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Research paper

TNF-alpha-induced metastasis gene changes in MCF-7 cells ☆

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Abstract

Objective: Studies have shown that TNF-α secreted by tumor cells and macrophages infiltrated into the tumor microenvironment might promote the metastasis of a variety of malignant cancers, including breast cancer. The present study was designed to detect global metastasis-related gene expression changes of MCF-7 cells treated by low dose TNF-α and to further explore the mechanisms by which TNF-α contributes to metastasis. **Methods:** MCF-7 cells were cultured and treated with low dose TNF-α (20 ng/ml). cDNA array analysis was applied to detect the metastasis related gene expressions. **Results:** A total of 36 gene expressions were significantly regulated by TNF-α. Functional analysis indicates that the altered genes belong to different functional group. Most of the genes changed may promote the metastasis of MCF-7 cells while the others may inhibit metastasis. The changes observed in gene expression following TNF-α were somewhat time dependent. **Conclusion:** TNF-α can enhance the invasive ability of MCF-7 cells, partly by regulating a series of metastasis related genes, and these genes may take part in every step of metastasis. Some of the genes deserve further study.

Key words: Tumor necrosis factor-α; metastasis; cDNA array

INTRUDUCTION

Metastasis is the spread of tumor cells from the primary site to form secondary tumors at other sites in the body. It is a fairly complicated process involving multiple steps:① detachment of cells from the primary tumor; ②penetration of the basement membrane;③migration through the stroma; ④ penetration of lymphatics and vasculature; ⑤ escape from immune surveillance; survival in the circulation and arrest in capillary beds of distant organs; ⑥penetration of lymphatic or blood vessel wall; ⑦ extravasation into tissue and growth of metastatic deposits in the new location. Various biochemical substances are involved in these steps, such as cell adhesion molecules, matrix metalloproteinases, vascular endothelial growth factor and chemokines. The formation of these factors is influenced by activated macrophages which surround the tumor cells, and chemokines secreted by these macrophages e.g. tumor necrosis factor-α (TNF-α).

Tumor necrosis factor(TNF) α is a potent pleiotropic proinflammatory cytokine produced by macrophages, neutrophils, fibroblasts, keratinocytes, NK cells, T and B lymphocytes and tumor cells. It plays critical roles not only in inflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease, but also in malignant diseases. Locally administered high dose TNF-α had been shown to cause vessel regression and hemorrhagic necrosis and it has been studied as a anticancer agent[1]. However, the effects of endogenous TNF-α are opposite to the effects observed with high dose TNF-α therapy. Instead of causing tumor regression, cancer-derived TNF-α can mediate tumor progression by inducing growth, proliferation, invasion and metastasis of cancer cells. A series of reports have proved this concept both in animal models and in clinical observations. To date, TNF-α expression has been observed in the tumor micro-environment in a variety of malignancies[2-7], including breast, pancreatic, kidney, lung, prostate, colorectal, bladder, and esophageal

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cancers, and melanoma , lymphoma and leukemias. Moreover, higher levels of TNF-α have been correlated with advanced tumor stage, greater paraneoplastic complications and shorter survival time in patients with the same cancer type. Accordingly, TNF-α has been considered a potential target for the therapy of solid tumors. The various mechanisms by which TNF-α might promote cancer invasion and metastasis had been summarized[8]. However, the complicated intra and inter cellular interactions make it difficult to elucidate the effects of physiological levels of TNF-α on the tumor cells.

Gene expression microarrays allows us to analyze the alterations of a variety of genes at the same time, also they are useful for discovery of new genes that are critically involved in any process. Thus, it has gained popularity among scientists worldwide as the most favored technique for global differential gene expression analysis. Panels of genes regulated by TNF-α had been reported for some cell types[9], and some genes associated with lymph node metastasis and distant metastasis have also been identified for several cancer types, including breast cancer, using this technique[10-16]. In the present work, MCF-7 cells, an estrogen positive breast cancer cell line, was treated with low dose TNF-α Microarray analysis was used to identify the altered gene expressions profile associated with the metastatic phenotypes.

MATERIALS AND METHODS

Cell culture

MCF-7 cells, a human estrogen receptor-positive breast cancer cell line, were purchased from American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium(DMEM) containing 10%(v/v) fetal calf serum supplemented with 0.3 mg/ml glutamine, 5 ng/ml bovine insulin and 100 U/ml penicillin plus 100 ug/ml streptomycin. Cells were seeded with approximately 5×10^5/ml and were cultivated at 37℃ and 5% CO_2.Cells were treated with TNF-α (R&D Laboratories, Inc. St. Paul, MN, USA) at a final concentration of 20 ng/ml for 3 h, 24 h or they were left untreated as control.

