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Cytokine production in mammary adenocarcinoma and its microenvironmental cells in patients with or without metastases in regional lymph nodes

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Abstract

In recent years, the concept of formation of a sufficiently autonomous cytokine network in a malignant tumour has emerged. In this regard, the data on the role of this network and its signalling pathways in the process of metastasis are an interesting topic. The aim of this study was to evaluate the in vitro cytokine-producing potential of mammary adenocarcinoma (MAC; and cells of its microenvironment) from patients with or without metastases in regional lymph nodes (LNs). By enzyme-linked immunosorbent assays of culture supernatants, we studied the cytokine production by biopsy samples of MAC: spontaneous and stimulated by polyclonal activators (PAs: phytohaemagglutinin, concanavalin A and lipopolysaccharide). The levels of spontaneous production of interleukin (IL)-10 and granulocyte colony-stimulating factor (G-CSF) and the amounts of IL-2, IL-10, G-CSF and monocyte chemoattractant protein-1 (MCP-1) produced during stimulation by PAs, as well as the index of stimulation by polyclonal activators (ISPA) for IL-2 production, were lower for MAC with LN metastasis than for MAC without LN metastasis. The levels of spontaneous production of IL-2 and interferon (IFN)-γ and the ISPA for granulocyte–macrophage colony-stimulating factor (GM-CSF) production were higher for MAC with LN metastasis. There were only three pairwise correlations between the produced cytokines that were specific to MAC with LN metastasis: IL-2 and IFN-γ, IL-6 and GM-CSF, and IL-8 and GM-CSF. There were 10 pairwise correlations between the produced cytokines that were specific to nonmetastasising MAC: IL-6 and IL-10, IL-6 and MCP-1, IL-8 and IL-10, IL-8 and MCP-1, IL-10 and G-CSF, IL-10 and MCP-1, IFN-γ and MCP-1, MCP-1 and G-CSF, G-CSF and IL-1Ra, and GM-CSF and tumour necrosis factor (TNF)-α. Our data indicate that metastatic tumours show desynchronisation of many pathways of induction and synthesis of cytokines that are characteristic of nonmetastatic tumours.

Keywords

cytokine, cytokine-producing potential, mammary adenocarcinoma, metastasis into regional lymph nodes, polyclonal activators

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Introduction

According to the modern understanding of tumour biology, cytokines can be secreted both by a tumour and by cells of its microenvironment, thus having autocrine and paracrine effects.[1–4] It is known, for example, that cytokines can not only inhibit (interferon (IFN)-γ, tumour necrosis factor (TNF)-α) but also enhance (interleukin (IL)-1β, IL-6, IL-8, transforming growth factor (TGF)-β, monocyte chemoattractant protein-1 (MCP-1), colony-stimulating factor (CSF)-1 and vascular endothelial growth factor (VEGF)) the growth and metastasis of a malignant tumour including breast cancer.[1,2,5–7] There are correlations between the concentration of certain cytokines in blood (IFN-γ, IL-10 and VEGF) and the degree of tumour vascularisation, tumour cell proliferation, tumour invasiveness and metastasis.[6,8,9] Nonetheless, there is little data on the extent to which the growth and metastasis of malignant tumours depends on the cytokines produced by immune system cells, how effectively these cytokines enter the tumour through its vascular network and to which degree endogenous (i.e. intratumoral) cytokine production is not sufficient for tumour growth. The evidence accumulated to date supports the notion that an autonomous cytokine network may be produced inside a tumour[4,10] and that this network is one of the multi-component regulators of tumour progression and metastasis. Nevertheless, the mechanisms underlying the functioning of this network are poorly studied. This statement is especially true for breast cancer, in particular, it is not known how a change in the cytokine profile and the cytokine-producing potential of breast cancer cells or cells of its microenvironment are associated with various stages of tumour progression and metastasis.

