



TNF- α , IL-6 and Their Soluble Receptor Serum Levels and Secretion by Neutrophils in Cancer Patients

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Abstract. Simultaneous evaluation of cytokines and their soluble receptor production and the serum levels can be helpful in understanding the local and systemic immune response of a tumor-bearing host. In the present study we examined serum levels of TNF- α , IL-6 and their soluble receptors: sTNFRp55, sTNFRp75 and sIL-6R confronted with their production by the polymorphonuclear neutrophils (PMN) from cancer patients. Examinations were carried out in patients with adenocarcinoma breast cancer and squamous cell carcinoma of the oral cavity and related to the clinical course and to different phases of therapy. Secretion of IL-6, sTNFRp55 and sTNFRp75 by PMN appeared to be dependent on tumor type, clinical progression of disease as well as on therapy, suggesting a significant role of these cells at different phases of the immune response to cancer associated with these mediators. Changes in values of TNF- α , IL-6 and their soluble receptors in sera of both cancer groups, dependent on tumor type, clinical progression and cancer therapy, could have a diagnostic and prognostic role in cancer disease.

Key words: tumor necrosis factor α ; interleukin 6; soluble IL-6 receptor; soluble TNF receptor; breast cancer; oral cavity cancer.

Introduction

Numerous experimental studies have underlined the key importance of cytokines for an effective anticancer immune response^{1, 6, 23}. IL-6 and TNF- α , strong pro-inflammatory mediators, play a crucial role in the regulation of both the humoral and cellular antitumor defense systems^{10, 15}. They exert a variety of functions, involving differentiation and proliferation of B and T cells, induction of NK and cytotoxic T cells and

stimulation of macrophages and neutrophil activity^{2, 10, 15}. TNF- α and IL-6 are also capable of cross-regulating one another: TNF- α induces IL-6 production by macrophages and other cell types and, conversely, IL-6 can inhibit TNF- α secretion by mononuclear cells^{2, 17}. Thus, IL-6 can also play an anti-inflammatory role by controlling the level of other pro-inflammatory cytokines. Moreover, it has been well documented that these cytokines may have the ability to inhibit the growth of different cancer cell lines directly^{1, 10, 15}.

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On the other hand, it has also been shown that IL-6 and TNF- α are involved in the pathogenesis of a variety of malignant diseases. IL-6 has been identified as a growth factor for human myeloma, plasmacytoma, leukemia, lymphoma and Kaposi's sarcoma^{15, 21}. TNF- α has been implicated in the pathogenesis of multiple myeloma⁷.

The functional reactivity of TNF- α and IL-6 is regulated by synthesis, membrane-bound receptors expression and the presence of naturally occurring factors, such as soluble cytokine receptors^{5, 9, 15}.

Soluble receptors exert their biological effects through mechanisms of action that are defined by their relationship to ligand and to membrane-bound receptors. The soluble TNF receptors sTNFRp55 (120a) and sTNFRp75 (120b) at high concentrations compete with the surface receptors for TNF and prevent TNF activity. Since sTNFRs retain their affinity for TNF but do not induce signal transduction, they may be regarded as natural inhibitors of TNF. In contrast, sTNFRs at lower concentration can increase TNF activity by stabilizing its trimeric structure and preventing its dissociation into inactive monomers^{3, 9}.

The soluble IL-6 receptor has been reported to act agonistically by increasing the sensitivity of IL-6 – responsive cells and by transmitting IL-6 responsiveness to cells that lack the α -chain (gp80) but express the β -chain (gp130)^{5, 15}.

The main source of TNF- α , IL-6 and their soluble receptors in the peripheral blood are mononuclear cells, involving lymphocytes and monocytes^{3, 10, 15}. Much less is known about the role of neutrophils in the release of these mediators. Since polymorphonuclear neutrophils (PMN) quantitatively dominate in the peripheral blood, dysregulation of mediator production by these cells can lead to various effects in the cellular- and antibody-mediated reactions of a host to a tumor.

