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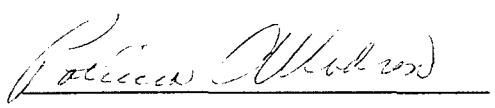

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To determine if a correlation exists between the expression profile of interleukin-8 (IL-8), and the metastatic phenotype of breast tumor cells, the IL-8 levels produced by four breast tumor cell lines were assayed by ELISA. The constitutive levels of IL-8 were quantified for two metastatic and two non-metastatic human breast tumor cell lines. The data from these assays show the metastatic cells released much higher levels of IL-8 into their media than did the non-metastatic tumor cells. The higher levels of IL-8 mRNA produced by the metastatic tumor cells, as determined by RT-PCR, correlate with the amount of IL-8 released by these cells. A correlation was also observed between cellular morphology and IL-8 profile. Metastatic cell lines are more motile and fusiform in appearance, where the non-metastatic lines have a non-migratory, more epithelial appearance. These correlations suggest a possible enhancing or promoting role for IL-8 in the metastatic potential of breast tumor cells.

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Joseph E DeLuca 8/13/99

PI - Signature Date

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INTRODUCTION

The experiments outlined in this proposal were designed to determine if a correlation exits between the metastatic potential of breast tumor cell lines and their levels of ectopic IL-8 expression. Since IL-8 is an angiogenic factor its ectopic expression by breast tumor cells *in vivo* may lead to an increase in the neovascularization of these tumors. The increase in neovascularization is likely to lead to an increase in tumor growth and metastasis. The immediate goal of the experiments out lined in this proposal was to ascertain if IL-8 is more highly expressed in the metastatic breast tumor cells than in non-metastatic breast tumor cells. If it was found that metastatic cells express more IL-8, it would create long term goals directed at understanding the molecular reasons fo these differences in the level of expression. The endpoint would be the development of means to inhibit the synthesis and release of ectopic IL-8 by tumor cells. The ability to attenuate or inhibit the expression of IL-8 by tumor cells may well diminish their angiogenic, growth and metastatic potentials. The research in this proposal was designed to examine the levels of expression of IL-8 by metastatic and non-metastatic breast tumor lines. If differences existed, we were to look for autocrine factors that would upregulate IL-8 expression in the metastatic cells. The last aspect of this research examined the differential in induction of IL-8 by inflammatory mediators likely to be released by tumor associated inflammatory cells, i.e., IL-1β and TNF-α.

The data obtained from these experiments indicate the constitutive expression of IL-8 by metastatic breast cell lines is much greater than the constitutive levels released by non-metastatic cells. The IL-1β and TNF-α are much more potent inducers of IL-8 in the metastatic cells than

in the non-metastatic cells. Upon examination of the conditioned media from the metastatic cells there was no indication of an autocrine factor contributing to the high levels of expression of IL-8 in the metastatic cells.

BODY

The proposal had four tasks. They were: (1) To ascertain the degree of correlation between the metastatic potential of human breast lines and the amount of α-chemokine they release. (2) To purify and/or identify the major α-chemokine from the breast cancer cell line releasing the highest level of α-chemokine activity. (3) To determine if the major α-chemokine being expressed by one of these breast tumor cell lines is being enhanced by the ectopic release of autocrine factors produced by the tumor cell line. (4) To test if any of the classes of anti-inflammatory agents can inhibit the expression of any of the elements in the autocrine-paracrine cascade and thereby mitigate the expression of the α-chemokine by the metastatic tumor cells.

(1) To ascertain the degree of correlation between the metastatic potential of human breast lines and the amount of α-chemokine they release. The data obtained from these experiments indicate the constitutive expression of IL-8, an α-chemokine, by metastatic breast cell lines is much greater than the constitutive levels released by non-metastatic cells, both constitutively and under induction conditions. Details of our findings and methodology are contained in the manuscript comprising Appendix A.

(2) To purify and/or identify the major α-chemokine from the breast cancer cell line releasing the highest level of α-chemokine activity. We found the metastatic cells are releasing the α-chemokine, IL-8, constitutively , as well as, under induction conditions. The

metastatic cells were not found to release other factors, such as, GRO α, GRO β, or GRO γ. Also constitutively and under induction conditions, mRNA for IL-8 was seen, but not so for GRO α, GRO β, or GRO γ.

(3) To determine if the major α-chemokine being expressed by one of these breast tumor cell lines is being enhanced by the ectopic release of autocrine factors produced by the tumor cell line. We were unable to demonstrate the presence of such an autocrine factor in the conditional media of the four cell lines examined. We could not demonstrate the presence of such a factor in either the unfractionated conditioned media or concentrated fractions of the conditioned media. The fractionation was carried out using several different HPLC columns. The lack of detection of an autocrine factor that enhances IL-8 expression does not mean the activity isn't present, it only means the methods we employed failed to detect the activity.

(4) To test if any of the classes of anti-inflammatory agents can inhibit the expression of any of the elements in the autocrine-paracrine cascade and thereby mitigate the expression of the α-chemokine by the metastatic tumor cells. In essence, since IL-8 was the only chemokine released by these cells, its expression was the only one followed. All compounds were first screened in a dose dependent 72 hour toxicity assay. Compounds were tested at non-toxic doses to see if they could inhibit IL-8 expression. Since the IL-8 promoter has an "essential" NFκB consensus sequence we tested a large series of anti-oxidants at non-toxic doses. The only compounds that worked in this series were resveratrol and curcumin. The resveratrol gave a 20-35% maximal inhibition while the curcumin gave a maximal response between 40 and 60% maximal inhibition. While the resveratrol inhibited IL-8 expression in both

the MD-231 and MD-435s cells, the inhibitory effects of curcumin were only observed on the MD-231 cells. The only other compound tested that inhibited IL-8 expression was dexamethasone, a steroidal anti-inflammatory. Dexamethasone inhibits IL-8 expression approximately 50%.

DISCUSSION

Problems: The Twin Cities area has an extremely low unemployment rate, i.e., 1.6%, and several biotech firms including R&D Systems, that pay much higher rates than the University does at this point. The above combination makes it difficult to recruit and maintain junior scientists. For the two periods in which this grant was in effect we lost one junior scientist to graduate school and two to R&D Systems.

