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Research Article

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Induction of Increased Levels of Matrix Metalloproteinase-2 (MMP-2) and -9 in Human Breast Cancer Cell Lines by Activation of GM-CSF Receptor Bc via C-Fos – ERK 1/2 Signaling

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Abstract

Background and objectives: Matrix metalloproteinase (MMP) -2 and -9 play important roles in the invasion and metastasis of breast cancer, but the mechanism of their regulation is not clearly understood. GM-CSF has been shown to be associated with cancer invasion and metastasis. The goal of our study was to examine the stimulation of *GM-CSF*/ interleukin 3 *(IL-3)*/*IL-5 receptor* common β-chain (βc) and its effects on MMP-2 and -9 regulation in human breast cancer cells.

Methods: The constitutive expression of the *GM-CSF*/*IL-3*/*IL-5 receptor* common βc and GM-CSF production were analyzed in BT 549, MCF-7, and MDA-MB 231 human breast cancer cell lines. We studied the effects of recombinant IL-3, IL-5 and GM-CSF on the gene expression and enzyme activity of MMP-2, and -9 in the aforementioned cell lines. The signaling pathway activated by these cytokines, the blocking of this pathway, and the effect on MMP-2 and -9 productions were also assessed. The downregulation of the GM-CSF receptor βc gene (CSF2RB) expression and its response to cytokine stimulation were also studied.

Results: We observed that the human breast cancer cell lines BT 549, MCF-7, and MDA-MB 231 constitutively produce GM-CSF and express the *GM-CSF*/*IL-3*/*IL-5 receptor* common βc. When these cell lines were treated with recombinant human (rh) GM-CSF, IL-3, and IL-5, enzyme activity and gene expression of MMP-2, and -9 were increased.

Conclusions: Our findings indicate that the activation of the c-Fos – ERK 1/2 signaling pathway upregulates MMP-2 in response to exogenous GM-CSF, IL-3 or IL-5 cytokines. Clinically relevant concentrations of GM-CSF (as low as 10 ng/mL) were sufficient to stimulate MMP-2 and -9. Our results suggest a potential mechanism by which GM-CSF may promote tumor invasion and metastases.

Keywords

Matrix metalloproteinase; GM-CSF/IL-3/IL-5 receptor common β-chain; BT 549, C-Fos; *CSF2RB*; MCF-7; MDA-MB 231; ERK 1/2

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Abbreviations: BT 549: Human Breast Tumor Cell Line 549; C-Fos: FBJ Osteosarcoma Oncogene; ERK 1/2: Extracellular Signal-Regulated Kinase 1/2; ECM: Extracellular Matrix; MMP: Matrix Metalloproteinase; MAPK: Mitogen Activated Protein Kinase; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; IL-3: Interleukin 3; IL-5: Interleukin 5; MCF-7: Michigan Cancer Foundation-7 Human Cell Lines; MDA-MB 231: M. D. Anderson Cancer Center, Human Breast Cancer Cell Line

Introduction

Breast cancer is the most frequently diagnosed cancer, with 1.67 million cases worldwide and the leading cause of cancer death among women, accounting for 25.2% of the total cancer cases and 14.7% of the cancer deaths [1,2]. In order for cancer to metastasize, cells must escape from the primary tumor, migrate and then invade a secondary site. This process requires the degradation of the extracellular matrix (ECM), allowing tumor cells to invade local tissue, intravasate and extravasate blood vessels, and begin new metastatic formation. Such tumor invasion and migration is dependent on proteolytic degradation of the basement membranes and interstitial matrices that encapsulate the tumor and the secondary site [3]. Evidence suggests that basement membrane degradation is primarily mediated by proteinases secreted by the tumor [4-6].

Matrix metalloproteinase (MMPs) are one such family of proteinases, best known for their ability to degrade ECM components and for their role in the process of long-term tissue remodeling. MMPs are upregulated in almost every type of cancer, and their expression is often associated with a poor prognosis for patients with gastric, pancreatic, prostate, breast and cervical squamous cell cancers [7-9]. The expression and activity of MMPs have been linked to advanced stages of breast cancer, increased invasion of tumor cells, and building metastatic formations [8,10]. Gelatinases, MMP-2 and -9 are produced by the reactive stromal cells around breast tumors [11,12] and by breast cancer cell lines which have adopted mesenchymal traits indicative of an epithelial to mesenchymal transition [13].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein growth factor [14] that stimulates proliferation and maturation of myeloid progenitor cells. Recombinant GM-CSF (rGM-CSF) is now routinely used to increase dangerously low white blood cell levels in millions of cancer patients undergoing intensive chemotherapy [15]. GM-CSF has been prescribed as sargramostin (aka Leukine™) at a dose of 250 μg/m^2/day, leading to corresponding peak blood levels as high as 29 ng/ml and sustained levels in the 1 to 10 ng/ml range [16-18].

Of concern are findings that high levels of circulating GM-CSF correlated with poor prognosis of patients with non-small cell lung cancer [19]. Moreover, when human cell lines of breast carcinoma, osteosarcoma [20], melanoma [21], lung cancer [22-24], squamous cell carcinoma [25], small cell carcinoma [26], colon carcinoma [27] and renal carcinoma [28] were treated with rGM-CSF they exhibited growth, migration and invasion. The precise mechanism of these GM-CSF effects is not known, however the ability of GM-CSF to upregulate MMPs in cancer cells may contribute to tumor cell growth [16,29-32]. Among several signaling transduction pathways, mitogen

activated protein kinase (MAPK) pathway components, such as ERK 1/2, have been correlated to MMP activation and expression, mainly MMP-2 [33-35].

