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Report

Tumour necrosis factor-α and interleukin-1 and -6 in fibrocystic breast disease

Alfonso Herruzo[1], Jose Antonio Castilla[2], Angel Ramírez[3], Cristina Marcos[4], and Salomón Menjón[1]
[1]*Department of Obstetrics and Gynaecology,* [2]*Clinical Analysis Service,* [3]*Nuclear Medicine Service,* [4]*Pathological Anatomy Service, "Virgen de las Nieves" University Hospital, Granada, Spain*

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Summary

The risk of developing breast cancer is higher in women presenting gross cystic disease (cysts > 3 mm in diameter) of the breast with intracystic K+/Na+ > 3 as compared with K+/Na+ < 3. The present study reports the levels of tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) in the breast cyst fluid of women with gross cystic disease and analyses the relationship between the intracystic concentration of these cytokines, sex steroid hormones, and the K+/Na+ ratio. The concentration of these cytokines, estradiol, testosterone, dehydroepiandrosterone sulfate (DHEA-S), and 17-OH-progesterone were determined in the breast cyst fluid of 54 women with gross cystic disease. No significant differences were found in the cystic levels of IL-1 between cysts with intracystic K+/Na+ < 3 and > 3. However, in cysts with intracystic K+/Na+ > 3 we found a lower concentration of IL-6 and TNF-α than in those with intracystic K+/Na+ < 3. Stepwise multiple linear regression analysis demonstrated that the concentration of IL-6 in breast cyst fluid was predicted statistically by a negative regression coefficient for the concentration of estradiol and DHEA-S, and by a positive regression coefficient for the concentration of TNF-α. The concentration of TNF-α in breast cyst fluid was predicted statistically by a positive regression coefficient for the concentration of IL-6, and by a negative regression coefficient for the concentration of estradiol. No candidate variable was included in the model to predict concentrations of IL-1 in breast cyst fluid. Our results indicate that IL-6 and TNF-α could have a local 'protector' role in gross cystic disease, and that they could be used as a marker to identify cyst type.

Introduction

Women with gross cystic disease (cysts > 3 mm in diameter) of the breast, which are lined by apocrine epithelium, have a higher risk of developing breast cancer than women with cysts which are lined by flattened epithelium [1–3]. The ratios of breast cyst fluid concentrations of potassium to sodium for cysts lined by flattened epithelium (< 3) have been shown to be lower than the values obtained for cysts lined by apocrine epithelium (> 3) [4, 5]. The intracystic levels of sex hormones and various growth factors were found to be unevenly higher in K+/Na+ > 3 cysts as compared with K/Na< 3 cysts, suggesting a possible biological explanation for the higher risk of developing breast cancer observed in women presenting apocrine cysts [6–10].

Wide ranging concentrations of several cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) are present in breast cyst fluid [11–13]. These cytokines regulate breast fibroblast aromatase activity [12], and previous studies have revealed an association between the presence of a breast tumour and aromatase activity in the breast quadrant in which the tumour is located [14]. Alterations of IL-6 expression are associated with pathogenesis in breast cancer [15], although their role in carcinogenesis is controversial [16–21].

The present study reports the levels of IL-1, IL-6, and TNF-α in the breast cyst fluid of women with fibrocystic breast disease, and analyses the relationship between the concentration of these cytokines and sex steroid hormones.

Materials and methods

A total of 54 patients aged between 19 and 53 years, attending the breast pathology unit of our hospital for

a routine check-up or with mastalgia, were studied. All patients were subjected to a physical examination, mammography, echography, and fine needle aspiration cytology (FNAC). Informed consent was obtained from the women. After FNAC the women were divided into two groups: group A – 29 women aged between 19 and 51 years, mean age 40.8, diagnosed with gross cystic breast disease and a cyst fluid ratio K+/Na+ < 3; and group B – 25 women aged between 31 and 53 years, mean age 42.6, with macrocysts and a ratio K+/Na+ in the aspirated fluid of > 3.