The original hypothesis states "tumor cells are releasing diffusible molecules that attract inflammatory cells to the site of the tumor. Once at the site of the tumor, the inflammatory cells can release products that enhance the tumor's chances of progressing and metastasizing". The data generated in the course of carrying out the experiments outlined in this proposal are totally consistent with IL-8 being an enabler of the metastatic process in tumors that are ectopically releasing IL-8. Since the submission of this proposal it has been shown that IL-8 can be both an autocrine factor for tumor cells and a chemoattractant for endothelial cells; these are consistent with ectopic IL-8 expression contributing to the growth and progression of a tumor.

This work has lead us to believe IL-8 is strongly correlated with the metastatic potentials of tumor cells. Since we have also noticed a strong correlation between a cell's metastatic phenotype and morphology displayed by the tumor cell, it seems judicious to only note this as a

correlation. A correlation although consistent with cause and effect does not prove there is a causal relationship. It seems obvious that one would want to know if there is a direct cause and effect relationship between IL-8 expression and a tumor's growth and metastatic potential. This might be best done using molecular biological techniques to either add an IL-8 expression system to a tumor cell line that is neither secreting IL-8 nor is metastatic, or attenuating IL-8 expression, via a stable construct, in metastatic cells which are high producers of IL-8. The metastatic potential of these cells could then be ascertained to determine if the metastatic potential changes with the alteration of IL-8 expression. To determine if the host's inflammatory cells, in this case the most likely the neutrophils since IL-8 is a potent neutrophil chemoattractant, are contributing to the metastatic process one could determine if a neutropenic nude athymic mouse is less likely to develop metastatic tumor from an implanted IL-8 secreting metastatic tumor.

KEY RESEARCH ACCOMPLISHMENTS

- ❑ Found metastatic breast carcinoma lines constitutively release much higher levels of IL-8 than do non-metastatic breast carcinoma lines.
- ❑ Found the metastatic breast carcinomas are induced to produce and release much more IL-8 than the non-metastatic breast carcinoma cell lines when treated with the inflammatory mediators IL-1β and TNF-α .
- ❑ Found that a metastatic clone (MCF-7-ADR) arising from the non-metastatic breast carcinoma line MCF-7 has an IL-8 expression pattern similar to the patterns seen in the other metastatic breast carcinoma lines. This suggests the correlation of the high levels of IL-8 expression with the metastatic potential of the cells in the metastatic lines is likely a result of their expressed phenotype rather than being due to a mere coincidence of cell selection.
- ❑ Noted the constitutive expression of IL-8 correlates with the expressed morphology of the breast carcinoma line. The epithelioid non-metastatic, non-migratory cells release little if any IL-8. The fusiform metastatic, poorly associated, migratory cells release much higher levels of IL-8.

REPORTABLE OUTCOMES

- ❑ The enclosed submitted manuscript is the direct outcome of this grant.
- ❑ Single cell clones of MCF-7, MD-231, and MD-435 were obtained as well as drug resistant populations and clones of each of the above.
- ❑ Created a data base on breast tumors and metastasis to be used in the preparation of future papers and grants.
- ❑ Designed experiments to determine if IL-8 is directly involved as an enabler in the metastatic process.
- ❑ Applied for an NIH grant to support the animal work necessary to ascertain if IL-8 is acting as a direct enabler in the metastatic process of breast carcinomas.
- ❑ During the process have trained two undergraduate bioengineering summer interns that were awarded positions in the University of Minnesota's Bioengineering Program.
- ❑ Added to the training of three junior scientists.

CONCLUSIONS

The results of the experiments outlined in this proposal indicate that there is a strong correlation between the ectopic IL-8 expression and the metastatic potential of breast carcinoma lines phenotype. We also demonstrated that IL-1β and TNF-α are much more potent inducers of IL-8 expression in metastatic breast carcinoma lines than in non-metastatic breast carcinoma lines. The differences in the constitutive expression are much larger than previously reported in either cell lines or clones. The induction had not been reported to the best of my knowledge. These data are the basis of further studies to determine the cause of these differences in the constitutive expression of IL-8 and if the aberrantly high expression of IL-8 by the metastatic cells might be a novel target for the intervention of tumor growth, progression and metastasis.

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APPENDIX A

TITLE

A Potential Role for Interleukin-8 in the Metastatic Phenotype of Breast Carcinoma Cells[1]

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[3] The abbreviations used are: IL, interleukin; TNF-α, tumor necrosis factor-α; RT-PCR, reverse transcription-PCR; SCID, severe combined immunodeficiency; NFκB, nuclear factor-κB; FBS, fetal bovine serum.

ABSTRACT

To determine if a correlation exists between the expression profile of the α-chemokine, interleukin-8 (IL-8), and the metastatic phenotype of breast tumor cells, the IL-8 levels produced by four breast tumor cell lines were assayed by ELISA. Initially, the constitutive, IL-1β induced, and TNF-α induced levels of IL-8 were quantified for two metastatic and two non-metastatic human breast tumor cell lines. The data from these assays show the metastatic cells, under either basal or induced conditions, released much higher levels of IL-8 into their media than did the non-metastatic tumor cells. The higher levels of IL-8 mRNA produced by the metastatic tumor cells, as determined by RT-PCR, correlate with the amount of IL-8 released by these cells. To eliminate the possibility the observed differences are a result of selection of the tumor cell lines, we examined the IL-8 profiles of two clonal isolates of the MCF-7 tumor cell line, the metastatic or invasive line being derived from an original isolate of the non-metastatic line. The non-metastatic or parental cell line showed an IL-8 profile similar to the non-metastatic lines examined in earlier studies, producing little if any IL-8 constitutively and releasing low levels of IL-8 when induced with inflammatory mediators, IL-1β or tumor necrosis factor-α (TNF-α). The invasive derivative yielded an IL-8 profile consistent with those of the metastatic cell lines tested in earlier studies. These data are consistent with the earlier correlation between IL-8 expression and metastatic phenotype. A correlation was also observed between cell morphology and IL-8 profile. Metastatic cell lines are more motile and fusiform in appearance. By contrast, the non-metastatic lines have a non-migratory and more epithelial appearance. The higher levels of IL-8 produced by metastatic tumor cells and their massive response to the inflammatory mediators, suggest a possible enhancing or promoting role for IL-8 in the metastatic potential of breast tumor cells.

INTRODUCTION

Metastasis is the process by which tumor cells spread from a tumor site to remote sites after gaining access to the circulatory system culminating in the establishment of one or more tumors at distant sites (1). The better the mechanisms contributing to the metastatic potential of a tumor cell are understood, the more likely we will be able to develop means to intervene in the progression of this process. It has been established that a major, if not essential, requirement for metastasis, is angiogenesis, the recruitment of new blood vessels to supply the tumor (2). The newly acquired vessels supply tumor cells with oxygen and nutrients required for sustained growth. A number of known mediators of angiogenesis contributing to tumor neovascularization, can be derived either directly from the tumor cells or may be products of tumor associated inflammatory cells (3).

