CELLS IN RESPONSE TO LIVER REGENERATIVE FACTORS IN VITRO. Donna Beer Stolz, Wendy M. Mars and George K. Michalopoulos. University of Pittsburgh School of Medicine, Department of Cellular and Molecular Pathology, Pittsburgh, PA 15261

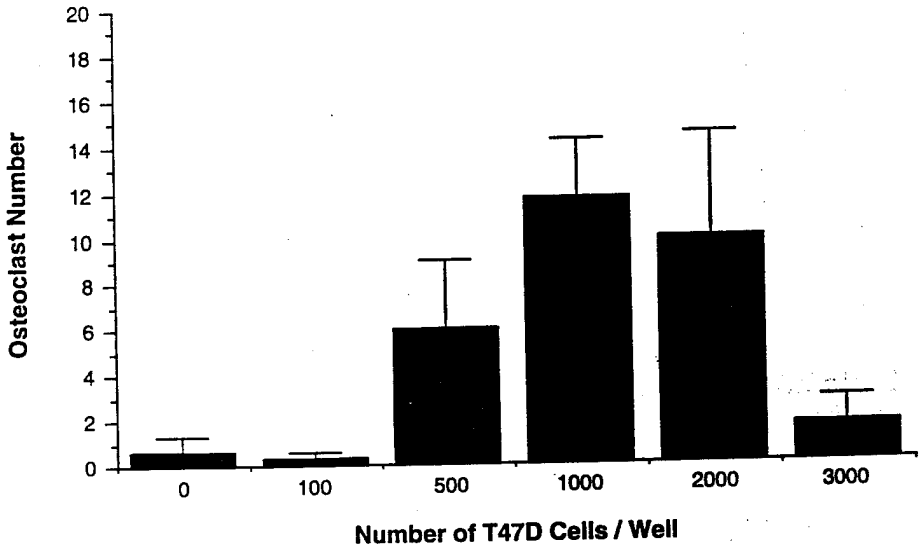
As a result of mechanical or chemical injury to the liver causing hepatocyte loss, the liver responds by regeneration to its original mass. We have investigated the effect of the growth factors hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1) and the serine protease urokinase-type plasminogen activator (uPA) on primary rat and human hepatocytes as well as two human hepatocellular carcinoma cell lines in order to examine their role in motility during the regenerative process. In human and rat primary hepatocyte cultures grown on collagen type I in serum-free medium, 50 ng/ml HGF or EGF stimulated the motility 5.5 and 4 fold respectively and DNA synthesis 2 fold at 24 hr. Although DNA synthesis was abrogated to near control levels upon concomitant addition of 1 ng/ml TGF-β1 to these cultures, the HGF-induced motility was not affected and the EGF-induced motility increased to 7 fold over controls. Therefore, simultaneous addition of TGF-β1 to either HGF or EGF-stimulated cultures shuts down the proliferative effects of the growth factors, but not the motogenic responses in primary hepatocytes. Additionally, when uPA was added to cultures stimulated with either HGF or EGF, HGF-induced motility was significantly enhanced, but EGF motility remained unaffected. HepG2 and Hep3B human hepatocellular carcinoma cell lines were also examined for their response to HGF, EGF and TGF-β1. Hep3B were hyper-motile under all assay conditions. HepG2 cells, however, displayed 3 fold increased motility with HGF and nearly 20 fold with EGF. Addition of TGF-β1 slightly enhanced both HGF and EGF-induced motility. Parallel DNA synthesis assays indicated that hepatocellular carcinomas did not respond to any cytokine. The data suggest that separate growth factors involved in liver regeneration act synergistically to modulate normal primary and transformed hepatocyte motility in vitro. These results indicate that two distinct pathways exist within hepatocytes to uniquely modulate motogenic and proliferative responses.

B1-415 HUMAN BREAST CANCER CELL LINES SUPPORT OSTEOCLAST DIFFERENTIATION IN VITRO, M. Mehrdad Tondravi, Marisol Quiroz, F. Patrick Ross and Steven L. Teitelbaum, Department of Pathology, Jewish Hospital, Washington University, St. Louis, MO 63110

Breast cancer often metastasizes to bone. These bone metastases cause osteolysis resulting in bone fractures and hypercalcemia. Although the precise mechanisms of breast cancer metastasis to bone is not understood, osteoclasts probably play a critical role. Several lines of evidence indicate that the cancer cells do not resorb bone per se, and have led us to the hypothesis that once a tumor has been established in the bone marrow environment, the cancer cells promote the differentiation of marrow-residing osteoclast precursors into mature, bone-resorbing, polykaryons. As a test of this hypothesis, we have established a cell culture system whereby breast cancer cells promote osteoclast differentiation in vitro. The osteoclast is a member of the monocyte/macrophage lineage. In mammals, differentiation of macrophage precursors into osteoclasts requires the presence of accessory cells which include stromal cells or osteoblasts. We have established that several human breast cancer cell lines (MCF-7, T47D, MDA-MB-231) can assume the role of the accessory cells in osteoclastogenesis. We are addressing whether these cancer cell lines induce osteoclast differentiation through a secratory factor by culturing the osteoclast precursors in the bottom of transwell dishes and the cancer cells on the separating filter. The results thus far, with one of the cell lines indicates that a soluble factor is not involved. We have begun to generate clonal sublines of some of our breast cancer cells and have identified clones of T47Ds which do not support osteoclastogenesis in vitro while other sublines do. Experiments are underway to test the ability of these sublines to establish tumors and cause osteolysis in vivo, in the bone marrow of nude mice.