To date, the effect of GM-CSF on human breast cancer cell MMP production has not been investigated. We hypothesized that the GM-CSF/IL-3/IL-5 receptor common βc can be activated by exogenous GM-CSF, IL-3, and IL-5, and that this activation stimulates the production of MMP-2 and -9 in human breast cancer cell lines through the ERK 1/2 signaling pathway.

Methods

Cell lines and culture conditions

Cell lines were obtained from and authenticated by the American Type Culture Collection (ATCC, Rockville, MD). The human breast cancer cell line BT 549 (ATCC® HTB-122™) was maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Wisent Technologies, Rocklin, CA) while MCF-7 (ATCC® HTB-22™) and MDA-MB 231 (ATCC® HTB-26™) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent Technologies, Rocklin, CA). Both culture media contained 10% FBS, 100 IU/ml penicillin, and 100 g/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Biological reagents

Recombinant human (rh) GM-CSF, IL-3, and IL-5 were purchased from R&D Systems (Minneapolis, MN). Anti-β Tubulin, c-Fos, phosphorylated ERK 1/2 (p-ERK 1/2) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA), and total ERK 1/2 from Santa Cruz Biotechnology (Dallas, TX). GM-CSF, IL-3 and IL-5 ELISA kits were purchased from eBioscience Inc. (San Diego, CA).

GM-CSF production

Cells were cultured in a 6 well plate, at initial concentration 0.25×106 cells in 2 mL of serum free media. After 24 hr. incubation, culture supernatants were collected and cleared of debris by centrifugation at 3000 rpm for 5 min and kept at -20°C until later use. GM-CSF, IL-3 and IL-5 concentrations were measured by ELISA (eBioscience ELISA kit, San Diego, CA) following instructions provided by the company. Cell counting was performed after each experiment on a Coulter Z2 counter and analyzed by the AccuComp software (Beckman Coulter, Fullerton, CA). All experiments were run in quadruplicate and repeated three times.

Analysis of *GM-CSF/IL-3/IL-5 receptor* common βc in human breast cancer cell lines by Western Blotting

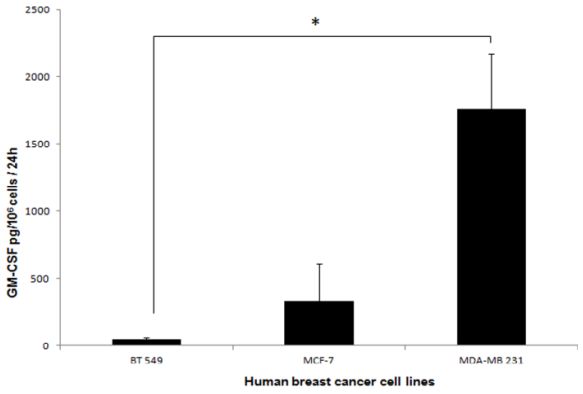
Western Blotting was performed to corroborate the presence of the GM-CSF/IL-3/IL-5 receptor common β-chain (βc) in human breast cancer cell lines. These cell lines were cultured as mentioned above and, when they reached 80% confluency, the cells were solubilized in lysis buffer [50 mM TBS (pH 8.0), 150 mM NaCl, 0.002% sodium azide, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin, 200 mM sodium orthovanadate, 1% (octylphenoxy) polyethoxyethanol, 0.5% sodium deoxycholate] and phosphatase inhibitors for 30 min on ice, followed by centrifugation (12 000 rpm) at 4°C for 5 min to sediment the particulate material. The protein content of these lysates was determined by the Bradford method (BioRad, Hercules, CA). Following protein quantification, 35 µg total protein was loaded and electrophoresed under reducing conditions on 4-20% Tris-Glycine precast gels (BioRad, Hercules, CA) and transblotted onto nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked in 5% milk-TTBS (0.01 M Tris, pH 7.6, 0.1% Tween, 0.1 M NaCl) for 1 hr. at room temperature and probed with rabbit polyclonal anti-Cytokine Receptor Common β-Chain antibody (Cell Signaling Technology Inc., Beverly, MA). After overnight incubation with the first antibody at 4°C, it was followed by a second incubation with horseradish peroxidase conjugated rabbit antibody (Bethyl Laboratories, Montgomery, TX) for 1 hr. at room temperature. Bands were visualized with a Western Lightning Plus-ECL (Enhanced Chemiluminescence) Substrate kit (Perkin Elmer, Waltham, MA). For a positive control, we used a human monocytic cell line, THP-1, which has functional GM-CSF/IL-3/IL-5 receptor common βc [36].