Our previous examinations demonstrated changes in the release of TNF- α , IL-6 and their soluble receptors by PMN from breast and oral cavity cancer patients *in vitro*^{11–13}. The results obtained revealed that cells from both cancer groups exerted different capacities for the release of TNF- α and sTNFRs¹³. In other studies we showed that sIL-6R and sTNFRp55 and sTNFRp75 concentrations in the culture supernatants of PMN from breast cancer were dependent on the phase of cancer therapy¹¹. Subsequent examinations revealed that the PMN from oral cavity cancer patients released different amount of sTNFRp55 and sTNFRp75 with regard to the stage of disease progression¹².

In the present study we compared the TNF- α , IL-6, their soluble receptor serum levels and secretion by

PMN of breast cancer and oral cavity cancer patients. To investigate whether the examined mediators might have a prognostic importance in both cancer groups, their values were related to the clinical course of patients and to different phases of therapy.

The simultaneous evaluation of cytokines and their regulatory soluble receptor production by PMN and the serum levels can be helpful in understanding the local and systemic immune responses of a tumor-bearing host.

Materials and Methods

Patients. We examined 23 patients (16 males and 4 females) with squamous cell carcinoma of the oral cavity aged between 44 and 72 years (mean 58) and 23 patients (females) with adenocarcinoma breast cancer aged between 38 and 62 years (mean 50). No infection or fever was present in any of the patients during examination. Blood samples were taken from each patient at presentation before surgery. They had not received chemotherapy before admission to the study. All patients did not have increased leukocytosis or other signs of inflammation.

The control subjects (n=12) were normal healthy persons whose ages ranged from 32 to 53 years (mean 42.5 year).

Blood samples. Blood samples were taken from each patient at presentation, during and after cancer therapy, which involved surgery and adjuvant chemotherapy (CMF – cisplatin, metotrexate and fluorouracil) in breast cancer patients. In contrast, therapy in the oral cavity cancer patients first involved adjuvant chemotherapy (cisplatin and fluorouracil) and later surgery.

Sera. Sera obtained from venous blood samples were stored frozen (from –20 to –70°C) until tested.

PMN preparation. Cells were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient (1.115 g/ml)²⁴. This method enables simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMC), containing 95% lymphocytes, and PMNs, containing 94% PMNs. The purity of the isolated PMNs was determined by May-Grunewald-Giemsa-staining. Cells were washed three times with RPMI-1640 medium (RPMI-1640 Medium, Gibco) and were suspended in the culture medium to provide 5×10^6 cells/ml.

After culture, the viability of the PMN was >92% as determined by trypan blue exclusion.

Culture conditions. The culture medium consisted of RPMI-1640 medium supplemented with 10% fetal

calf serum (Pro Animal, Wrocław, Poland), 100 U/ml penicillin and 50 ng/ml streptomycin.

The PMN in medium were distributed into 96-well microtitre plates (Microtest III-FALCON). Cell cultures were performed in duplicate. The cultures were incubated at 37°C in humidified atmosphere of 5% CO₂ in the air. After 18 h of incubation supernatant was removed, diluted ten fold, and then assayed for TNF- α , IL-6, sTNFRs and sIL-6R.

Cytokine and receptor assays

TNF- α assay. TNF- α was measured with the Quantikine TM TNF- α Immunoassay from R&D Systems (Minneapolis, MN, USA), in duplicate. This assay employs the quantitative sandwich enzyme immunoassay technique and recognizes both natural human TNF- α and recombinant human TNF- α . Recombinant TNF- α was used as standard. The assay range is from 15.6 pg/ml to 1000 pg/ml TNF- α . No significant cross-reactivity of interference was observed with the other cytokines and rhTNF- β , rhTNFRI and rhTNFRII.