For the past two decades, members of the α-chemokine family have been studied as ectopic tumor cell products thought to contribute to growth and progression of tumor cells (4). IL-8[3], a member of this family of small basic peptides, was first purified on the basis of its neutrophil chemoattractant activity (5). The ectopic expression of IL-8 by melanoma cells has been reported to be a contributing factor to both their growth rate and metastatic potential *in vivo*. Preliminary screening of eight human melanoma cell lines show that six of the eight cell lines produce significant levels of IL-8 (6). In an experimental metastasis model, it was established that the "metastatic" potential of several clones derived from human melanoma cell lines, correlates with the level of IL-8 produced (7). It has been demonstrated for an IL-8 secreting non-small cell lung carcinoma, the *in vivo* growth rate and number of spontaneous lung metastases formed *in vivo* is dependent upon the level of IL-8 secreted. Passive immunization of SCID mice bearing these tumors with a neutralizing anti-IL-8 monoclonal antibody depressed the rate of tumor growth by greater than 40% with an accompanying decline in lung metastases.

However, this anti-IL8 antibody did not inhibit the *in vitro* growth of these cells, demonstrating that IL-8 can enhance tumor growth and metastasis *in vivo*, independently of an autocrine role (8). A further assessment of the role of IL-8 expression in tumor growth and metastasis was accomplished by transfecting an IL-8 expression system into a poorly tumorgenic, non-metastatic, human melanoma line that was not expressing α-chemokines. Transfected clones, producing IL-8, formed tumors faster in nude mice than the parental line or mock transfected clones. The *in vivo* growth rate of tumors produced by these clones correlates with their level of IL-8 expression. The IL-8 producing, transfected clones are metastatic whereas the parental line is not (9). These data indicate the ectopic expression of IL-8 can contribute to both an increased *in vivo* growth rate and increased metastatic potential.

Since the original purification and characterization of IL-8 as a neutrophil chemoattractant, it has also been found to be an endothelial cell chemoattractant *in vitro* and an angiogenic factor *in vivo* (10,11). The current paradigm explaining the correlation between increased growth and metastatic potential of a tumor cell line with its ectopic release of IL-8, involves the angiogenic properties of IL-8 which enhance tumor neovascularization. It is thought that these newly acquired blood vessels will contribute to the growth of these tumors both by increasing the rate at which oxygen and nutrients are supplied to the tumor cells, as well as, facilitating the removal of byproducts of cellular metabolism from the tumor environment. The proximity of new tumor vessels can facilitate the metastatic potential of cells within the tumor by presenting a route with immature barriers of new endothelial cell basement membranes through which the tumor cells can enter the circulation. Once in the circulation, these tumor cells are carried to distant sites where they can extravasate through post-capillary venules and establish metastatic centers in distal tissues.

In view of the potential role of IL-8 during metastasis, the expression of IL-8 by six

human breast carcinoma cell lines was examined to ascertain if the levels of IL-8 produced correlate with their metastatic potentials. Our findings indicate a strong correlation between the reported metastatic phenotype of the cell lines and the basal levels of IL-8 released *in vitro*. A correlation between cellular morphology and invasive or metastatic potential was also observed.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The MDA MB-231 and MCF-7 cell lines (American Type Culture Collection, Rockville, MD), and L-MCF-7 and L-MCF-7-ADR cell lines (12) were cultured with Dulbecco's Modification of Eagle's Medium (Mediatech #10-017-CV) + 10% FBS in sterile tissue culture flasks and incubated at 37°C/7%CO_2. The MDA MB-435S and T47D cell lines (ATCC, Rockville, MD) were cultured with RPMI 1640 (Mediatech #10-040-CV) + 10% FBS in sterile tissue culture flasks (Falcon #353111) and incubated as stated above. Cells were passed at room temperature in a laminar flow hood during their logarithmic phase of growth.

ELISA for Human IL-8

The six cell lines were seeded in a total of twelve 6-well plates containing 5ml of their respective media containing 10% FBS. At 80% confluency, the media was aspirated and 2ml of fresh media containing 10% FBS was introduced to each well. One of the plates for each cell line was treated with the following concentrations of IL-1β (a gift from Jim Cone of Otsuka Pharmaceutical Company): 0.1ng/ml, 0.2ng/ml, 0.4ng/ml, and 0.8ng/ml. The second plate for each cell line was treated with the following concentrations of TNF-α (Peprotech Incorporated, Rocky Hill, NJ): 0.5ng/ml, 1ng/ml, 2ng/ml, and 4ng/ml. At 24 hours, 1ml of media was collected from each well, clarified of cells and cellular organelles, stored at -20°C, and the number of cells per well was determined. The IL-8 ELISA was performed according to the manufacturer's instructions (PharMigen's OptEIA Human IL-8).

cDNA Synthesis

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Gibco BRL) from IL-1β treated, TNF-α treated, and non-treated subconfluent cultures of all four cell lines. To determine IL-8 mRNA production, MDA MB-231, MDA MB-435S, MCF-7, and T47D cell lines were either treated with 1ng/ml IL-1β, 4ng/ml TNF-α, or not treated. At 3h, the cellular RNA was harvested with Trizol reagent according to the manufacturer's protocol. For cDNA, 1 μg of RNA, determined by UV spectroscopy, was reverse transcribed with MLV reverse transcriptase according to the Superscript II reverse transcriptase manufacturer's protocol (Gibco BRL).

Oligonucleotides

Primers were designed using a human IL-8 published sequence (13). The 283bp human IL-8 product was amplified using the following sequences: 5 - ATG ACT TCC AAG CTG GCC GT- 3 and 5 - CCT CTT CAA AAA CTT CTC CAC ACC -3 . β-actin primers producing a 643bp product were also designed using the following sequences: 5 - CAT GGA TGA TGA TAT CGC CG -3 and 5 - TCT CCT TAA TGT CAC GCA CGA -3 . Both sets of primers were constructed at the University of Minnesota Microchemical Facility.