Measurement of MMP-2 and -9 potential enzyme activities by zymography

Gelatin zymography was performed on conditioned breast cancer cell media to assess MMP-2 and MMP-9 activity. In brief, a total of 0.25×10^6 cells/well were seeded in a 24-well culture plate. After incubation for 24 hr. in regular culture media, the BT 549, MCF-7, and MDA-MB 231 were washed and placed in culture with serum free medium in the absence or presence of rhGM-CSF, rhIL-3 or rhIL-5 in varying concentrations (0, 5, 10, 50, 100 ng/ml; according to Tomita et al. [29]). After the following 24 hr. culture supernatants were collected and cleared of debris by centrifugation at 3000 rpm for 5 min. The protein content was determined by the Bradford method (BioRad, Hercules, CA). These supernatants were mixed with non-reducing sample loading buffer and loaded onto 10% polyacrylamide gels co-polymerized with 2 mg/mL gelatin. 20 µg of supernatant was loaded into each lane. Following electrophoresis, gels were rinsed in 2.5% Triton X-100 (3 x 20 min) and then kept in incubation buffer overnight (18 hr.) at 37°C. Composition of incubation buffer was (mM): Tris-HCl (50), $CaCl_2$ (5), NaCl (150), and 0.05% NaN_3. After incubation, gels were stained in 0.05% Coomassie Brilliant blue for 2 hr. and destained. The intensity of each gelatinolytic band was scanned and semi-quantified by Image J (National Institutes of Health, USA, http://imagej.nih.gov/ij). The resulting intensities were normalized with respect to untreated cells for each cytokine group, and expressed as a ratio of intensity relative to the untreated cells; all measurements were performed in triplicate.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from 0.25×10^6 cells in the absence or presence of rhGM-CSF, rhIL-3 or rhIL-5 in varying concentrations (0, 5,10,50,100 ng/ml), by using Trizol (Life Technologies, Thermo Fisher Scientific, Burlington, ON) according to the manufacturer's instructions. Total RNA (2µg) was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, Burlington, ON). Gene expression was quantified by real-time quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, and Burlington, ON). The DNA amplification was carried out using a 7500 Fast Real-Time PCR-System (Applied Biosystems, Thermo Fisher Scientific, and Burlington, ON). The PCR primers used were as follows: for MMP-2, 5'GCTGGCTGCCTTAGAACCTTTC-3', antisense primer 5'GAACCATCACTATGTGGGCTGAGA-3', for MMP-9, 5'GCACGACGTCTTCCAGTACC-3', antisense primer 5'GCACTGCAGGATGTCATAGGT-3', and for our internal control gene GAPDH, 5'GCACCGTCAAGGCTGAGAAC-3', antisense



*Note: Cells were cultured at an initial concentration of 1 X 10^6 cells in 3 mL of growing media, and after 24 hours incubation, conditioned media was collected and GM-CSF content was measured by ELISA. Data are expressed as the mean ± standard error of the mean (S.E.M). Experiments were done in triplicate. One-way ANOVA followed by Bonferroni's post hoc multiple comparisons test were conducted. Asterisk shows statistically significant higher GM-CSF production by MDA-MB 231 compared to the other two cell lines, P< 0.001.

Figure 1: GM-CSF protein production by the human breast cancer cell lines BT 549, MCF-7, and MDA-MB 231.

primer 5'TGGTGAAGACGCCAGTGGA-3'. All cell MMP mRNA levels were normalized by the GAPDH mRNA level in each sample.

Western blot analysis of treated breast cancer cell lines

Cells were seeded in a 24 well plate at 0.25×10^6 cells/well and cultured with serum-free medium in the absence or presence of rhGM-CSF, rhIL-3 or rhIL-5 in varying concentrations (0, 5,10,50,100 ng/ml; according to Tomita et al. [29]). After 30 min of incubation, cell stimulation was stopped by centrifugation and by washing the cells three times with ice-cold PBS. The cell pellets were resuspended and lysed in lysis buffer as mentioned above. After quantification, 10 µg total protein was loaded, electrophoresed under reducing conditions on 8% SDS-PAGE gels, and transblotted onto PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking for 1 hr. at room temperature, the membranes were probed with either polyclonal anti-β Tubulin, c-Fos, phosphorylated-ERK 1/2 (Cell Signaling Technology, Inc., Beverly, MA) or total ERK 1/2 (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, and then with the appropriate horseradish peroxidase conjugated secondary antibody (either anti-mouse or anti-rabbit, Bethyl Laboratories, Montgomery, TX) for 1 hr. at room temperature. Bands were visualized and their intensity was semi-quantified by Image J (National Institutes of Health, USA, http://imagej.nih.gov/ij).

Inhibition of the ERK 1/2 pathway

A total of 0.25×10^6 cells/well were seeded in a 24-well culture plate. After incubation for 24 hr. in regular culture media, the cells were washed and re-fed with serum-free medium in the absence or presence of ERK 1/2 inhibitor PD98059 (EMD Biosciences Inc, San Diego, CA) dissolved in 0.1% dimethyl sulfoxide (DMSO) at 10 or 25 µM for two hours prior to the addition of rhGM-CSF, rhIL-3 or rhIL-5 at 10 ng/ml. To ensure that DMSO did not affect cell viability, a control received culture medium which contained DMSO alone at the final concentration of 0.1% (v/v). After 24 hr. culture, supernatants were collected and cleared of debris by centrifugation at 3000 rpm for 5 min. Protein content was measured in these supernatants for zymography.

GM-CSF/*IL-3/IL-5 receptor* common β-chain gene down regulation

Small interfering RNAs targeted to *CSF2RB* gene, positive control (GAPDH gene) and negative control (scrambled siRNAs) were pre-designed and validated (Life Technologies, Thermo Fisher Scientific, and Burlington, ON). Transfection conditions were optimized using a GAPDH siRNA. Transfection of MDA-MB 231 cells (70% confluency) with siRNA was performed using Lipofectamine RNAiMAX (Life Technologies, Thermo Fisher Scientific, Burlington, ON) according to the recommendations of the manufacturer. Briefly, the transfection reagent was pre-incubated with the siRNA oligos either targeted to *CSF2RB*, GAPDH or the irrelevant control 5 min prior to the application to the cells. After 24 hr. incubation, the cells were washed and re-fed with serum-free medium in the absence or presence of rhGM-CSF (10 ng/ml). After the following 24 hr., culture supernatants were collected and cleared of debris by centrifugation at 3000 rpm for 5 min and used for zymography. For RNA isolation, cells were treated with Trizol (Life Technologies, Thermo Fisher Scientific, and Burlington, ON) according to the manufacturer's instructions.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post hoc test were used for all experiments of three or more groups. All analyses were performed with the SAS statistical software (SAS Institute Inc., Cary, NC). A value of $P<0.05$ (*) was considered statistically significant.