IL-6 assay. IL-6 was determined with the ENDOGEN human IL-6 ELISA (Cambridge, MA, USA), according to the manufacturer's instructions. Recombinant IL-6 was used as standard. The assay range is from 0 pg/ml to 400 pg/ml.

sIL-6R assay. Samples of culture supernatant and sera used to detect the presence of sIL-6R were tested with ELISA using a commercially available kit, the Quantikine Human IL-6 sR Immunoassay (R&D, Minneapolis, USA), according to the manufacturer's instructions. Human recombinant sIL-6R was used as a standard. The optical density of each sample was measured at 450 nm. The standard curve was used to determine the amount of sIL-6R in the samples. This method is specific for measurement of natural and human recombinant sIL-6R. This kit has an assay range of 31.3-2000 pg/ml of sIL-6R.

sTNFR p55 assay. The concentration of sTNFRp55 (sTNF RI) was detected with ELISA using the Quantikine Human sTNF RI Immunoassay from R&D System (Minneapolis, MN, USA), according to the instructions. Recombinant human sTNF RI was used as a standard. The standard curve was used to determine the amount of sTNF RI. The assay range is 7.8-500 pg/ml.

sTNFR p75 assay. The concentration of sTNFRp75 (sTNF RII) was determined with ELISA using the Quantikine Human sTNF RII immunoassay from R&D System (Minneapolis, MN, USA), according to the instructions. Recombinant sTNF RII was used as a standard. The range of this assay was 7.8-500 pg/ml.

Statistical analysis. The results are expressed as mean $\pm$ standard deviation. Data were analyzed accord-

ing to variance and Student's *t*-test. Correlation's were calculated using Pearson's test. A *p*-value less than 0.01 was considered to represent a statistically significant difference.

Results

Serum levels of TNF- α and IL-6 in breast cancer patients in stage II were significantly higher than in the control (*p*<0.05). The values of sTNFRp55, sTNFRp75 and sIL-6R in this patient group were unchanged (Table 1). Breast cancer patients in stage III/IV had increased serum levels of TNF- α , IL-6, sTNFRp55, sTNFRp75 compared with stage II (Table 1).

In the serum of oral cavity cancer patients at different stages, elevated concentrations of TNF- α , IL-6, sTNFRp55 and sTNFRp75 compared with the control were observed. The mean concentrations of TNF- α , IL-6 and sTNFRp55 in patients in stage III/IV were significantly higher than in patients in stage II (Table 1).

The values of all mediators were compared in both cancer groups. Concentrations of IL-6, sTNFRp55, and sTNFRp75 in the oral cavity cancer patients in stages II and III/IV were significantly higher than those in the breast cancer patients at the same stages (*p*<0.05).

We have found a positive correlation between the serum concentrations of sTNFRp55 and sTNFRp75 of both cancer groups (*r*=0.91, *p*<0.001 in breast cancer and *r*=0.58, *p*<0.005 in oral cavity cancer).

To investigate the PMN contribution to the release of TNF- α , IL-6, sTNFRp55, sTNFRp75 and sIL-6R into the circulation, we analyzed these mediators in the sera as well as in the culture supernatants of these cells. The mean concentration of sTNFRp75 in the culture supernatants of PMN from breast cancer patients with stage II was statistically higher than in the control. The mean sTNFRp55, sTNFRp75 and IL-6 production by PMN from breast cancer patients in stage III/IV were significantly higher than in stage II and the control (*p*<0.05) (Table 2).

In contrast, in oral cavity cancer in stage II we found increased concentrations of TNF- α , sTNFRp55 and sTNFRp75 in the culture supernatants of PMN as compared with the control. TNF- α and IL-6 release by PMN from these patients in stage III/IV was higher than in stage II (Table 2).