Determination of IL-8 mRNA Production in Metastatic and Non-Metastatic Cells

A PCR master mix containing PCR buffer, 25mM $MgCl_2$ (FisherBiotech), 10mM dNTPs, 10μM of each IL-8 primer, 1.25 units Taq polymerase (FisherBiotech), and sterile water to 49μl was added to 1μl of cDNA. Amplification was performed using a PTC-100 programmable thermal controller (MJ Research, Inc.) with the following cycle: 95°C for 5 min (1 cycle), followed by 35 cycles of 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 30 sec, and concluding with a 10 min extension at 72°C. The PCR products were analyzed on a 1%

agarose gel stained with ethidium bromide.

RESULTS

Four tumorgenic breast carcinoma lines were examined for IL-8 expression to determine if a correlation exists between the ectopic expression of IL-8 by a tumor cell and its potential to metastasize. The metastatic cell lines, MDA MB-231 and MBA MB-435S, are both estrogen receptor negative (14). The cells used to establish the MDA MB-231 line were isolated from a pleural effusion taken from a patient having a breast adenocarcinoma and the MDA MB-435S line was derived from a pleural effusion obtained from a patient with metastatic breast ductal carcinoma. The non-metastatic lines, MCF-7 and T47D, are estrogen receptor positive cells (14). The MCF-7 tumorgenic breast carcinoma line was initiated using cells from a pleural effusion that originated as a breast adenocarcinoma. A pleural effusion from a breast ductal carcinoma gave rise to the T47D breast carcinoma line.

Conditioned media from these four tumor lines was examined for the presence of IL-8. The conditioned media from the metastatic lines showed that both cell lines constitutively produce substantial quantities of IL-8, between 25 and 700 picograms per ml per 1.25 X 10^5 cell per 24 hours. The basal level of IL-8 released by the non-metastatic tumor cell lines was very low or undetectable by ELISA, i.e., less than 5 picograms of human IL-8 per ml per 1.25 X 10^5 cells per 24 hours. Tumors often contain infiltrating monocytes and macrophages, which can be a source of the inflammatory mediators including IL-1β and TNF-α (3). Since these mediators have the potential to act as inducers of IL-8, their effects on IL-8 expression were tested on each of the four tumor cell lines. The responsiveness of the metastatic cell lines, MDA MB-231and MDA MB-435S, was robust (Fig. 2A). However, the response of the non-metastatic tumor cell lines, MCF-7 and T47D, to these mediators was minimal (Fig. 2B). The ordinates in Fig. 2 differ measurably. It was found that the constitutive levels of IL-8 expression in the metastatic cells

were higher than the highest induced levels in the non-metastatic cells. Conditioned media from the non-metastatic cells of the T47D line contained little if any IL-8 even under inducing conditions. The TNF-α induced MDA MB-435S cells produced the highest level of IL-8 under the conditions tested. Although both metastatic lines, MDA MB-231 and MDA MB-435S, appeared to have similarly shaped dose response curves to IL-1β, cells from the MDA MB-435S line appeared to be much more responsive to TNF-α than cells of the MDA MB-231 line.

The morphologies exhibited by the metastatic cell lines are bipolar or fusiform in nature with seemingly weak cell-cell interactions. In addition, both of the metastatic cell lines have motile, mesenchymal appearing morphologies and neither form distinct colonies in monolayer cultures (Fig. 1A and 1B). In contrast, the morphologies expressed by the non-metastatic lines are more epithelioid, with cells forming distinct colonies or islands of nonmigratory cells (Fig. 1C and 1D). These non-metastatic cells also express E-cadherin at their cell-cell junctions (14).

These data suggest a correlation exists between either the morphology exhibited by the cell types and/or the metastatic potential and the level of IL-8 expression by the respective cells. To rule out a coincidental or serendipitous IL-8 expression due to tumor variation, i.e., neither related to the morphology nor metastatic potential, we next examined a pair of breast cancer clones, L-MCF-7 and L-MCF-7-ADR (12). The latter clone being derived from the former. The L-MCF-7 cells are estrogen receptor positive and non-invasive in the nude mouse model while the L-MCF-7-ADR cells are estrogen receptor negative and invasive. Examination of the conditioned media from this pair of cell isolates showed that the parental clone expressed low levels of IL-8 constitutively and its IL-8 induction was relatively non-responsive to either IL-1β or TNF-α. In contrast, the induction of IL-8 in the L-MCF-7-ADR cells was very responsive to both IL-1β and TNF-α (Fig. 2C). We also observed the parental line, L-MCF-7, were epitheliod

in appearance, whereas cells from the adriamycin resistant line, L-MCF-7-ADR, were less epitheliod and more mesenchymal in appearance.

The apparent levels of IL-8 mRNA expressed in these cells was estimated using RT-PCR under constitutive and induced conditions (Fig. 3). The two metastatic cell lines produced low levels of IL-8 mRNA constitutively. The levels were enhanced by the addition of either IL-1β or TNF-α. In the case of MDA MB-231 cells, the IL-1β appeared to have a greater inductive effect on the IL-8 mRNA. This is consistent with ELISA data in which treatment of cells with IL-1β induced higher levels of IL-8 protein released into the conditioned media than did treatment with TNF-α. In the case of the MDA MB-435S cells, TNF-α appeared to induce higher levels of IL-8 mRNA, as well as, higher levels of IL-8 proteins to be released into the conditioned media. The non-metastatic cell lines did not produce measurable levels of IL-8 mRNA constitutively. When the non-metastatic cells were induced with IL-1β, both the MCF-7 and the T47D cells expressed low levels of IL-8 mRNA. The ELISA data from these cells indicated they are both stimulated to produce low levels of IL-8 in response to IL-1β. Treatment of the non-metastatic tumor lines by TNF-α showed only the MCF-7 cells were induced to express IL-8 mRNA, the T47D cells did n¹t express measurable quantities of IL-8 mRNA under these conditions. The data on mRNA levels were consistent with our ELISA data. Treatment of MCF-7 cells with TNF-α led to elevated expression of IL-8 mRNA and IL-8 protein in their media. In contrast, T47D cells treated with TNF-α under these conditions, did not express measurable quantities of IL-8 mRNA nor did they release IL-8 protein into their media.

DISCUSSION

Many reports have speculated and provided data supporting a major role for IL-8 in tumor metastasis. It has been demonstrated that the ectopic expression of IL-8 by tumor cells

contributes to increased growth potential, vascularization, and metastatic potential in a number of tumor models (7,8,9,15). Research heretofore has not shown how prevalent the ectopic expression of IL-8 is in breast cancer cells derived from primary tumors. We examined four breast cancer cell lines, two metastatic cell lines and two non-metastatic cell lines, to determine if a correlation exists between the level of IL-8 expression and metastatic potential. The data indicate a strong correlation between the level of IL-8 expressed by these breast tumor cells and their metastatic potential as observed in experimental metastasis assays. The level of IL-8 expression is higher in both metastatic cell lines constitutively and considerably higher when the cells are treated with either IL-1β or TNF-α when compared to non-metastatic cell lines.