Results

GM-CSF production and expression of the *GM-CSF/IL-3/IL-5 receptor* common β-chain by human breast cancer cell lines

Several studies have demonstrated the production of GM-CSF by various tumor cell lines of no hematopoietic origin [14,23]. We first determined if the human breast cancer cell lines BT 549, MCF-7 and MDA-MB 231 produce GM-CSF. After 24 hr. of culture in serum-free media, the supernatant of all three human breast cancer cell lines were tested by ELISA and showed detectable levels of GM-CSF (Figure 1). GM-CSF levels produced by 1×10^6 BT 549 and MCF-7 cell lines were not statistically different (53 ± 7 pg/mL; 337 ± 227 pg/mL, respectively). However, 1×10^6 MDA-MB 231 cells produced significantly higher GM-CSF levels than BT 549 (1756 ± 413 pg/mL, $P<0.01$). We also determined if there was constitutive IL-3 and IL-5 production by these cell lines since they share with GM-CSF the same receptor common βc. However, IL-3 and IL-5 protein levels were not detected by ELISA in any of the three cell lines tested (data not shown). Determinations were done in triplicate in four independent experiments.

Given the differing production of GM-CSF between cell lines, we next determined if BT-549, MCF-7 and MDA-MB 231 breast cancer cell lines express the GM-CSF/IL-3/IL-5 receptor common βc. All three cell lines expressed the 100 kDa of the receptor common βc as observed in the positive control cell line THP-1 (Figure 2).

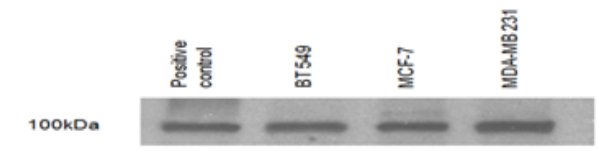
Effects of rhGM-CSF, rhIL-3, and rhIL-5 on MMP secretion and activity

To study the effects of rhGM-CSF, rhIL-3, and rhIL-5 on MMP-2 or MMP-9 secretion, tumor cells were cultured in the presence or absence of each recombinant cytokine for 24 hr. The BT 549 cell line showed production of the 92 kDa MMP-9 only after stimulation

with rhIL-3 at 10-100 ng/mL, while the increased production of the 72 kDa MMP-2 was evident with rhGM-CSF and rhIL-3 stimulation (Figure 3a) and to a lesser extent with the rhIL-5. The MCF-7 cell line demonstrated gelatinolytic activities of the 72 kDa MMP-2 with all three recombinant proteins. The activity of the 92 kDa MMP-9 was mainly stimulated by rhGM-CSF in a dose-dependent manner, while the increased activity of the 72 kDa MMP-2 was stimulated by rhGM-CSF, followed by rhIL-3, and rhIL-5 (Figure 3b). The cell line MDA-MB 231 only showed increase of the 72 kDa MMP-2 enzyme activity in a dose-dependent manner after treatment with the three cytokines at 10 to 100 ng/mL (Figure 3c).

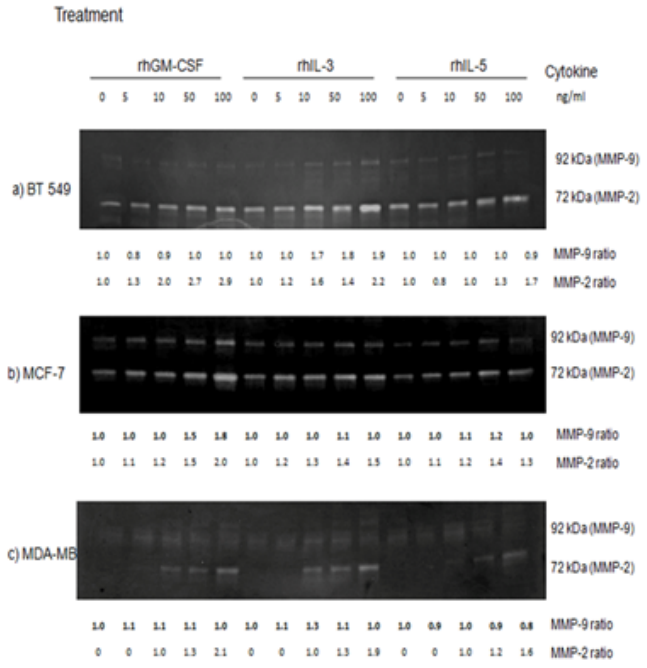
Recombinant human GM-CSF, IL-3 and IL-5 can induce MMP-2 and MMP-9 gene expression in human breast cancer cell lines

MMP-2 and MMP-9 gene expression was studied in BT 549, MCF-7 and MDA-MB 231 cells after receiving rhGM-CSF, rhIL-3 and rhIL-5 (Figure 4). The BT 549 cell line treated with rhGM-CSF only showed a statistically significant increase of MMP-2 in a dose-dependent manner ($P<0.0001$) (Figure 4a). The changes observed for MMP-2 and MMP-9 gene expression after rhIL-3, were not statistically significant, and no changes were observed after rhIL-5 treatment. The MCF-7 cell line showed statistically increased expression of the MMP-2 gene in a dose-dependent manner when the cells were treated with rhGM-CSF and rhIL-3 ($P<0.001$) (Figure 4b). On the other hand, MMP-9 gene expression remained unchanged after treatment. Furthermore, rhIL-5 treatment did not induce MMP-2 or -9 gene expression changes in the MCF-7 cell line. The MDA-MB 231 cell line showed almost the same MMP-2 and -9 gene expression after rhGM-CSF treatment (Figure 4c). However, this cell line treated with rhIL-5 showed a statistically significant difference in MMP-9 gene expression ($P<0.03$) (Figure 4c). MMP-9 gene expression was significantly higher in cells treated with high concentrations of rhIL-5 (100 ng/ml) than cells treated with 10 ng/ml ($P<0.05$).