Comparison of the values of all mediators in the culture supernatants of both cancer groups revealed that the concentration of TNF- α in oral cavity cancer in stage II was significantly higher than in breast cancer in stage II. In contrast, the mean levels of sTNFRp75

Table 1. Concentrations of serum parameters examined according to TNM classification

Parameter	Control		Breast cancer				Oral cavity cancer			
			II stage n=9		III, IV stage n=11		II stage n=7		III, IV stage n=13	
	x	SD	x	SD	x	SD	x	SD	x	SD
TNF- α pg/ml	13.1	8.9	21.6*	14.5	37.1** ^a	16.3	29.9*	10.5	53.9** ^a	23.7
sTNFRp55 ng/ml	0.64	0.18	0.59	0.33	1.03** ^a	0.33	3.54** ^b	1.88	6.75** ^{ab}	3.78
sTNFRp75 ng/ml	1.93	0.52	1.61	1.47	3.37** ^a	1.41	6.22** ^b	3.21	8.17** ^b	5.16
IL-6 pg/ml	9.81	3.96	16.7	8.7	29.4** ^a	12.9	53.5** ^b	29.3	92.5** ^a	61.4
sIL-6R ng/ml	41.2	21.75	42.3	26.67	77.7*	35.3	59.7	26.5	76.3*	38.4

* Statistical differences with control ($p < 0.05$).^a Statistical differences between patients in II and III, IV stages ($p < 0.05$).^b Statistical differences between breast and oral cavity cancer ($p < 0.05$).**Table 2.** Concentrations of mediators examined in the culture supernatants of PMN from control and cancer patients

Parameter	Control		Breast cancer				Oral cavity cancer			
			II stage n=9		III, IV stage n=11		II stage n=7		III, IV stage n=13	
	x	SD	x	SD	x	SD	x	SD	x	SD
TNF- α pg/ml	382	214	368	201	227	134	670** ^b	443	1296** ^a	588
sTNFRp55 ng/ml	271	113	396	186	1043** ^a	589	475	267	324	174
sTNFRp75 ng/ml	565	266	1476*	1077	2545** ^a	1355	1254*	689	890** ^b	495
IL-6 pg/ml	1403	747	1587	1130	2991** ^a	1321	1485	854	2477** ^a	1466
sIL-6R ng/ml	311	153	181*	114	356*	163	298	187	351	158

* Statistical differences with control ($p < 0.05$).^a Statistical differences between II and III/IV stage ($p < 0.05$).^b Statistical differences between breast cancer and oral cavity cancer ($p < 0.05$).**Table 3.** Concentrations of all mediators in the serum of breast cancer patients depending on phase of therapy

Parameter	Control		Patients before treatment		Patients after surgery		Patients after chemotherapy	
	x	SD	x	SD	x	SD	x	SD
TNF- α pg/ml	13.1	8.9	23.1*	13.7	21.9*	14.5	9.2** ^a	6.34
sTNFRp55 ng/ml	0.69	0.13	0.76	0.23	0.92	0.36	0.68	0.39
sTNFRp75 ng/ml	1.96	0.35	2.46	0.59	2.82	1.46	2.1	0.77
IL-6 pg/ml	9.81	3.96	23.7*	17.0	33.6*	17.8	18.5** ^a	14.3
sIL-6R ng/ml	39.05	19.8	59.7*	28.7	61.0*	31.2	26.8** ^a	12.4

* Statistical differences with control ($p < 0.05$).^a Statistical differences between values before and after treatment ($p < 0.05$).**Table 4.** Concentrations of all mediators in the serum of oral cavity cancer patients at various phases of therapy

Parameter	Control		Patients before treatment		Patients after chemotherapy		Patients after surgery	
	x	SD	x	SD	x	SD	x	SD
TNF- α pg/ml	13.1	8.9	45.8*	37.01	32.6*	21.4	41.3*	26.8
sTNFRp55 ng/ml	0.69	0.13	5.54*	2.61	5.57*	2.64	6.47*	3.08
sTNFRp75 ng/ml	1.96	0.35	7.41*	5.23	6.73*	4.05	7.22*	4.17
IL-6 pg/ml	9.81	3.96	79.6*	42.1	51.3*	29.4	69.5*	37.2
sIL-6R ng/ml	39.05	19.8	67.8*	39.8	58.7	3.07	61.2	36.2

* Statistical differences with control ($p < 0.05$).