APPENDIX

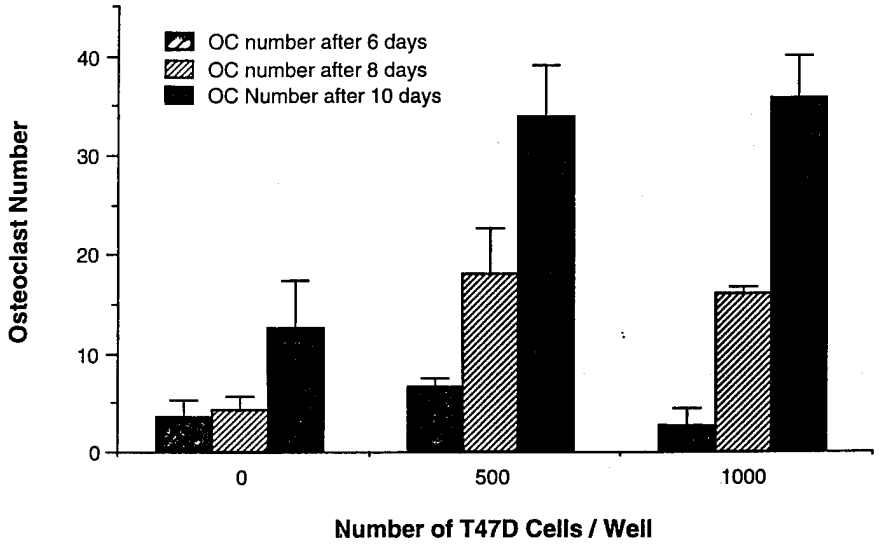
Figure 1A
The Process by which Human Breast Cancer Cells promote Osteoclast Differentiation *in vitro* is Concentration Dependent



Indicated number of T47D cells were co-cultured with 10^4 osteoclast progenitors for 10 days. The cells were fixed, stained for tartrate resistant acid phosphatase (TRAP, an osteoclast marker) and the number of TRAP positive cells counted. The number of osteoclasts observed initially increases with increasing number of T47D cells. With higher input number of T47D cells, the osteoclast number decreases which likely represents competition between the cancer cells and the osteoclasts for anchorage to the dish and/or nutrients.

Figure 1B

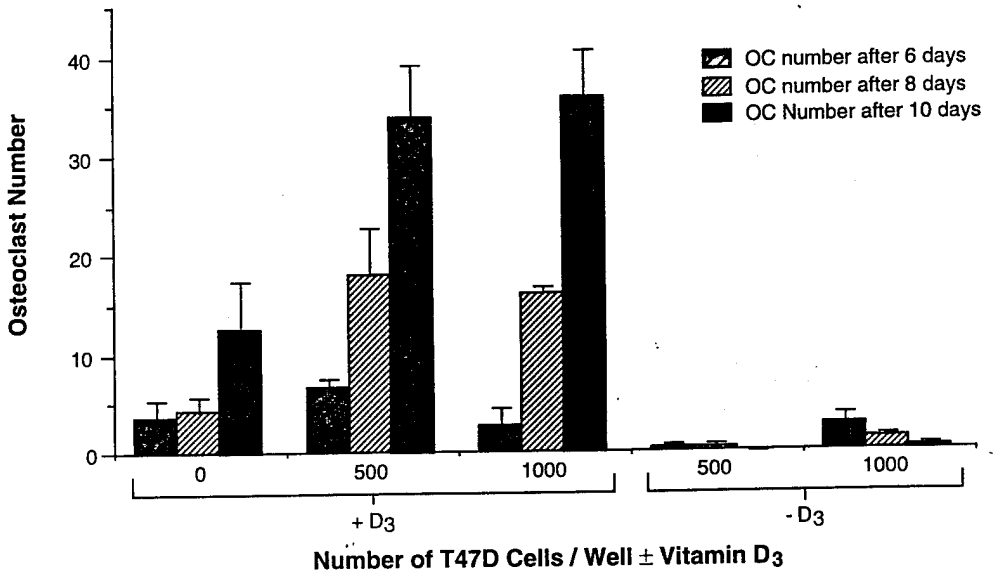
T47D Human Breast Cancer Cells promote Osteoclast Differentiation *in vitro* by 8 Days following Co-culture



Indicated number of T47D cells were co-cultured with 10^4 osteoclast progenitors. After 6, 8, or 10 days of co-culture the cells were fixed, stained for TRAP and the TRAP positive cells counted. Significant numbers of osteoclasts appear after day six.