We hypothesised that the cytokine profile of a mammary adenocarcinoma (MAC) that metastasised may be different from that of a MAC without metastases because most of the processes regulating tumour growth and invasion are regulated by cytokines.

The aim of this study was to evaluate the cytokine profiles and the cytokine-producing potential of MAC and of cells in its microenvironment among patients with or without metastases in regional lymph nodes (LNs).

Materials and methods

Patients

The research materials included tumour biopsy samples from 40 women aged 40–67 years with invasive ductal carcinoma that was classified as grade II to III adenocarcinoma. The exclusion criteria from the study were signs of haematogenous metastasis to distant organs and the presence of concomitant hormonal, chronic, inflammatory and infectious diseases. The patients were subdivided into two groups. The first group included 25 patients without detectable metastases in regional LNs at the time of the biopsy procedure (mean age of the patients in this group was 58.0 years). The second group included 15 patients with metastases in regional LNs (mean age of the patients in the group was 56.3 years). All the studies were conducted in accordance with the Helsinki Declaration (Brazil, Fortaleza, 2013).[11] All recommendations of the International Committee of Medical Journal Editors (ICMJE) were taken into account. Each patient was informed about the study being conducted and its objectives and methods. Written informed consent for participation in the study and for the tumour biopsy procedure was signed by each patient and verified by a physician. The study protocol was approved by the Ethics Committee of the Institute of Molecular Biology and Biophysics.

Measurement of cytokine production

The cytokine-producing activity of a tumour, its microenvironment profile and cytokine-producing potential were assessed using the CYTOKINE-STIMUL-BEST standardised assay kit (Vector-Best Inc., Novosibirsk Region). Tumour biopsy samples (8 mm^3) obtained by trepanobiopsy[9,12] were placed into two glass vials containing 1 mL of a liquid each; one vial contained only the DMEM/F12 medium (analysis of spontaneous production of cytokines), while the second vial contained a mixture of polyclonal activators (PAs; consisting of 4 μg/mL phytohaemagglutinin, 4 μg/mL concanavalin A and 2 μg/mL lipopolysaccharide) in an equivalent amount of the medium (analysis of PA-stimulated cytokine production). The incubation of tumour biopsy samples lasted for

Table 1. Levels of spontaneous cytokine production in the culture supernatant of biopsy samples of mammary adenocarcinoma from patients with and without metastases to regional lymph nodes.

Cytokine	Groups of patients with mammary adenocarcinoma		Statistical significance, *P*
	Without metastases	With metastases	
	Cytokine concentration in supernatant (spontaneous production, pg/mL), Me (25; 75)		
G-CSF	2714.00 (637.20; 2947.50)	649.00 (606.70; 2140.00)	0.0462
GM-CSF	41.80 (22.00; 73.70)	33.70 (12.70; 96.60)	0.4502
IFN-γ	8.80 (6.20; 13.65)	16.75 (9.30; 39.90)	0.0328
IL-1b	49.60 (27.60; 92.00)	22.80 (15.40; 86.00)	0.3042
IL-1Ra	9110.00 (5260.00; 21,375.00)	9600.00 (1659.10; 15,785.00)	0.3929
IL-2	2.00 (2.00; 2.05)	5.05 (3.00; 10.20)	0.0449
IL-6	46,100.00 (33,340.00; 69,340.00)	52,440.00 (35,480.00; 63,980.00)	0.9113
IL-8	25,000.00 (15,420.00; 37,520.00)	24,800.00 (12,560.00; 45,460.00)	0.8140
IL-10	14.90 (11.00; 24.90)	7.05 (2.80; 15.60)	0.0303
IL-17	2.00 (2.00; 5.10)	3.10 (2.00; 5.20)	0.3661
IL-18	108.70 (53.50; 213.50)	84.70 (31.90; 251.30)	0.4069
MCP-1	5220.80 (1915.00; 10,516.00)	2245.00 (1370.10; 6471.40)	0.1545
TNF-α	12.40 (8.40; 23.30)	11.50 (7.70; 18.10)	0.4210
VEGF-A	2362.40 (1843.60; 3384.60)	3686.80 (2175.00; 4402.20)	0.1694

G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte–macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TNF: tumour necrosis factor; VEGF-A: vascular endothelial growth factor A.