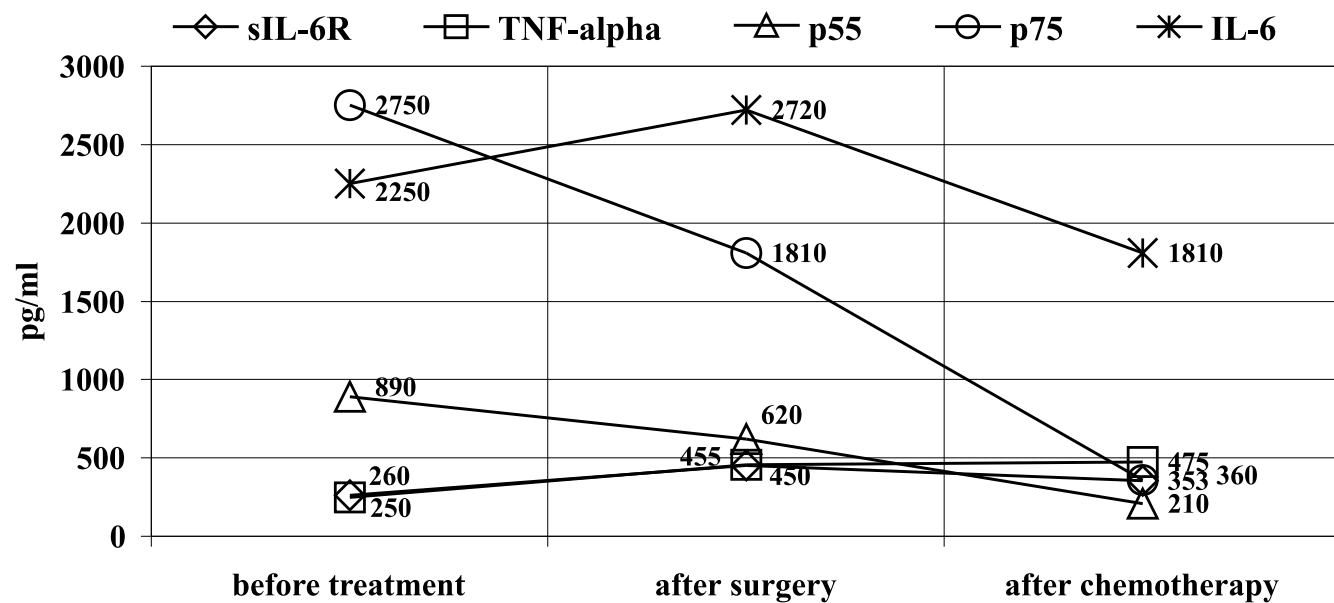


Fig. 1. Changes in release of examined mediators by PMN from breast cancer patients before and after treatment