Microarray analysis

mRNA from MCF-7 cells treated and untreated with TNF-α were isolated with the ArrayGrade total RNA isolation kit(Superarray, Frederick, USA) according to the manufacturer's recommendations. The quality of total RNA was evaluated by measuring the A_{260}/A_{280} ratio, which was at least 2.0, and by the gel-electrophoresis pattern, which revealed two major bands of 28S and 18S RNA. Using the TrueLabeling-AMP™ 2.0 Kit(GA-030)(Superarray), the mRNA was reversely transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP(Roche, Mannheim, Germany)by *in vitro* transcription. Prior to hybridization, the cRNA probes were purified with the ArrayGrade cRNA cleanup kit(Superarray) and quantified by UV absorbance. The purified cRNA probes were then hybridized to the pretreated Oligo GEArray Human Cancer metastasis Microarrays OHS-028(Superarray), which cover 92 tumor metastasis-related genes. For each reaction, 8 μg biotinylated cRNA was used for hybridization. Following several washing times, array spots binding cRNA were detected using alkaline phosphatase-conjugated streptavidin and CDP-Star as chemiluminescent substrate. Data acquisition and quantification of spot intensities were performed by using the GEArray Expression Analysis suite. Experimental data were compared with baseline data. The raw data were filtered so that individual spots had to pass several quality criterias, including minimum-intensity levels and minimum signal-to-background ratios. Genes that passed these criteria were used for further data analysis. Each gene signal was normalized by 10 % of the M6PR signal in the same membrane, and only those gene signals which were well above background were considered specific gene signals. Genes were considered to be differentially expressed when their signal intensities were found to be altered by $\geqslant$2.0 folds in TNF-α treated cells compared with TNF-α untreated cells. Other comparative data analysis was done in an Excel spreadsheet.

Statistical methods

All values are presented as mean ± SD. A *P* value less than 0.05 was considered statistically significant.

RESULTS

The MCF-7 cells were cultured without or with TNF-α for 3 h and 24 h. At the end of treatment, total RNA was isolated and subjected to cDNA microarray analysis. A total of 84 genes related to tumor metastasis which had been studied previously were analyzed. In response to TNF-α 31 gene expressions were up-regulated and 5 gene expressions were down-regulated by two or more folds in at least one test period when compared with those derived from TNF-α free cells(***Table 1***). Although these gene expression changes occurred over a wide range, most of them were within 10-fold. Only 5 gene expressions were up-regulated beyond 10-fold. The altered genes belong to different functional groups, suggesting that TNF-α was involved in multiple processes of the metastasis of MCF-7 cells. A gene may belong to several different groups because it is multifunctional. Here we briefly divided them into 3 groups: genes related to cell growth and proliferation, genes related to migration, and other genes related to metastasis. Among the 31 up-regulated genes, 15 genes responded

to TNF-α rapidly with changes in gene expression of > or = 2 occurring in 3 h. Six of the 15 were significantly decreased in 24 h, while the other 9 showed constitutively increased expression. The expression changes of 16 out of the 31 genes only attained statistical significance after 24 h.

Table 1 **Genes with significantly altered expression after treatment with TNF-α**