All women had a regular menstrual cycle (3–7 days/25–33 days) and had not taken any contraceptives or medication that might influence their hormonal state for at least 6 months. The FNAC was carried out between 08.00 and 10.00 h, after the patient had fasted since 00.00 h. The cyst fluid obtained by FNAC was immediately centrifuged at $1500 \times g$ for 10 min. The supernatant was separated and divided into aliquot parts which were frozen at -80°C until analysis was carried out. The intracystic Na+ and K+ concentrations were determined by indirect potentiometry with commercial kits (Beckman Instruments Inc, Brea, CA, USA). The ranges of K+ and Na+ in breast fluid cyst fluid were 3.9–136.6 and 22–164.5 mmol/l, respectively.

Levels of IL-1 were measured by radioimmunoassay (RIA) using the MEDGENIX IL-1β-IRMA kit (MEDGENIX, Fleurus, Belgium). The kit utilizes [^{125}I]-anti-IL-1β monoclonal antibodies, is specific for human IL-1β, and demonstrates negligible crossreactivity with human interferon α, β and γ, IL-2, and TNF-α and -β. Concentrations of IL-6 were measured by RIA using the MEDGENIX IL-6-IRMA kit (MEDGENIX, Fleurus, Belgium). The kit utilizes [^{125}I]-anti-IL-6 monoclonal antibodies, is specific for human IL-6, and demonstrates negligible crossreactivity with human interferon α, β and γ, IL-1α, IL-1β, IL-2, IL-4, and TNF-α and -β. Levels of TNF-α were measured by RIA using the MEDGENIX TNF-α-IRMA kit (MEDGENIX, Fleurus, Belgium). The kit utilizes [^{125}I]-anti-TNF-α monoclonal antibodies, is specific for human TNF-α, and demonstrates negligible crossreactivity with human interferon α, β and γ, IL-1, IL-2 and TNF-β. Breast cyst fluid samples were assayed at a minimum of two concentrations in duplicates. The intra-assay and inter-assay coefficients of variation were less than 10%. Parallelism was obtained between the competitive binding curves generated by dilutions of breast cyst fluid samples and the standard curve. Sensitivities of IL-1, IL-6 and TNF-α assays were 5, 6, and 5 pg/ml, respectively.

Estradiol and testosterone determinations were performed using RIA kits (Sorin Biomédica, Vercelli, Italy), and DHEA-S and 17-OH-progesterone levels using kits from Immuchem (ICN Biomedicals, CA, USA). Respective intra- and inter-assay coefficients of variation were 6.8% and 9.9% for estradiol, 6.3% and 9.1% for testosterone, 8.0% and 8.6% for DHEA-S, and 7.2% and 9.8% for 17-OH-progesterone.

Data are presented as the mean ± standard deviations. Shapiro-Wilk's test was used to check normal distribution. All variables were mathematically transformed prior to analysis because the data did not present a normal distribution. Logarithmic transformations normalized the estradiol, testosterone, DHEA-S, 17-OH-progesterone, IL-6, and TNF-α data, and root square transformations normalized the IL-1 data. Differences between group means were calculated using multivariate analysis (Hotelling T^2), and the comparisons were performed using Welch's test or the Student's *t*-test. Simple linear regression analysis was used to show the degree of linear association between the different cytokines and hormones studied, and a two-tailed probability level of 0.05 was considered significant. Stepwise multiple regression was used to predict cytokine levels in breast cyst fluid. The variable testosterone was found to be strongly correlated with the other explanatory variables; therefore to avoid problems of multi-colinearity testosterone was omitted. In the stepwise multiple regression analysis a variable was included if its partial regression coefficient was significant at the 0.05 level, and was eliminated if its partial regression coefficient failed to be significant at the 0.10 level.

Results

In the cysts with intracystic K+/Na+ >3 we found a higher concentration of estradiol, testosterone, DHEA-S, and 17-OH-progesterone than in those with intracystic K+/Na+ < 3 (Figures 1 and 2). No significant differences were found in the cystic levels of IL-1. However, in the cysts with intracystic K+/Na+ > 3 we found a lower concentration of IL-6 and TNF-α than in those with intracystic K+/Na+ < 3 (Figure 3).

A positive correlation was found between IL-6 and TNF-α, and a negative correlation was found between IL-6 and estradiol, 17-OH-progesterone, DHEA-S, and testosterone. There was a negative correlation between the levels of TNF-α and the levels of estradiol and testosterone. All steroid hormones analysed presented a positive correlation between each other (Table 1).