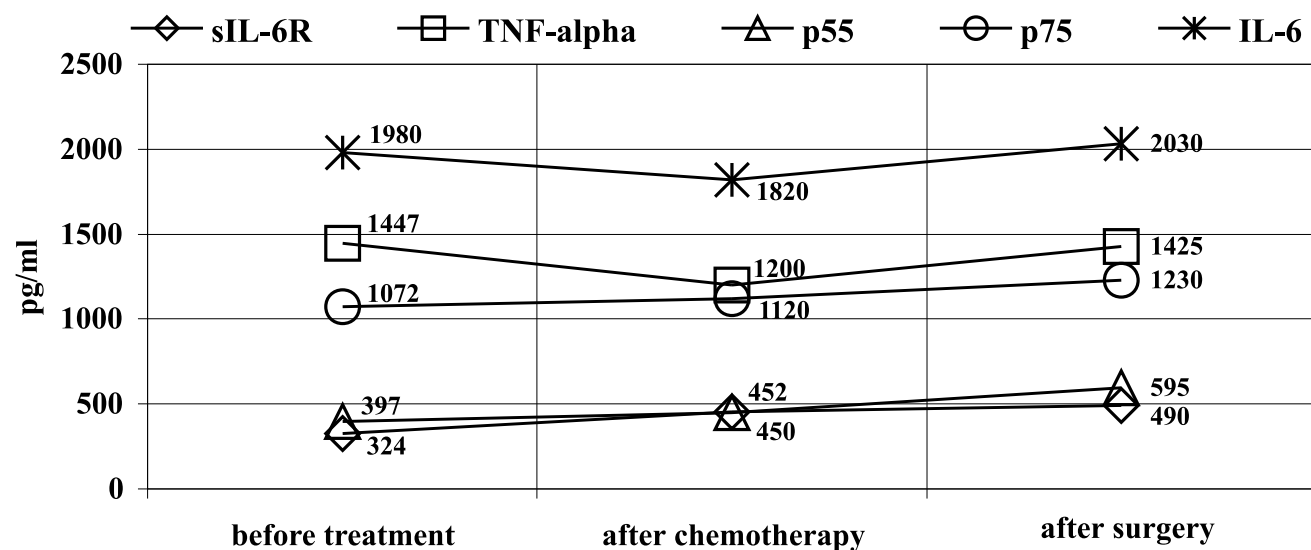


Fig. 2. Changes in release of examined mediators by PMN from oral cavity cancer patients before and after treatment

in the culture supernatants of PMN from oral cavity cancer in stage III/IV was significant lower than in breast cancer in stage III/IV.

The values of mediators examined in the culture supernatants and sera of both cancer patient groups were analyzed according to the different stages of therapy.

When the phases of treatment were considered, in breast cancer patients the changes in serum concentrations of TNF- α , IL-6, sTNFRp55, sTNFRp75 were not significant. However, the values of most mediators were higher than in the control. We have found decreased the serum concentrations of TNF- α and sIL-6R

after adjuvant CMF treatment in comparison with their determination before treatment (Table 3).

In contrast, in the culture supernatants of breast cancer PMN we observed changes in the concentrations of some mediators examined during therapy. The amounts of sTNFRp55 and sTNFRp75 after surgery were lower than those before treatment (Fig. 1). TNF- α , IL-6 and sIL-6R production after surgery was insignificantly higher than before treatment. Decreased concentrations of IL-6, sTNFRp55, sTNFRp75 and sIL-6R after adjuvants CMF therapy were seen as compared with data before treatment.

In oral cavity cancer patients no significant differences were seen in the sera and culture supernatants regarding the phase of therapy (Table 4, Fig. 2).

In the present study we have analyzed the relationship between the concentrations of TNF- α , IL-6, sTNFRp55, sTNFRp75 and sIL-6R in the sera and culture supernatants of PMN. A direct correlation was shown between the release of sIL-6R by PMN and serum levels in breast cancer before treatment ($r=59$, $p<0.05$). There were no correlations among the rest of the mediators examined.

Discussion

The present study demonstrated for the first time an interesting relationship between serum levels of pro-inflammatory and anti-inflammatory mediators in cancer patients.

In breast cancer patients at early stage of disease, high concentrations of pro-inflammatory TNF- α and IL-6 were associated with unchanged levels of anti-inflammatory sTNFRp55 and sTNFRp75. However, in patients with advanced stages, elevated expressions of TNF- α appear to be balanced by a simultaneous increase of sTNFRp55 and sTNFRp75 levels.

In contrast, in all oral cavity cancer patients increased serum TNF- α was associated with high concentrations of sTNFRp55 and sTNFRp75.

Interestingly, the relationships between sTNFRs and TNF- α changed in both cancer groups. The serum ratios of sTNFRp55 to TNF- α and sTNFRp75 to TNF- α (37.4 and 102.6, respectively) in breast cancer patients were lower than those in oral cavity cancer (78.6 and 164.6, respectively) and control (49.2 and 148.5 respectively).