72 h. To obtain a culture supernatant, each tumour biopsy sample was taken out of the vial, and the remaining cells were pelleted by centrifugation at 900*g* for 15 min. Using enzyme-linked immunosorbent assays, the concentrations of IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, TNF-α, IFN-γ, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF) and VEGF-A in the supernatant were determined. The index of stimulation by polyclonal activators (ISPA) for production of cytokines by a tumour and its microenvironment was calculated using the following formula: ISPA = A/B, where A is a cytokine concentration after stimulation of the tumour by PAs and B denotes the cytokine concentration in the supernatant without stimulation (spontaneous production). High ISPA can be interpreted as a substantial cytokine-producing reserve, whereas low values indicate limited capacity for cytokine production.

Statistical analysis

Statistical processing of the data was performed by the Mann–Whitney U test. The results were expressed as a median (Me) and the higher and lower quartile (25; 75). Correlations between the values of interest were determined using Spearman's rank correlation (r), taking into account its statistical significance (*P*).

Results

Cytokine production by cultured biopsy samples of MAC

There were significant differences in IL-2, IL-10, IFN-γ and G-CSF concentrations (spontaneous production) in the MAC culture supernatant between the groups of patients with and without regional LN metastasis (Table 1). Besides, there were differences between these groups in IL-2, IL-10, MCP-1 and G-CSF concentrations in the culture supernatants of the tumour biopsy samples after incubation with PAs (Table 2), as well as differences in the ISPA for IL-2 and GM-CSF production (Figure 1). In the group of patients with regional LN metastasis, the values of spontaneous production of IL-10 and G-CSF; the levels of IL-2, IL-10, G-CSF and MCP-1 production stimulated by PA; and the ISPAs for the production of IL-2 were lower than those in the group

Table 2. Cytokine production stimulated by PAs, in biopsy samples of mammary adenocarcinoma from patients with and without metastases in regional lymph nodes.

Cytokine	Groups of patients with mammary adenocarcinoma		Statistical significance, *P*
	Without metastases	With metastases	
	Cytokine concentration in supernatant after stimulation by PAs (pg/mL), Me (25; 75)		
G-CSF	2836.00 (2645.00; 2985.00)	1352.00 (1262.80; 2876.00)	0.0284
GM-CSF	125.60 (79.20; 207.00)	133.30 (33.80; 457.70)	0.9901
IFN-γ	21.70 (8.50; 45.50)	17.50 (8.90; 64.50)	0.6919
IL-1b	590.00 (265.00; 855.00)	575.00 (485.00; 855.00)	0.5197
IL-1Ra	16,810.00 (10,755.00; 27,150.00)	16,779.70 (10,853.40; 40,690.50)	0.7196
IL-2	7.40 (4.80; 16.40)	3.95 (2.00; 6.90)	0.0462
IL-6	116,350.00 (66,750.00; 165,000.00)	87,950.00 (17,650.00; 141,250.0)	0.2396
IL-8	42,750.0 (25,500.00; 65,300.0)	46,900.00 (7850.00; 75,850.0)	0.9309
IL-10	23.90 (10.20; 87.00)	9.10 (1.10; 28.40)	0.0259
IL-17	9.40 (3.10; 23.30)	4.00 (2.00; 7.60)	0.0519
IL-18	183.10 (99.20; 436.80)	234.20 (78.30; 412.60)	0.9901
MCP-1	6640.00 (2.386.40; 4.439.00)	1896.00 (1.053.00; 646.00)	0.0062
TNF-α	46.90 (20.00; 81.40)	22.00 (11.50; 98.30)	0.5859
VEGF-A	1789.0 (1181.00; 2715.20)	1664.4 (714.00; 3392.00)	0.6031

G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte–macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TNF: tumour necrosis factor; VEGF-A: vascular endothelial growth factor A; PAs: polyclonal activators.