*Note: Whole cell lysates were prepared and analyzed by Western blot using an anti-rabbit polyclonal antibody against the common β-chain. Expression was compared to the positive control THP-1 cell line.

Figure 2: Expression of *GM-CSFIIL-3IIL-5 receptor* common β-chain in BT 549, MCF-7 and MDA-MB 231 cell lines by immunoblot analysis.



*Note: After incubation with recombinant human IL-3, IL-5 or GM-CSF, conditioned media were collected and analyzed by gelatin zymography, and compared with untreated controls (lanes 1, 6, 11). The intensity of each gelatinolytic band was scanned and semi-quantified by NIH Image J software. Relative gelatinolytic activities corresponding to pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa) from triplicate determinations are shown.

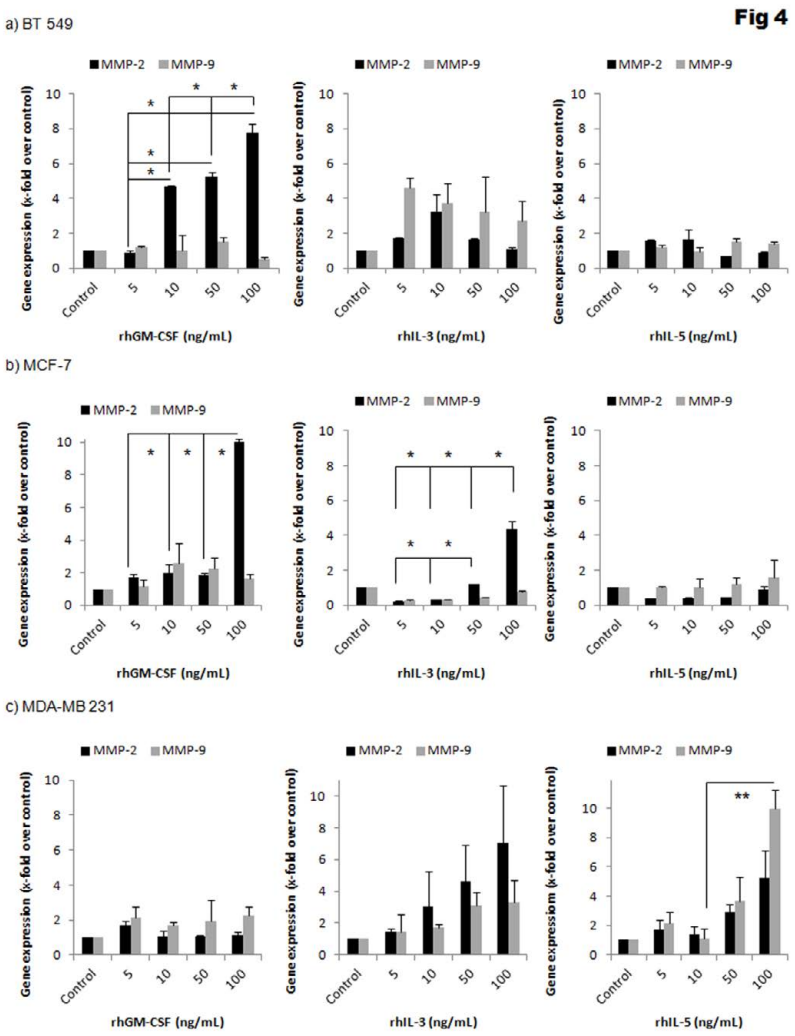
Figure 3: Effects of rhGM-CSF, rhIL-3 and rhIL-5 on gelatinase activity in human breast cancer cell lines a) BT 549, b) MCF-7 and c) MDA-MB 231.

Recombinant human GM-CSF, IL-3 and IL-5 can induce c-Fos and ERK 1/2 expression in human breast cancer cell lines

MMP genes are regulated by the transcription factor AP-1 [37]. AP-1 complexes are protein heterodimers of the two proto-oncogene families Jun and Fos. We investigated whether c-Fos and/or c-Jun protein expressions were increased after rhGM-CSF, rhIL-3 or rhIL-5 treatment in breast cancer cell lines. The expression of c-Fos at the protein level was analyzed by Western blot using the anti-c-Fos antibody. In the human breast cancer cell line BT 549, we observed a clear dose-dependent increase in the expression of c-Fos when the cells were activated with rhGM-CSF and rhIL-3 up to 50 ng/mL, and an increased c-Fos expression with rhIL-5 at 100 ng/mL (Figure 5a). For the MCF-7 cell line, the three cytokines increased the expression of c-Fos (Figure 5b). The MDA-MB 231 cell line showed modest increased expression of c-Fos when the cells were treated with the cytokines (Figure 5c). Western Blot analysis of the three treated cell lines tested with c-Jun antibody did not show any differences with the various concentrations of rhGM-CSF, rhIL-3 or rhIL-5 (data not shown). We next investigated signaling pathways for c-Fos in MMP-2 and -9 activation after rhGM-CSF, rhIL-3, or rhIL-5 treatment. The distal C-terminus of the GM-CSF receptor βc has been demonstrated to couple to a Ras-dependent signal transduction pathway [38] leading to the activation of AP-1[39]. This pathway includes many proteins, among them ERK 1/2, which is upstream of c-Fos. Thus, ERK 1/2 activation was investigated. As shown in Figure 5, rhGM-CSF, rhIL-3 and rhIL-5 increased ERK 1/2 phosphorylation, as compared with the untreated cells. The BT 549, MCF-7, and MDA-MB 231 cell lines treated with rhGM-CSF, and rhIL-3 showed increased ratios of p-ERK 1/2 in a dose-dependent manner. On the other hand, rhIL-5 treatment increased the expression of p-ERK 1/2 in BT 549 and MDA-MB 231 cell lines at 10 ng/mL and MCF-7 at 50 ng/mL (Figure 5). It has been determined in some cancer models that phosphorylation of ERK 1/2 modulates MMP production [9,40,41]. Other pathways that can be activated after GM-CSF receptor βc activation include the JAK/STAT and the JNK/SAPK pathways [42]. However, there were no changes in expression in any of the three cell lines studied (data not shown).