Available data indicate that changes in the relationship between TNF- α and its soluble receptors may have different implications. The excess of circulating sTNFRs can block the TNF/TNFR modulation, which is necessary for the effective functioning of the respective cells and may reduce TNF- α activity in response to tumor^{3,9,16}. In consequence, this may lead to the lower cytotoxic capacity observed in cancer patients. Moreover, increased IL-6 concentrations, observed in the present study, can be responsible for TNF- α synthesis inhibition¹⁷. It was also reported that changes in the TNF and sTNFRs relationships can lead to increased IL-1, IL-6 and GM-CSF production as well as influence macrophage activity⁴.

On the other hand, the decreased TNF activity appears to be a mechanism preventing the recurrence of

cancer, related to an incomplete eradication of tumors. It has been postulated that pro-inflammatory cytokines such as TNF- α and IL-6 could exert a potential role in promoting the local recurrence of breast cancer as well as head and neck cancer^{14, 18}.

Additionally, since TNF- α and IL-6 are involved in the development of cancer cachexia, it is possible that the presence of high concentrations of sTNFRs may have favorable effects on the host.

The immune response in cancer patients is often associated with progression of disease^{11, 16, 22}. The highest concentrations of TNF- α , IL-6, sTNFRp55 and sTNFRp75 presented in this study were found in those breast cancer patients whose clinical stage of disease was advanced. In the oral cavity cancer group, similar of changes involved the values of TNF- α , IL-6 and sTNFRp75. The increased serum concentrations of the mediators examined with progression of disease are probably caused by release from activated immune cells and/or directly by tumor cells.

In the present study we also demonstrated changes between pro-inflammatory and anti-inflammatory mediators in the culture supernatants of PMN isolated from both cancer groups. In contrast to serum, sTNFRp55 to TNF- α and sTNFRp75 to TNF- α ratios in the culture supernatants of PMN from breast cancer patients (2.4 and 6.86, respectively) were higher compared with the oral cavity cancer (0.38 and 1.04, respectively) and control group (0.71 and 1.47, respectively).

There are various explanations of the possible mechanisms responsible for the differences in the sTNFRs to TNF- α relationships observed in both cancer groups. One of them may be a contribution of other cells in the secretion of these mediators into the circulation of cancer patients²². The other reason may be an increased expression of suppressing cytokines, involving IL-10 or IL-4, which influence TNF- α and sTNFRs expression¹⁴.

The alterations observed in the present study between pro-inflammatory and anti-inflammatory mediators may also be caused by their release from tumor cells. The behavior of some parameters during therapy appears to confirm this suggestion. However, in the subsequent treatment, involving surgery and chemotherapy, the serum values of all mediators were unchanged in breast cancer patients. In contrast, the amounts of IL-6, sTNFRp55 and sTNFRp75 in the culture supernatants of breast cancer patient PMN during therapy altered and after treatment returned to the levels of the control.

The decreased ability of PMN to release sTNFRp55 and sTNFRp75 after surgery may be caused by the

removal of the tumor mass and the absence of the effects of tumor cells on PMN activity. The altered PMN function after adjuvant chemotherapy may be the reason for the lower release of sTNFRs by PMN after terminal treatment. However, in oral cavity cancer there were no significant differences during the therapy used. Thus, the observations presented suggest more transient changes of the mediators examined in the culture supernatants of PMN from breast cancer patients as compared with oral cavity cancer.

In conclusion, secretion of IL-6, sTNFRp55 and sTNFRp75 by PMN appeared to be more characteristic of the tumor-host interaction in breast cancer patients than oral cavity cancer patients. In contrast, the serum levels of TNF- α , IL-6 and their soluble receptors seemed to be more specific for the oral cavity cancer group. This appears to confirm that some cellular- and antibody-mediated responses in squamous cell carcinoma are different from those in adenocarcinoma. However, the above results require further examination, including cancer and non-cancer diseases with the same location.

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