Stepwise multiple linear regression analysis demonstrated that the concentration of IL-6 in breast cyst fluid was predicted statistically by a negative re-

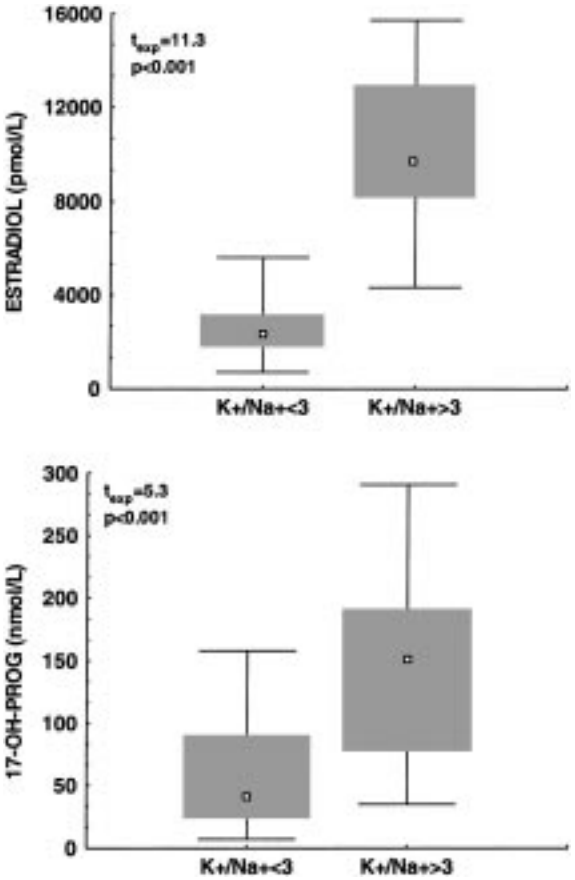

Figure 1. Intracystic estradiol and 17-OH-progesterone in K+/Na+ < 3 and K+/Na+ > 3 breast cyst fluid.

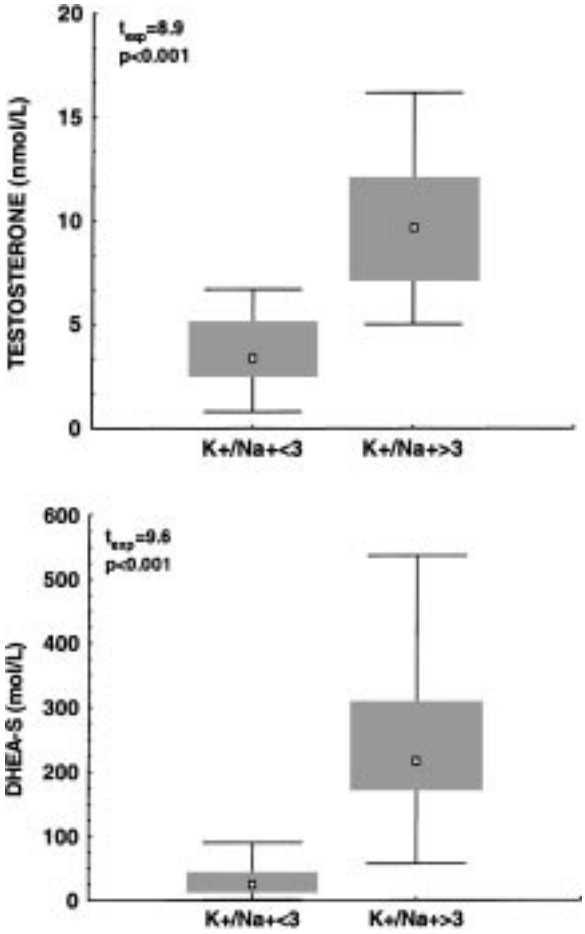

Figure 2. Intracystic testosterone and DHEA-S in K+/Na+ < 3 and K+/Na+ > 3 breast cyst fluid.

gression coefficient for the concentration of estradiol ($R^2 = 0.44$, Fexp $= 42.53$, g.l. $= 1.52$) and DHEA-S ($R^2 = 0.57$, Fexp $= 5.43$, g.l. $= 3.50$), and by a positive regression coefficient for the concentration of TNF-α ($R^2 = 0.52$, Fexp $= 8.61$, g.l. $= 2.51$). The concentration of TNF-α in breast cyst fluid was predicted statistically by a positive regression coefficient for the concentration of IL-6 ($R^2 = 0.39$, Fexp $= 33.65$, g.l. $= 1.52$), and by a negative regression coefficient for the concentration of estradiol ($R^2 = 0.44$, Fexp $= 5.28$, g.l. $= 2.51$). No candidate variable was included in the model to predict concentrations of IL-1 in breast cyst fluid.