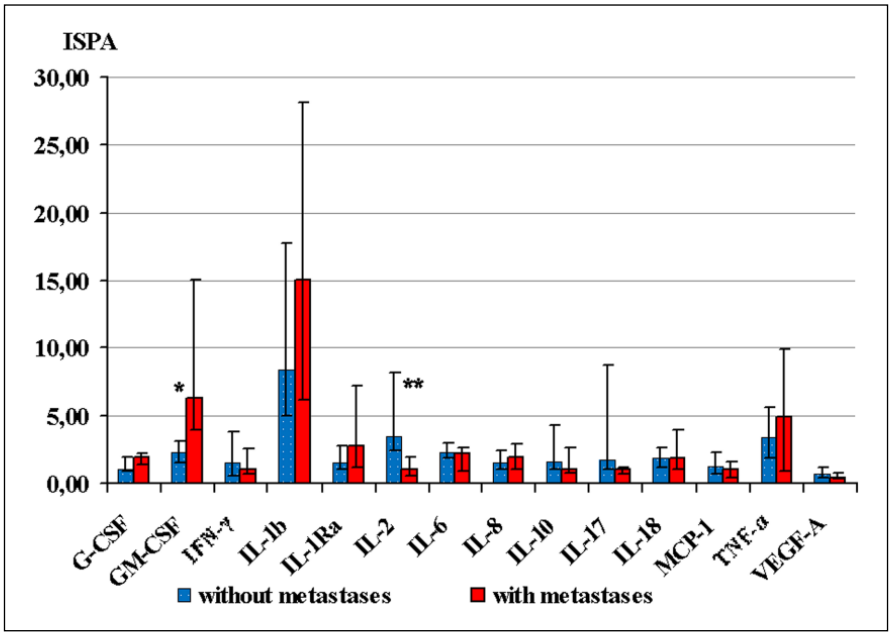

Figure 1. The index of stimulation by polyclonal activators (ISPA) for production of cytokines by a tumour and its microenvironment according to the analysis of culture supernatants of biopsy samples of mammary adenocarcinoma from patients with and without metastases in regional lymph nodes. The results are expressed as medians and the higher and lower quartile (25; 75). *$P < 0.05$, **$P < 0.01$. Significance of differences between two medians was evaluated by the Mann–Whitney U test.

of patients without regional LN metastasis. In the group with regional LN metastasis, the levels of spontaneous production of IL-2 and IFN-γ (Table 1) and ISPA for GM-CSF production were higher (Figure 1).

Correlations between cytokine production levels in biopsy samples of MAC

The correlations between the cytokine production in MAC samples from patients without LN

Table 3. Pairwise correlations between the cytokine production levels in a culture supernatant of biopsy samples of mammary adenocarcinoma from patients without and with metastases in regional lymph nodes.

Cytokine concentration in supernatant (spontaneous production, pg/mL)		Correlation coefficient, r (*P*)
In patients without metastases		
IL-2	IL-17	0.40 (0.0455)
IL-6	IL-8	0.71 (0.0001)
IL-6	IL-10	0.51 (0.0088)
IL-6	MCP-1	0.66 (0.0086)
IL-8	IL-10	0.43 (0.0339)
IL-8	MCP-1	0.77 (0.0001)
IL-10	G-CSF	0.61 (0.0012)
IL-10	MCP-1	0.61 (0.0011)
IL-17	IFN-γ	0.50 (0.0104)
IFN-γ	MCP-1	−0.43 (0.0312)
MCP-1	G-CSF	0.59 (0.0030)
G-CSF	IL-1Ra	0.42 (0.0371)
GM-CSF	TNF-α	0.53 (0.0062)
In patients with metastases		
IL-2	IL-17	0.71 (0.0031)
IL-2	IFN-γ	0.74 (0.0016)
IL-6	IL-8	0.75 (0.0014)
IL-6	GM-CSF	0.68 (0.0058)
IL-8	GM-CSF	0.66 (0.0078)
IL-17	IFN-γ	0.64 (0.0102)