Inhibition of MMP-2 and -9 production by PD98059

These results suggested that rhGM-CSF, rhIL-3, and to some extent rhIL-5 upregulated MMP-2, c-Fos, and activated the ERK



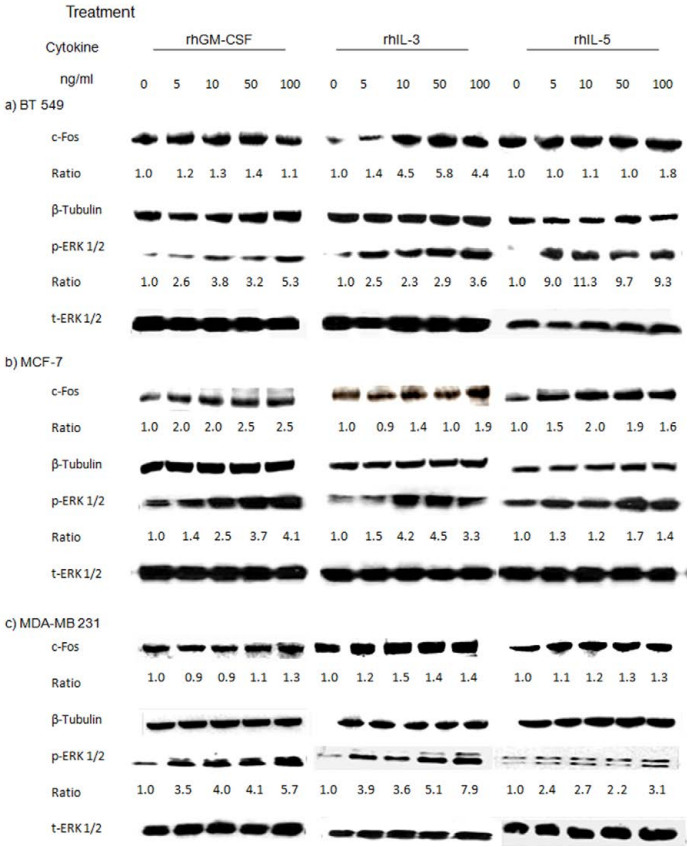
*Note: Human breast cancer cell lines were treated with varying concentrations of cytokines in serum-free media for 18 hours. Total RNA was extracted from the cells and qPCR was performed to assess expression of MMP-2 and -9 transcripts. Values were normalized over GAPDH and are the mean ± SEM of triplicate values from one out of two representative experiments. One-way ANOVA followed by Bonferroni's post hoc multiple comparisons test was conducted (*P<0.01, **P<0.05).

Figure 4: MMP-2 and -9 gene expression after cytokine treatment.

1/2 pathway in the three breast cancer cell lines, and MMP-9 in the MCF-7 cell line. To further examine the link between the activation of ERK 1/2 and the upregulation of MMP-2 and -9, the ERK 1/2 specific inhibitor (PD98059) was used. For these experiments, the cells were activated with 10 ng/ml of rhGM-CSF, rhIL-3 or rhIL-5, since sustained levels of GM-CSF in the 1 to 10 ng/ml range were reported in patients who received GM-CSF therapy [16-18]. In general, as seen in Figure 6, when the inhibitor was added to the cell lines 2 hr. prior to cytokine treatment, MMP-2 and/or -9 upregulation were significantly suppressed by PD98059 at 10 and/or 25 μM. These results suggested that MMP-2 and -9 inductions by rhGM-CSF, rhIL-3 or rhIL-5 depended on the activation of ERK 1/2 (Figure 6).

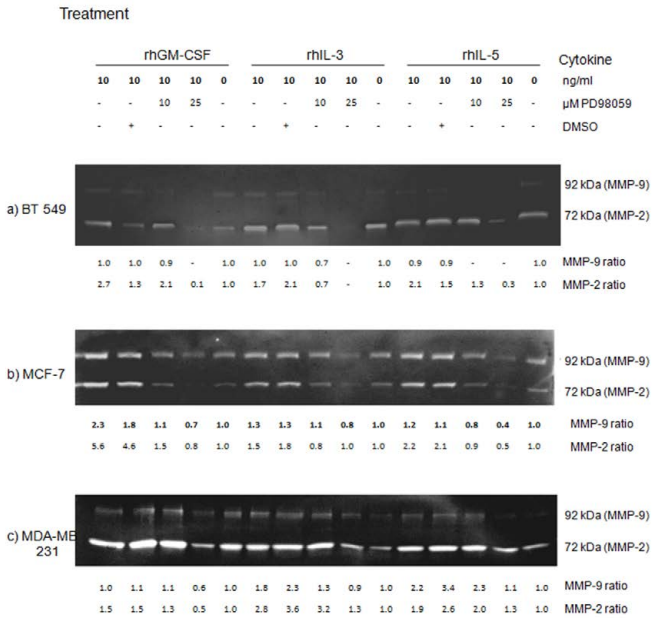
Reduced MMP-2 activity by downregulation of the GM-CSF receptor βc gene (*CSF2RB*)