Gene symbol	Gene bank	description	Fold3hr	fold24hr
Cell Growth and Proliferation Genes:				
kRAS	NM_004985	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	2.7	2.6
MYC	NM_002467	V-myc myelocytomatosis viral oncogene homolog(avian)	1.2	2.2
pTEN	NM_000314	Phosphatase and tensin homolog(mutated in multiple advanced cancers 1)	1.8	2.1
RB1	NM_000321	Retinoblastoma 1(including osteosarcoma)	2.8	6.2
CCL7	NM_006273	Chemokine(C-C motif) ligand 7	−1.1	7.8
CXCL12	NM_000609	Chemokine(C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.4	2.1
CXCR4	NM_003467	Chemokine(C-X-C motif) receptor 4	2.8	2.8
EPHB2	NM_004442	EPH receptor B2	−1.3	2.2
FGFR4	NM_002011	Fibroblast growth factor receptor 4	−2.1	−2.8
GNRH1	NM_000825	Gonadotropin-releasing hormone 1(luteinizing-releasing hormone)	5.3	2.6
IGF1	NM_000618	Insulin-like growth factor 1 (somatomedin C)	−1.9	−2.1
IL18	NM_001562	Interleukin 18 (interferon-gamma-inducing factor)	2.8	1.4
IL−1b	NM_000576	Interleukin 1, beta	18.1	21.3
IL−8rb	NM_001557	Interleukin 8 receptor, beta	−3.0	−1.2
MET	NM_000245	Met proto-oncogene (hepatocyte growth factor receptor)	1.7	3.7
PLaur	NM_002659	Plasminogen activator, urokinase receptor	1.1	2.2
Set	NM_003011	SET translocation (myeloid leukemia-associated)	2.5	2.5
SSTR2	NM_001050	Somatostatin receptor 2	1.7	2.5
VEGF	NM_003376	Vascular endothelial growth factor A	1.7	2.6
NR4A3	NM_006981	Nuclear receptor subfamily 4, group A, member 3	4.0	4.2
SMAD2	NM_005901	SMAD family member 2	1.8	2.3
Migration related genes				
APC	NM_000038	Adenomatosis polyposis coli	3.4	5.5
CD44	NM_000610	CD44 molecule(Indian blood group)	1.18	2.1
FAT	NM_005245	FAT tumor suppressor homolog 1(Drosophila)	1.6	2.5
FN1	NM_002026	Fibronectin 1	1.7	3.3
PNN	NM_002687	Pinin, desmosome associated protein	2.1	1.9
mCAM	NM_006500	Melanoma cell adhesion molecule	3.0	2.4
ITGB3	NM_000212	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	−3.5	−1.9
MMP3	NM_002422	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	−2.3	3.6
MMp7	NM_002423	Matrix metallopeptidase 7 (matrilysin, uterine)	−1.5	11.1
MMP9	NM_004994	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	7.5	110.9
MMP13	NM_002427	Matrix metallopeptidase 13 (collagenase 3)	1.8	26.8
METAP2	NM_006838	Methionyl aminopeptidase 2	2.9	3.1
CTSK	NM_000396	Cathepsin K	3.4	3.0
CTSO	NM_001334	cathepsin O	2.1	2.8
KISS1	NM_002256	KiSS-1 metastasis-suppressor	−2.0	1.2

Notes:The gene expression changes were considered significant if a change of ⩾ 2.0 folods occurred in at least one of the times tested after TNF-α treatment. The genes were divided into different groups according to their main functions. Gene symbols and description and function were derived from the NCBI UniGene database. Expression ratio refers to TNF-α treated versus TNF-α untreated.

DISCUSSION

Metastatic disease is a major adverse prognostic factor in breast carcinoma. A better understanding of the mechanisms of breast carcinoma metastasis will greatly contribute to the development of therapy strategies and improvement of prognosis of breast cancer patients. A growing body of evidence has suggested that endogenous TNF-α derived from macrophages and tumor cells may promote the metastasis of breast carcinoma. Recent study showed that the invasiveness of breast cancer cell line, MCF-7, which had moderate invasive ability, was enhanced by co-culturing with macrophages. However the information of gene changes was limited. In the present experiment, MCF-7 cells were co-cultured with TNF-α at a relatively low concentration, 20 ng/ml, and microarray analysis was employed to

explore the mechanisms by which the TNF-α globally impinges on the metastasis phenotype of MCF-7 cells.

Our study showed that the expression of a total of 36 of the genes tested was significantly changed following TNF-α treatment. These genes were involved in a number of biological processes including extra-cellular matrix degradation, cell adhesion, cell cycle control, cell growth and proliferation, and transcription regulation. In another words, low dose TNF-α affects the two major processes of metastasis: proliferation and migration. All of these genes have previously been shown to take part in the tumorigenesis and/or metastasis of breast carcinoma. Some of these genes had ever been reported to be correlated with the pro-metastatic process of TNF-α on breast cancer, while most of them have not been studied in this particular context. We reviewed these genes in the Pubmed database and found confirmation that the majority of the up-regulated genes may play a role in promoting the metastatic phenotype of MCF-7 cells, since they were found to be overexpressed in invasive cancers and had been extensively studied in vitro and/or in vivo. On the other hand, high expressions of several genes may inhibit breast cancer metastasis, including EPHB2, mCAM, pTEN, RB1, SMAD2, and SSTR2. Four of the five genes down-regulated in our study have been shown to be metastasis promoting agents and down-expression of the mRNA of these genes may play a negative role in promoting the metastatic phenotype of MCF-7 cells. The down-expression of KISS1 gene may contribute to metastasis. However, this kind of functional classification of gene expression may be problematic because the role of one gene in metastasis is sophisticated and is regulated by many factors. Furthermore, cancers exhibit great heterogenicity and may differ from cell to cell. For example, it has been suggested that melanoma cell adhesion molecule (MCAM) plays an important role in tumor progression, implantation and placentation. MCAM expression can promote tumor progression in human melanoma[17,18], in contrast, it may act as a tumor suppressor in breast carcinoma. In summary, TNF-α altered the expression of many gene which might play opposite roles in the process of metastasis:promotion and depression, although the former seems to be predominant.