G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte–macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TNF: tumour necrosis factor.

metastasis and the cytokine production in MAC samples from patients with LN metastasis were studied next (Table 3). As shown in the table, there were three pairwise correlations between cytokine production levels that were characteristic of both groups of patients: IL-2 and IL-17, IL-6 and IL-8, and IL-17 and IFN-γ. In the assays of MAC without LN metastasis, 10 pairwise correlations between cytokine production levels, which are specific to nonmetastasising MAC only, were revealed at the time of the study: IL-6 and IL-10, IL-6 and MCP-1, IL-8 and IL-10, IL-8 and MCP-1, IL-10 and G-CSF, IL-10 and MCP-1, IFN-γ and MCP-1, MCP-1 and G-CSF, G-CSF and IL-1Ra, and GM-CSF and TNF-α. Besides, there were only three pairwise correlations between cytokine production levels that were specific to MAC with LN metastasis only at the time of study: IL-2 and IFN-γ, IL-6 and GM-CSF, and IL-8 and GM-CSF.

Discussion

In a number of papers, it has been shown that while the endogenous (tumour-produced) IL-2 stimulates proliferation of tumour cells and their invasiveness, exogenous (host's) IL-2 can inhibit tumour growth.[13–15] Our data indicate that IL-2 produced by tumour cells and the cells of its microenvironment at a relatively high concentration can stimulate the process of metastasis into regional LNs.

Numerous studies show that IFN-γ has a high antimetastatic activity in the treatment of various tumours.[16,17] On the other hand, IFN-γ-producing capacity of the cells in the tumour microenvironment can increase with metastatic spread to LNs.[18] The results of our study suggest that relatively high production of IFN-γ in the tumour (obviously, T-lymphocytes infiltrating the tumour) may create conditions that contribute to the metastasis to regional LNs.

Just as some other cytokines, IL-10 can exert a dual proliferative and inhibitory effect on breast tumour cells; this observation points to a complex role of IL-10 in breast cancer initiation and progression.[3,19] In our study, the concentration of IL-10 was lower in the culture supernatant of MAC from patients with LN metastasis as compared to patients without LN metastases. These data indicate that IL-10 can play the role of a suppressor in the cytokine-dependent mechanism underlying metastasis to LNs.

We observed 10 pairwise correlations between cytokine production levels that are specific to non-metastasising MAC only. There were only three pairwise correlations between cytokine production levels that were specific to MAC with LN metastasis: IL-2 and IFN-γ, IL-6 and GM-CSF, and IL-8 and GM-CSF. These data suggest that metastatic tumours show desynchronisation of many pathways of activation and synthesis of cytokines (IL-6 and IL-10, IL-6 and MCP-1, IL-8 and IL-10, IL-8 and MCP-1, IL-10 and G-CSF, IL-10 and MCP-1, IFN-γ and MCP-1, MCP-1 and G-CSF, G-CSF and IL-1Ra, and GM-CSF & TNF-α) that are characteristic of nonmetastatic tumours.

It was found that the ISPA was higher only for GM-CSF production (more than 2.5-fold) in the group of patients with LN metastases in comparison with the group of patients without LN metastases. This finding points to the involvement of this cytokine in the cytokine-dependent mechanisms underlying the regulation of metastasis to LN.

It can be assumed that in those tumours in which the desynchronisation of the production of many cytokines takes place (and, conversely, synchronisation of molecular and cellular processes that simultaneously increase the production of IL-2, IFN-γ, IL-6, IL-8 and GM-CSF), conditions are created that promote tumour metastasis. Additional studies are needed to clarify the specific mechanisms behind the 'segment' of the cytokine regulation of metastasis that we identified here.

Declaration of conflicting interests

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