To rule out the possibility that the differences in IL-8 expression between the metastatic and non-metastatic cells is a coincidence of sample selection, the IL-8 expression profiles of two clonal isolates of the MCF-7 cell line were examined. The pair of MCF-7 lines consisted of an adriamycin sensitive parental line, L-MCF-7, and an adriamycin resistant line, L-MCF-7-ADR, derived from the L-MCF-7. The L-MCF-7 is morphologically similar to the MCF-7 line used previously in this report; it is estrogen dependent, appears to have an epithelioid morphology, a non-migratory phenotype, and is non-invasive both *in vitro* and *in vivo* (12,16). The L-MCF-7-ADR line is estrogen independent, it tends to exhibit more of a mesenchymal morphology, appears to have a migratory phenotype, and is invasive both *in vitro* and *in vivo* (16). The data obtained from the clonal isolates of MCF-7 cells indicate the L-MCF-7-ADR cells express more IL-8 constitutively and show a significantly elevated response to the inflammatory mediators IL-1β and TNF-α, in comparison to levels seen in the parental, L-MCF-7 cells, under similar conditions. Hence, both the metastatic lines and the L-MCF-7-ADR line produce higher levels of IL-8 overall than do the non-metastatic cell lines, further strengthening the earlier correlation between metastatic potential and ectopic IL-8 production.

Further examination of the data reveals another correlation between the level of IL-8 released by tumor cells and cellular morphology. The morphology of these cell lines parallels their reported metastatic potential. The metastatic cells, which produce IL-8 constitutively at elevated levels, exhibit a more mesenchymal cellular morphology with less cell-cell interaction. The non-metastatic cells exhibit an epithelial morphology, forming tight adhesive colonies of seemingly non-migratory cells. Others have reported that *in vitro* expression of IL-8 by cultures of untransformed mesenchymal cells is highly inducible by either IL-1β or TNF-α (17). This raises the possibility that morphological appearance or "state of phenotypic differentiation" may be a contributing factor in the expression of IL-8. Alternatively IL-8 may be partially responsible for the altered morphology, however, the latter possibility is less likely since short term exposure of the non-metastatic cell lines to IL-8 did not induce a morphological change (data not shown). None the less, long term exposure to IL-8 might induce a morphological change.

The cells exhibiting a mesenchymal appearance may be expressing factors, not present in epithelial cells that enhance IL-8 expression. For example, these factors may be acting synergistically with transcription factors activated by either IL-1β or TNF-α, inducing agents of IL-8. These factors, which are active in metastatic cells, may be contributing to constitutive, as well as, IL-1β and TNF-α inducible levels of IL-8 expression. NFκB, a transcription factor that is activated by either IL-1β or TNF-α, is an example of such an element. Activated NFκB recognizes a consensus sequence in the promoter region of the IL-8 gene and is essential but not sufficient for the induction of IL-8 expression. Since both the metastatic and non-metastatic breast cancer lines express activated NFκB, it is possible that the metastatic breast cell lines have factors working either coordinately or synergistically with activated NFκB to enhance IL-8

expression (18). If the activation of cytoplasmic NFκB could be decreased or prevented in breast tumor cells, perhaps their expression of IL-8 would be inhibited or attenuated, diminishing the metastatic potential of these cells.

It is also possible epithelial or non-metastatic cells may either lack a component in the signal transduction pathway or express a factor(s) that attenuates the expression of IL-8. Although the several consensus sequences for the transcriptional activation of the IL-8 gene have been identified within the promoter region of IL-8, there is little known about the mechanisms regulating the repression of IL-8 expression. The binding of the POU-homeodomain transcription factor (Oct-1) to the IL-8 promoter represses IL-8 expression by binding to an element that overlaps one of the transcriptional activators of IL-8 expression (19). The differential regulation of IL-8 between the metastatic and non-metastatic breast cells may be due to any combination of the above possibilities, i.e., positive elements inducing IL-8 expression in the metastatic cells and/or the presence of a repressor(s) in the epithelial, non-metastatic breast tumor cells.

The current theory explaining the observed increased rate of metastases in tumor cells ectopically releasing IL-8, attributes the increase to the angiogenic properties of IL-8 (12,14). The angiogenic properties of IL-8 can enhance the metastatic potential of tumor cells by enhancing the vascular supply. The newly acquired vessels will enhance the growth rate of the tumor and present a proximal means for tumor cell dissemination. The neutrophil chemoattraction properties of IL-8 may also contribute to angiogenic and metastatic potentials. The neutrophils, during their journey from the vessels from which they extravasate, to the tumor to which they are migrating, are remodeling the extracellular matrix (ECM) by releasing proteases and a heparanase (20). In this process of remodeling, the neutrophils are likely to release many factors sequestered in the ECM, such as basic fibroblast growth factor (bFGF). These released factors can act as growth factors and chemoattractants for both endothelial cells

and tumor cells. The liberation of these sequestered factors may increase the metastatic potential of a tumor any number of ways, for example, by increasing angiogenesis, stimulating tumor progression, and enhancing tumor cell migration (21,22).

Understanding how the angiogenic properties of IL-8 work in concert with the other possible factors controlling IL-8 expression to increase the metastatic potential of a tumor cell will give insights into the process of tumor progression. Knowledge of these mechanisms will increase our understanding of not only tumor progression and metastasis, but also the developmental process involving epithelial to mesenchymal transition. Ideally, this information and knowledge will provide us with a means to design new approaches and compounds to attenuate neutrophil mediated inflammation and the ectopic expression of IL-8 by tumor cells expressing pre-metastatic or metastatic phenotypes. If this could be achieved, it may allow us to intervene in the progression of tumors or potentially, if caught at an early enough stage, prevent tumor metastasis.