In order to examine the involvement of the GM-CSF receptor βc for the production of MMP-2 and -9 in breast cancer cell lines, we repressed the expression of the GM-CSF receptor βc gene (aka *CSF2RB*) by transiently transfecting MDA-MB 231 cells with a siRNA against *CSF2RB* (si*CSF2RB*). The gene silencing efficacy for the *CSF2RB* was 34% compared to the irrelevant gene control (siScrambled) (Figure 7a). We aimed to verify if that observable *CSF2RB* downregulated expression would affect the response to rhGM-CSF. *CSF2RB* gene expression was not significantly higher in the si*CSF2RB* cells treated with rhGM-CSF (10 ng/ml) as compared to the untreated. However, the siScrambled RNA transfected cells (control) showed significantly increased *CSF2RB* gene expression when treated with rhGM-CSF ($P<0.01$). MMP-2 gene expression was statistically significantly lower in the *CSF2RB* downregulated cells than in the control ($P<0.01$). After rhGM-CSF treatment, although the same trend was observed, the difference was not significantly different (Figure 7b). The si*CSF2RB* cells did not show increased MMP-9 gene expression after rhGM-CSF treatment. On the other hand, after rhGM-CSF treatment, control cells showed statistically significant higher MMP-9 gene expression compared to si*CSF2RB* cells ($P<0.01$) (Figure 7c). After rhGM-CSF treatment (10 ng/ml), MMP-2 activity was lower in si*CSF2RB*



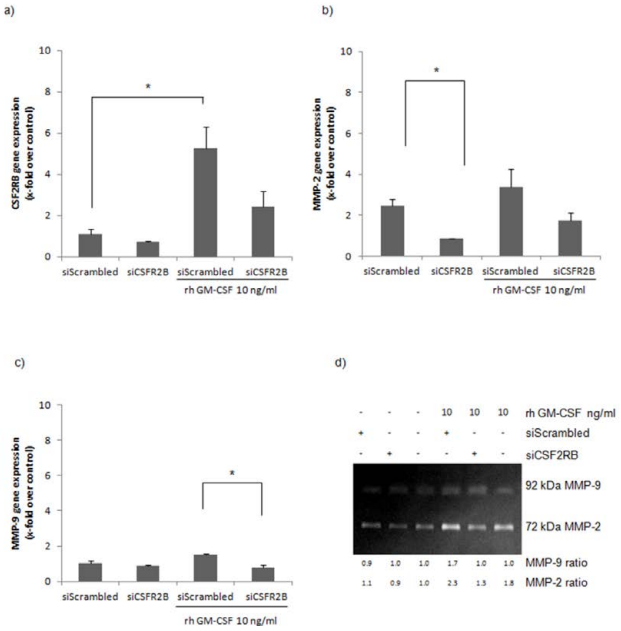
*__Note:__ Cells were cultured in the presence or absence of rhGM-CSF, rhIL-3 or rhIL-5 for 30 minutes. Whole cell lysates were prepared and analyzed by Western blot using an anti-c-Fos and p-ERK 1/2 antibodies. Gel loading was standardized for equal protein loading.

Figure 5: c-Fos, and p-ERK 1/2 expression in a) BT 549, b) MCF-7, and c) MDA-MB 231 human breast cancer cell lines.



*__Note:__ Two hours prior to stimulation with rhGM-CSF, rhIL-3 or rhIL-5 (10 ng/ml), a) BT 549, b) MCF-7 or c) MDA-MB 231 cells were treated with 10 or 25 μM PD98059 or left untreated. Conditioned media were collected 24 hr. later and analyzed by zymography as described in Materials and Methods. Control cells were unstimulated, untreated, or stimulated and treated with DMSO. Addition of DMSO in the culture medium had no effect on the induction of MMP-2 and -9 secretions. All these experiments were performed at least three times and gave the same results.

Figure 6: Effect of PD98059 on GM-CSF, IL-3 or IL-5 -induced MMP-2 and -9 expressions.



*Note: Cells were then treated with vehicle or with 10 ng/mL rhGM-CSF for 18 hours. Total RNA was extracted and qPCR was performed to assess a) *CSF2RB*, b) MMP-2 and c) MMP-9 transcript levels. Values were normalized over the expression of GAPDH, and are the mean ± S.E.M of triplicate values from one representative experiment. MMP-2 and -9 secretion and activity measurement by zymography d) after transient downregulation of CSF2RB. One-way ANOVA followed by Bonferroni's post hoc multiple comparisons test were conducted (*P<0.01).

Figure 7: The human breast cancer cell lines BT 549, MCF-7 and MDA-MB 231 were transiently transfected with siRNA against *CSF2RB* or a random sequence (siScrambled) as described in the Methods section.

cells compared to siScrambled or non-silenced cells, as shown by zymography (Figure 7d).