The expression of four matrix metalloproteinases (MMPs) genes was significantly increased in this study following TNFα. Remarkably, the MMP-9 and MMP-13 gene expressions were up-regulated by 110.9-fold and 26.8-fold, respectively, in our data. MMPs are the most important enzymes responsible for the degradation of ECM components, which is critical for tumor growth and migration. TNF-α plays a critical role in tumor progression, partly due to stimulating the production of matrix metalloproteinases[19–21]. In their recent experiments, Seeger and colleagues showed that TNF-α alone induced an increase of about 30% of MMP-9 concentrations in the supernatant of MCF-7 cells[22]. Co-cultivation of the human breast cancer cell lines MCF-7 and SK-BR-3 with macrophages leads to enhanced invasiveness of the malignant cells due to TNF-α dependent MMP(MMPs -2, -3, -7 and -9) induction in the macrophages[23]. The strikingly high levels of MMPs gene expression seen in our study following TNF-α are consistent with previous studies and may indi-cate the central role of MMPs in the pro-metastasic effect of TNF-α.

Adhesion molecules play an important role in tumor-environment interactions and the process of metastasis. CD44 is a multifunctional membrane receptor involved in cell adhesion, motility, and metastasis[24]. CD44s was up-regulated in both in situ and invasive breast ductal carcinoma, colocalizing with its ligand, hyaluronan, in the same cells[25]. Another group reported that the presence of CD44v3 significantly correlated with tumor infiltration by T lymphocytes and tumor metastases to draining lymph nodes, with loss of expression of TP53[26]. With a murine metastasis model transplanted with MCF-7 cells, Joshua et al[27] found that CD44 was sparsely expressed in primary tumor cells but homogeneously overexpressed in cells transiting the lymphatics and populating lymph nodes, suggesting that CD44 was involved in the metastasis of MCF-7 cells. TNF-α affects T cell migration by regulating CD44 expression through the activation of MAPK pathway, and alters the invasiveness by regulating the expression of CD44 in several kinds of ovarian cancer cells through JNK activation. Our results showed that CD44 gene expression in MCF-7 cells was up-regulated by TNF-α. Taken together with other studies, this suggests that the overexpression of CD44 may play a role in the metastasis promoted by TNF-α in MCF-7 cells.

In order to know which of these genes were already published as TNF-α related, Pubmed queries were made to test for association with TNF-α. The majority of gene expression changes have already been reported related to TNF-α, while several genes(CTSO, GNRH1, PNN, SET, SSTR2, FGFR4) have not. Moreover, some of them have been demonstrated to be tightly correlated with the metastasis and prognosis of breast cancer. For example, FGFR4 is a member of FGFR family which is critically involved in cell growth, differentiation, migration and tumorigenesis[28]. EGFR4 is highly expressed in mammary carcinomas as well as some other cancer types[29-31]. FGFR4 Arg388 is a natural occurring polymorphism of FGFR4. Study showed that

FGFR4 Arg388 genotype was significantly prevalent in node positive breast cancer patients and correlated with early recurrence and reduced DFS, suggesting a role of FGFR4 in metastasis. In a word, these breast cancer related genes have been reported to be regulated by TNF-α and they deserve further study to clarify their linkage.

These changed genes also showed a time dependence in their expression pattern. Expression of six genes was significantly changed with TNF-α at 3 h and lowered at 24 h. Biochemical function analysis showed that these genes were predominantly transcription related genes and they may be early response genes to TNF-α. In addition, 16 gene expressions were increased only at 24 h, and these genes encode proteins mostly consisting of adhesion molecules(eg.CD44, FAN, FN1), matrix metalloproteinases(eg.MMP3, MMP7, MMP13), cytokines(eg. CCL7, CXCL12), and receptors(eg. EPHB2, MET, PLAUR, SSTR2, VEGF). Furthermore, some genes were constitutively up-regulated throughout the experiment.. This time course pattern of the changed genes may indicate that an orderly series of biochemical changes took place following the treatment with TNF-α and consequently lead to the final pro-metastasis effect. In addition, some important gene changes may have been missed due to the limited number of time points that were tested.

In summary, we identified 36 genes that were regulated by TNF-α with signal intensity changes ≥2-fold relative to control cells. These changed genes are involved in many biochemical processes and may take part in every step of metastasis. Function analysis showed that most of the changed genes may facilitate the metastasis of MCF-7 cells while a few of them may inhibit. Some of them have never been reported to be regulated by TNF-α according to Pubmed database searches. The gene expression changes seen with TNF-α were somewhat time dependent. These findings will contribute to further understanding the mechanisms by which TNF-α promotes breast cancer metastasis and further study on some of these genes is warranted.

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