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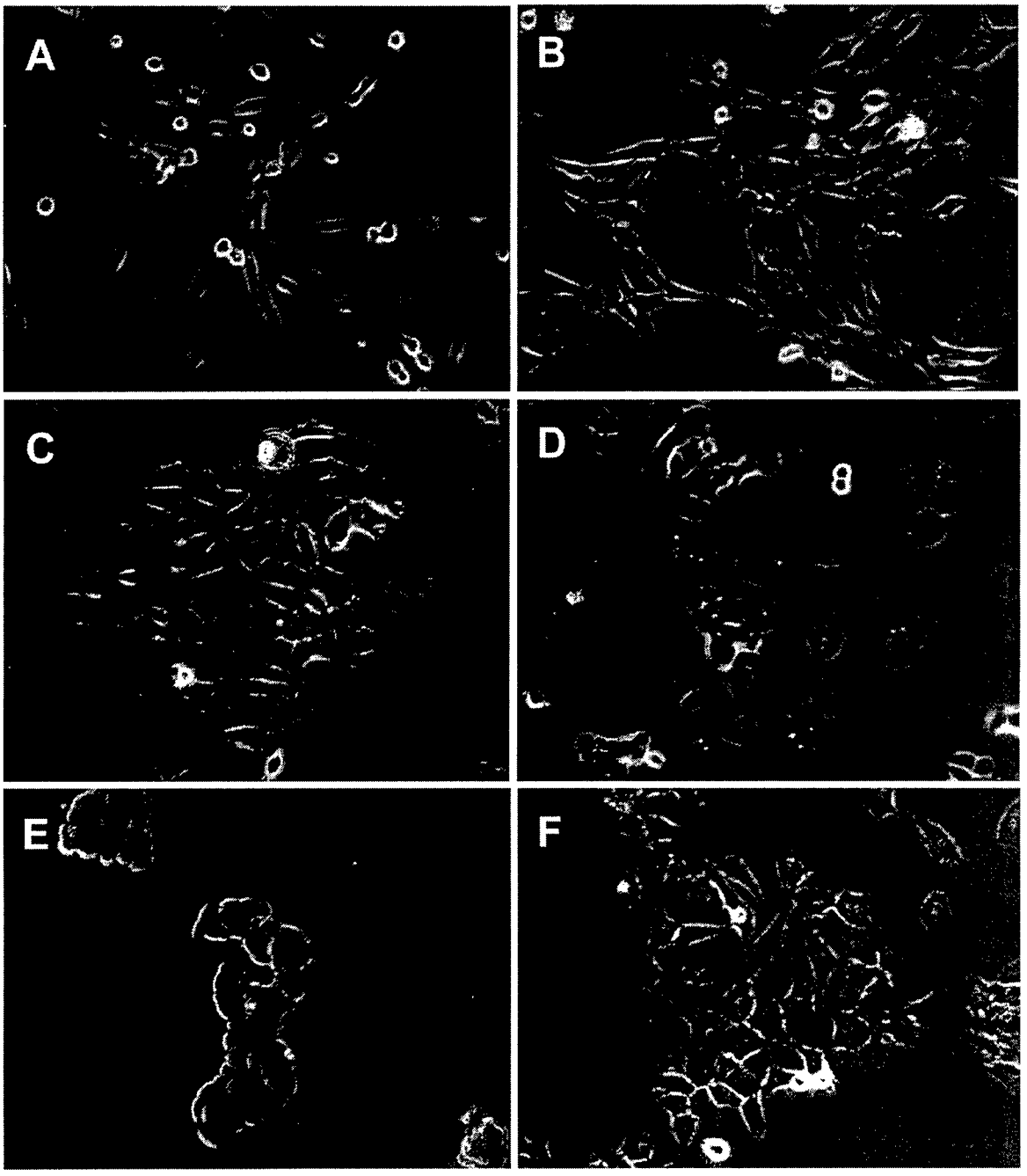


Figure 1: Morphologies of metastatic and non-metastatic cells.

Cells were plated, maintained for 72 hours in DMEM + 10% FBS or RPMI 1640 +10% FBS, and then visualized by phase contrast microscopy (200x).

A. MDA MB-231
B. MDA MB-435S
C. MCF-7
D. T47D
E. L-MCF-7
F. L-MCF-7-ADR

Fig. 2: IL-8 expression by metastatic and non-metastatic breast cancer cells.

Cells were seeded in six-well plates in DMEM containing 10% FBS or RPMI 1640 containing 10% FBS. At 80% confluency, various concentrations of IL-1β or TNF-α were added to each well. At 24 hr, 1ml of media was collected from each well, clarified of cells and organelles, and an IL-8 ELISA was performed. Approximately 125,000 cells were counted per well.