Figure 2

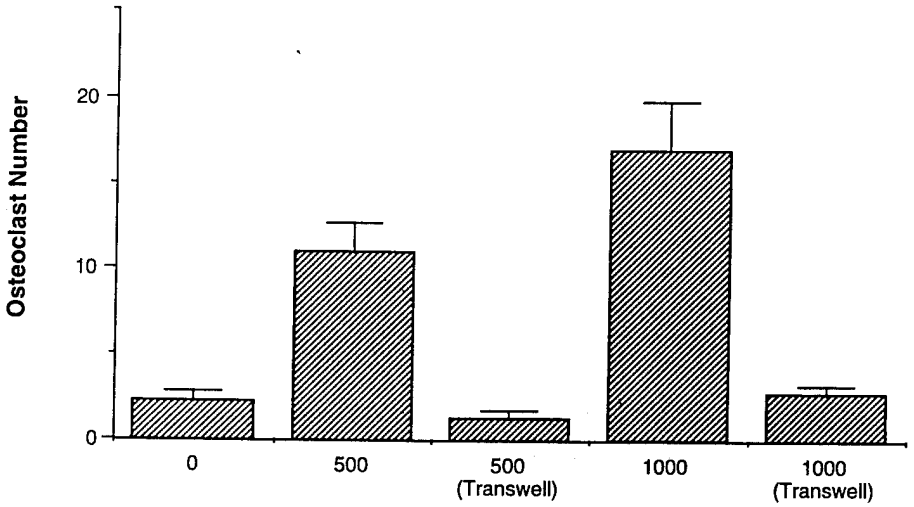
1,25-Dihydroxyvitamin D_3 is Essential for Human Breast Cancer Cell Induced Osteoclast Differentiation *in vitro*



Indicated number of T47D cells were co-cultured with 10^4 osteoclast progenitors. After 6, 8, or 10 days of co-culture the cells were fixed, stained for TRAP and TRAP positive cells were counted. Very few osteoclasts develop in the absence of the osteoclastogenic hormone vitamin D.

Figure 3

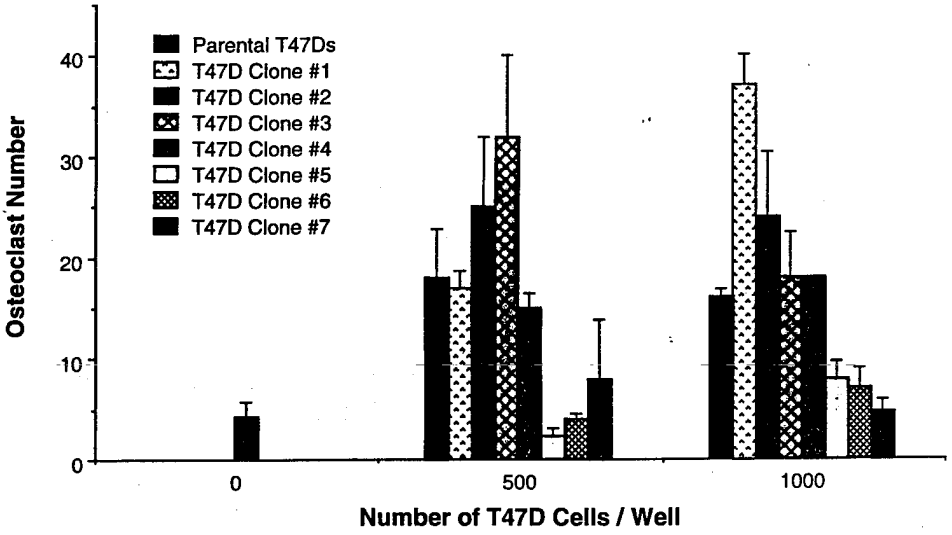
Osteoclast Induction by the T47D Human Breast Cancer Cell Line is Not Mediated by a Soluble Factor



To determine whether osteoclast induction by T47D cells is mediated through a soluble factoror cell contact, the indicated number of T47D cells were co-cultured with osteoclast progenitors as in previous figures or the two cells were seperated by a filter in transwell dishes. After 10 days the TRAP positive cells were counted. The number of osteoclasts generated from the transwell dishes is at background levels indicating that cell contact isrequired for osteoclast induction by T47D cells. Similar results were obtained when the osteoclast progenitors were fed with conditioned media from the T47D cells or when the co-cultures were set up in soft agar.

Figure 4

Identification of Independent T47D Subclones that either Do or Do Not promote Osteoclast Differentiation *in vitro*



Osteoclast induction of seven T47D clones was test by co-culturing zero, 500 or 1000 of each of the clones with 10^4 osteoclast precursors. After 8 days of co-culture the cells were fixed, stained for TRAP and counted. While T47D clones 1-4 are as osteoclastogenic as the parental T47D cells, clones 5-7 do not promote osteoclast differentiation above background.