Discussion

The three breast cancer cell lines studied here constitutively produce GM-CSF and express the GM-CSF/IL-3/IL-5 receptor common βc. Our data indicate that rhGM-CSF, rhIL-3 and to some extent rhIL-5 upregulated MMP-2, c-Fos, and activated the ERK 1/2 pathway in the BT 549, MCF-7 and MDA-MB 231 breast cancer cell lines, and MMP-9 in the MCF-7 cell line. GM-CSF therapy is prescribed to breast cancer patients who develop leucopenia after chemotherapy [15]. GM-CSF stimulates the proliferation and maturation of leucocytes; however, it could have the same effect on remaining cancer cells after chemotherapy. In this paper, we hypothesized that rhGM-CSF may stimulate breast cancer cells by activating the receptor common βc and induce MMP production. MMP-2 and -9 have been suggested to play a critical role in tumor invasiveness [8, 10]. These enzymes can degrade collagen type IV, and allow the escape of cancer cells, thus facilitating metastasis [43]. Here, we demonstrated that human breast cancer cell lines are able to produce GM-CSF. We observed that cells well-known for their invasiveness, i.e., MDA-MB 231 cell line, produced elevated levels of GM-CSF. All the three cell lines; BT 549, MCF-7 and MDA-MB 231, expressed the common receptor βc (Figure 2), indicating that these cell lines can be stimulated by GM-CSF.

In early GM-CSF receptor studies, it was observed that small cell carcinoma (SCC) cell lines were able to respond at 10 pmol/L of GM-CSF, even though these cells have 10 times less binding sites that hematopoietic cell [20]. In our study, we observed that all three breast cancer cell lines expressed the common receptor βc as compared to the hematopoietic cell line THP-1. We next determined that the activation of GM-CSF/IL-3/IL-5 receptor βc could stimulate the production of MMPs in human breast cancer cells. We observed by zymography that all three human breast cancer cell lines we tested were stimulated by rhGM-CSF, rhIL-3, and rhIL-5 to produce MMP-2 (Figure 3). MMP-2 mRNA levels coincided with the MMP-2 increased activity after rhGM-CSF treatment in the BT 549 cell line. The same trend was observed for the MCF-7 cell line when treated with rhGM-CSF and rhIL-3. The MDA-MB 231 cell line treated with rhGM-CSF showed a modest increase in the expression of c-Fos, increased expression of p-ERK 1/2, and increased MMP-2 protein activity, but not mRNA levels. MCF-7 cells showed significantly increasing levels of MMP-2 gene expression and a dose-dependent increase in MMP-2 and -9 enzyme activities after rhGM-CSF treatment. They also showed increases in c-Fos and p-ERK 1/2. This cell line has been reported to be very weakly invasive and essentially non-metastatic [44].

Studies have revealed the effect of GM-CSF on MMP expression [29,30,31,45], and that a large variety of mitogens and growth factors activate ERK 1/2 pathways. It was proposed by de Groot et al. [42] that the activation of the GM-CSF/IL-3/IL-5 receptor common βc could lead to an alternative signaling pathway: Y577 – Shc - Grb2/m SOS – Ras – Raf – Ek – ERK. Activation of this pathway would eventually enhance the transcription of c-Fos and c-Jun. Moreover, fairly recent reports have indicated that the MAPK pathways are involved in the regulation of MMP gene expression in human prostate cancer and non-small cell lung cancer cells [46,47]. In our study, the three human breast cancer cell lines we tested showed increased expression of p-ERK 1/2 after treatment (Figure 5). The results for MDA-MB 231 corroborate published data [48]. The fact that p-ERK 1/2 was detected confirms that c-Fos activity is also regulated by posttranscriptional modification caused by phosphorylation of different kinases like MAPK, as proposed by Gruda et al. [49]. By using PD98059, the

results suggest that rhGM-CSF, rhIL-3 and rhIL-5 utilize the classical ERK 1/2 signaling pathway for inducing MMP-2 secretion in the three cell lines, and suggest that transient activation of ERK 1/2 is necessary and sufficient to induce this event (data observed in time course experiment, not shown).

MDA-MB 231 is a well characterized cell line. It is human, metastatic, invasive, estrogen receptor-negative, progesterone receptor-negative, and does not overexpress HER2 [50]. Since MDA-MB 231 showed the highest GM-CSF production, this cell line was chosen to downregulate the gene for the GM-CSF receptor βc (aka *CSF2RB*). This gene was downregulated by only 34% compared to the control. However, with this downregulation, we observed decreased MMP-2 and -9 gene expression (Figures 7b,7c). Not only was decreased gene expression observed after *CSF2RB* downregulation, there was also decreased protein activity as shown by zymography (Figure 7d). Here we showed that activation of the GM-CSF receptor βc upregulated MMP-2 and -9 protein and gene expression.

Conclusions

Activation of the GM-CSF/IL-3/IL-5 receptor common βc modulates MMP-2 gene expression and enzyme activity of MMP-2 and -9 by activating the c-Fos – ERK 1/2 signaling pathway in human breast cancer cell lines. We demonstrated this by using the ERK 1/2 blocker PD98059, which significantly downregulated MMP-2 and, to a certain extent, MMP-9 enzyme activity. We also observed that partial *CSF2RB* downregulation can reduce MMP-2 protein activity.

Our findings indicate that the activation of the c-Fos – ERK 1/2 signaling pathway upregulates MMP-2 and, to a certain extent, MMP-9 in response to exogenous GM-CSF, IL-3 or IL-5 cytokines on the GM-CSF/IL-3/IL-5 receptor common βc. These preliminary results suggest that clinically relevant concentrations of GM-CSF (10 ng/mL) upregulate pathways known to be involved in tumor invasion and metastasis.

Availability of Data and Materials

All data generated and analyzed during this study are included in this published article.

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