Fig. 2A: IL-8 Production by Metastatic Cell Lines

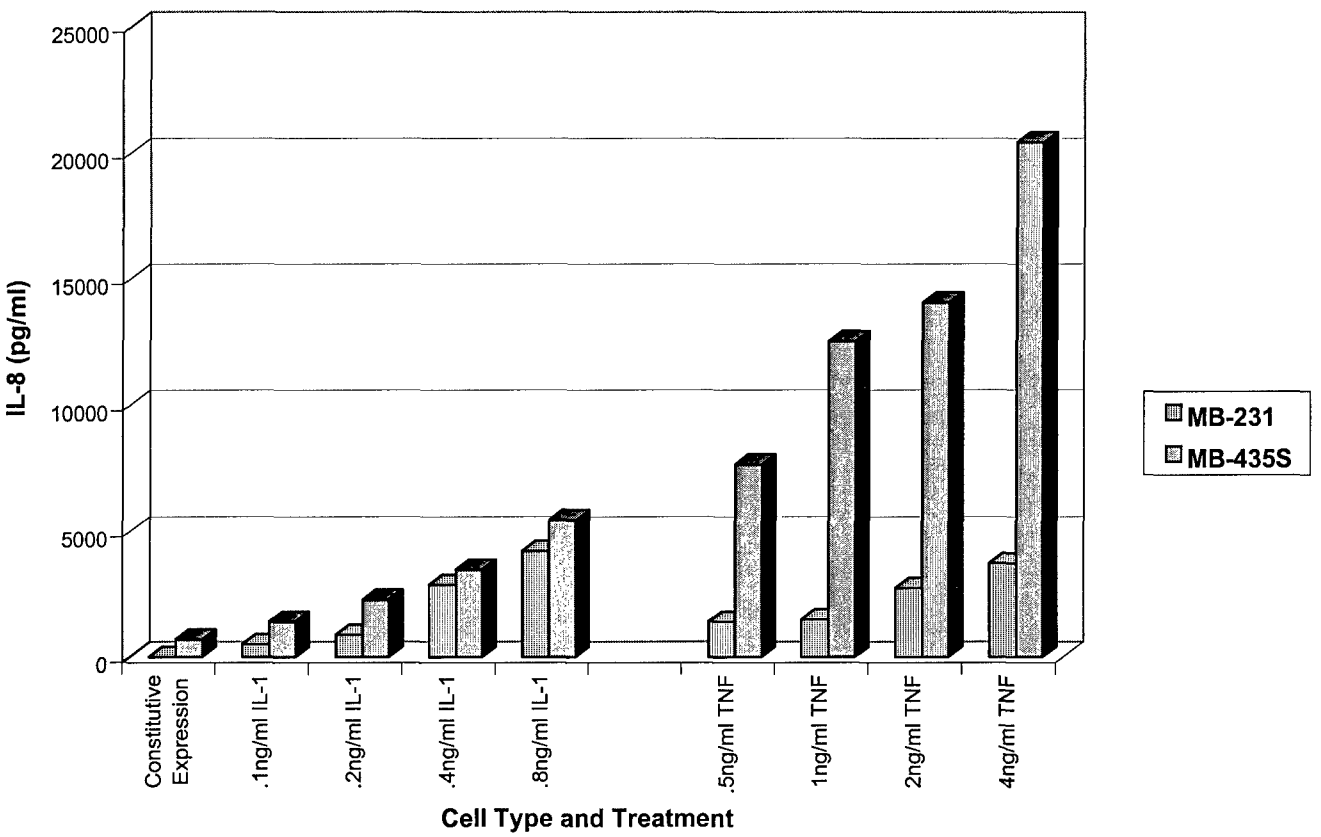


Fig. 2B: IL-8 Production by Non-Metastatic Cell Lines

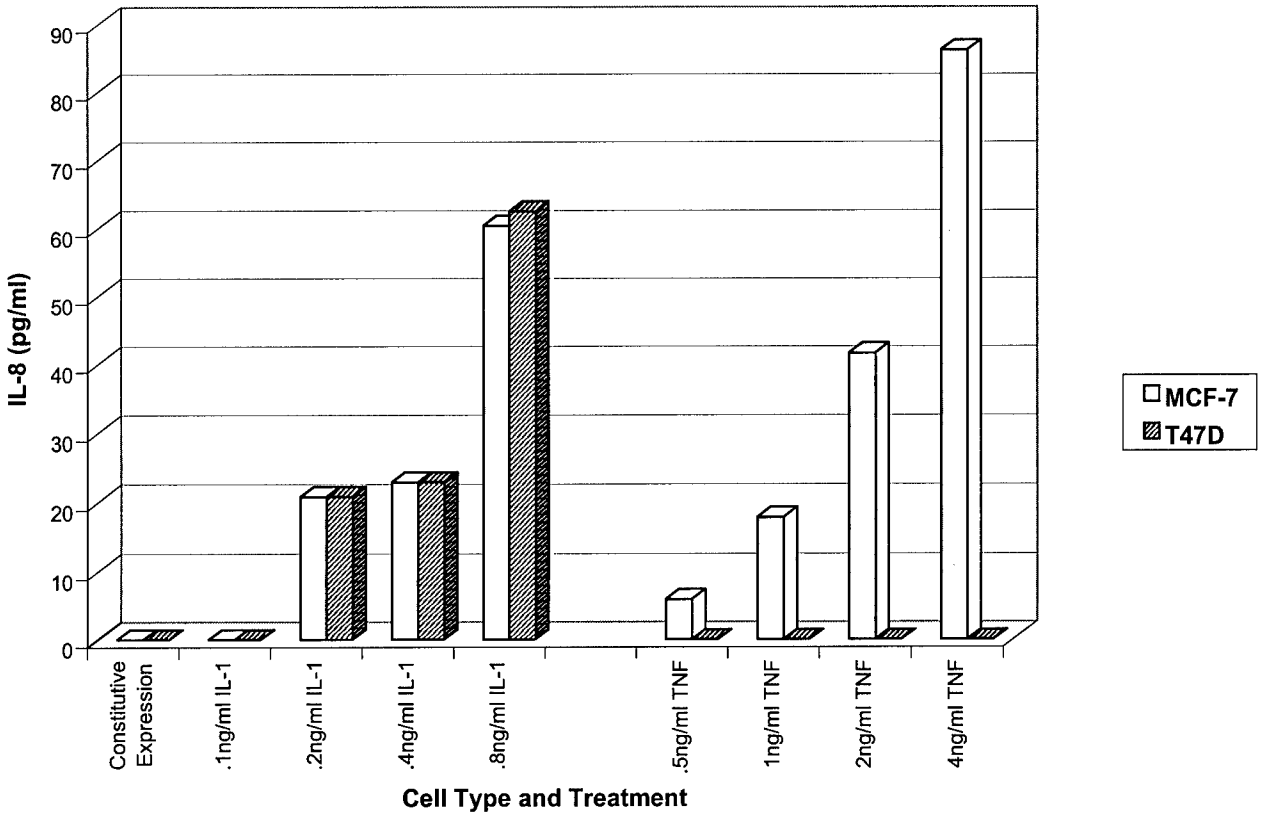
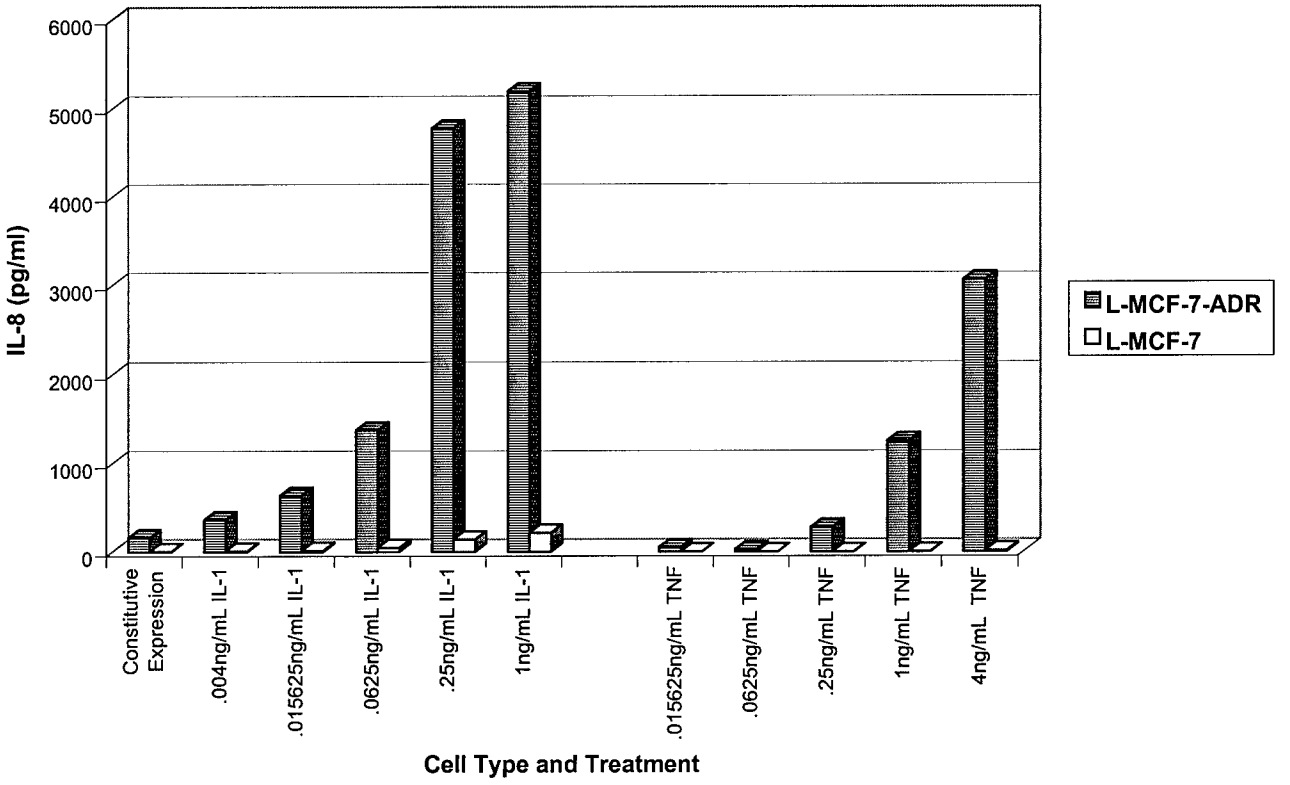