Discussion

The fact that no significant differences were found in the concentrations of IL-1 for the two types of macrocysts does not mean that this cytokine does not play an important role in physiopathological events that are common to both cysts, a role which is implied by the presence of intracystic levels higher than those normally detected in serum (< 15 pg/ml). IL-1 inhibits the growth of malignant breast cells in culture, despite increasing the insulin receptors and their RNA [22]. This paradoxical effect might be due to the fact that IL-1 blocks tyrosine kinase activity, which is a key factor in the pathway of the second intracellular messengers, which intervene in the effects of different growth factors.

The levels of IL-6, however, were higher in intracystic K+/Na+ < 3, which coincides with the findings of other authors [11, 13, 23, 24]. Moreover, and as in the case of IL-1, the intracystic levels of IL-6 were greatly above the values normally detected in serum (6–31 pg/ml), lending weight to the hypothesis of local production.

The negative correlation between androgens and IL-6 may be explained by the stimulating effect of

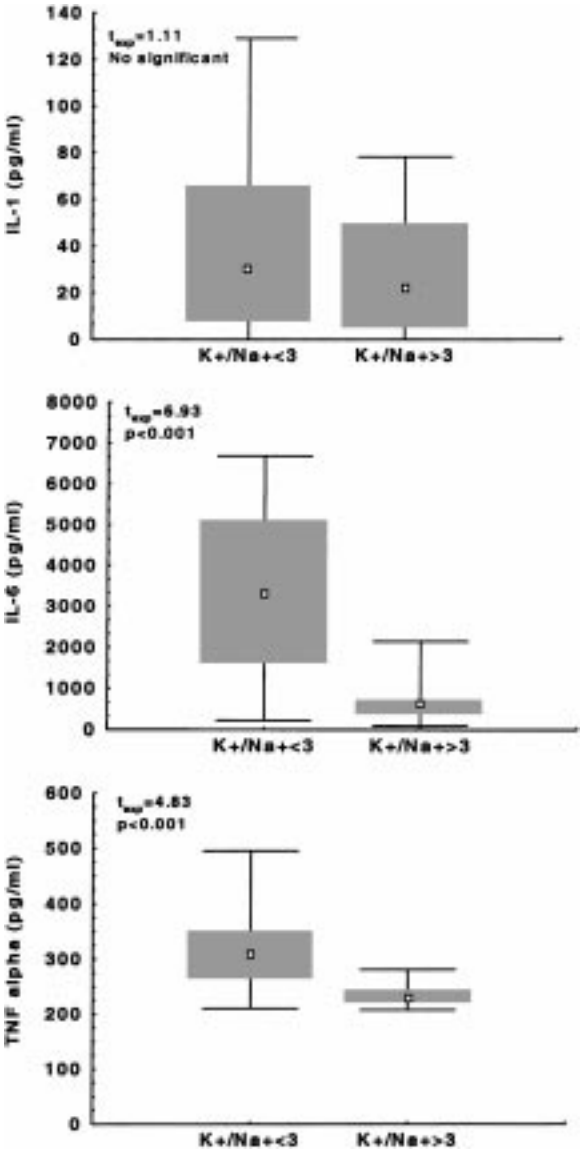

Figure 3. Intracystic IL-1, IL-6, and TNF-α in K+/Na+ < 3 and K+/Na+ > 3 breast cyst fluid.