Fig. 2C: IL-8 Production by L-MCF-7 Cell Lines



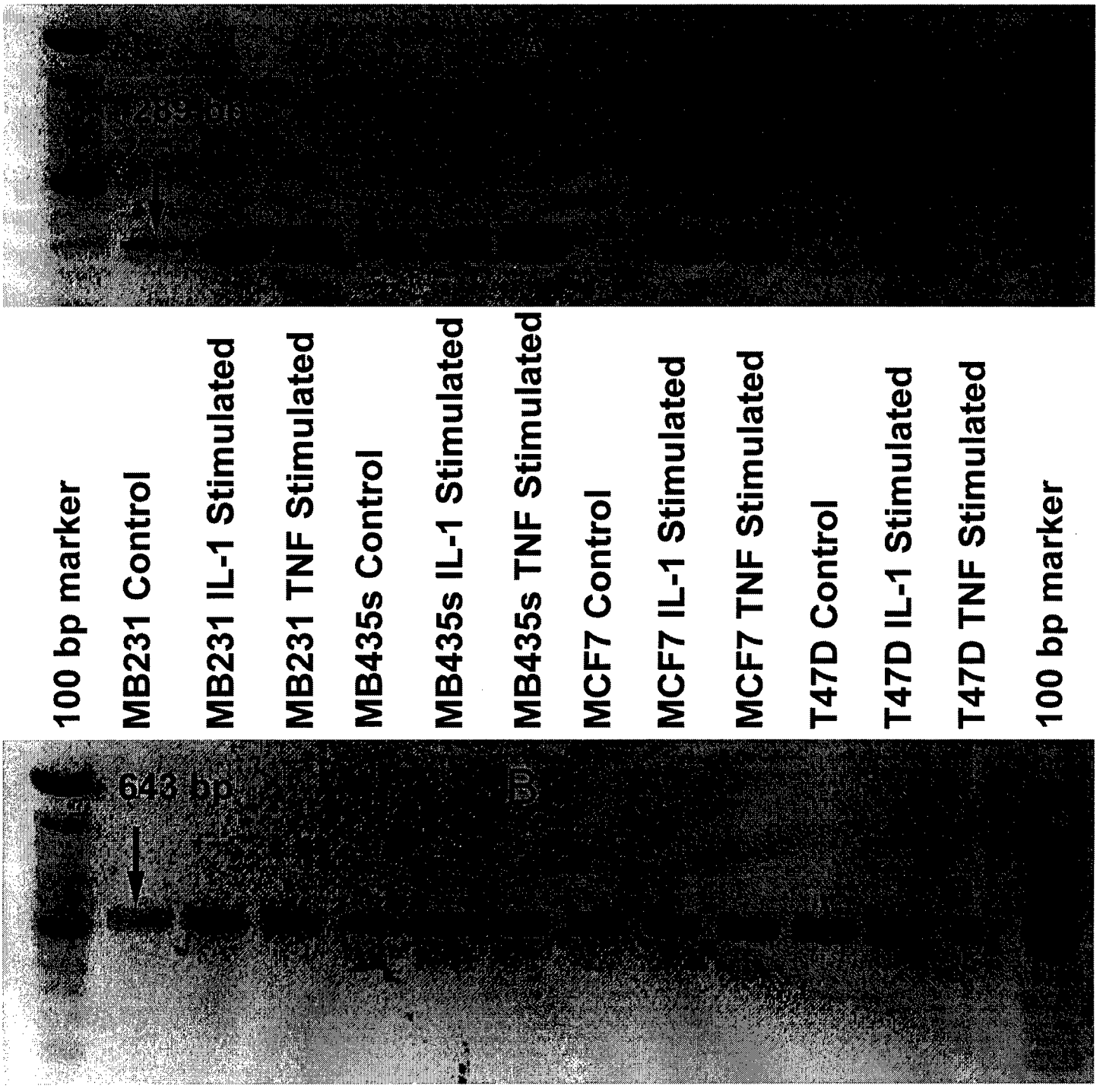


Fig. 3: IL-8 mRNA expression by metastatic and non-metastatic breast cancer cells. IL-8 mRNA production of stimulated and unstimulated metastatic and non-metastatic cells. Total RNA was extracted from untreated (Control) cells, and from cells that were stimulated for 3 hours with either 1ng/ml IL-1 beta, or 4ng/ml TNF-alpha. First-strand cDNA was produced by amplification with 1 microgram of RNA using oligo-dT primers. 35 cycles of PCR were performed using primer sets amplifying a 289 bp segment of the human IL-8 gene (A). Twenty-five cycles of PCR were performed using a beta-actin primer set amplifying a 643 bp segment of the human beta-actin gene (B).

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