IL-6 on mammary fibroblast aromatase [23, 25], thus provoking the transformation of androgens into estrogens. It therefore seems contradictory that both we and other authors [11, 13] should find a negative correlation between IL-6 levels and estrogens, considering, too, the stimulating effect of IL-6 on 17-ß estradiol oxido-reductase [17], the enzyme responsible for the conversion of estrone into 17-β estradiol. These discrepancies could be explained by the highly heterogeneous response of the different breast cell lines to stimulus by IL-6. For malignant cells, both mammary and of other origin, the following have been reported: (a) an indifferent response [16, 21], (b) a stimulating effect [17–19], and (c) an inhibitory effect [20, 21]. The behaviour of receptors for IL-6 seems to be involved in this varied response [26].

Normal breast epithelial cells [13, 15] and breast stroma fibroblasts [27] produce cytokines – among them IL-6. The transformation of these cells by various oncogenes leads to alterations in the production of cytokines and growth factors, particularly of IL-6. As the same authors found [15, 24, 25] levels of IL-6 fell histochemically in cases of invasive ductal carcinoma, but not in non-invasive carcinomas; they also found that IL-6 levels rise in lobular carcinomas. The above facts suggest that alterations in the synthesis of cytokines are present not only in mammary neoplasias but also in normal breast tissue.

Intracystic concentrations of TNF-α were also significantly higher in K+/Na+ < 3 cysts. This result does not coincide with that obtained by Macdiarmid et al. [12] who found no differences in the levels of TNF-α in the fluid of mammary cysts when these were analysed by electrolyte content. However, these authors analysed a total of seven cystic liquids, which is seven times less than the number we studied.

Basolo et al. [15] studied cultures of normal epithelial breast cells and found them to be an important source of IL-6 and IL-8 cytokines, producing a form of TNF related to TNF-α that behaved like a growth factor. This cytokine was also observed, however, in breast tumours where IL-6 rates were lower than in normal tissue.

Macdiarmid et al. [12] studied fibroblasts of benign and malignant breast cells, finding that TNF-α is a powerful dose-dependent stimulator of aromatase activity in which IL-1 and IL-6 might also interact. These observations, though, would seem to be in contradiction with the negative correlation between TNF-α and estradiol and testosterone, as commented above in the case of IL-6. Nevertheless, the *in vitro* effect of TNF-α on aromatase activity occurs in a minimum concentration of 2,000 pg/ml, and the TNF-α concentration observed by us and by others [11, 12] in cystic liquid was considerably lower than this level. Moreover, this effect of the stimulation by TNF-α of breast fibroblast aromatase, observed *in vitro*, is only produced in the presence of dexamethasone [12], and we have no data on the concentration of glucoconticoids in the surroundings of the breast macrocyst.

It might be considered that the concentration of TNF-α, which in our study is greater when intracystic K+/Na+ < 3, to some extent performs a 'protector' role against breast cancer. De Kossodo et al. [28] attempted to determine the possible anti-tumor mech-

Table 1. Significant ($p < 0.05$) simple linear correlation coefficients between cytokines and steroid hormones

	17-OH-progesterone	Testosterone	DHEA-S	IL-1	IL-6	TNF-α
Estradiol	0.54	0.78	0.64	–	−0.68	−0.59
17-OH progesterone	–	0.55	0.47	–	−0.55	–
Testosterone	–	–	0.64	–	−0.64	−0.53
DHEA-S	–	–	–	–	−0.60	–
IL-1	–	–	–	–	–	–
IL-6	–	–	–	–	–	–
TNF-α	–	–	–	–	0.62	–

anisms in which TNF-α might intervene, associating it with an interferon, in a model of breast cancer, in the belief that it could play a part in the selective destruction of tumoural vascularisation and in the increase in the expression of stromal cytokines, cytokine receptors, and adhesion molecules with which it would be related and would interact.

Finally, concerning the origin of TNF-α in cystic fluid, Lewis et al. [29] investigated the relation of this product to the development of angiogenesis in tumor tissue. They found that the tumor-associated macrophages in the stromal section, together with malignant epithelial cells, were the greatest cellular producers of cytokines, while TNF-α receptors were expressed by leukocytes, malignant cells, and endothelial cells in the tumoural vessels. These findings support the theory of vascular activity previously proposed by these authors.

These results demonstrate that different cytokines that are normally produced in noncancerous breast tissue, as in the case of gross cystic disease, affect the metabolism of steroid hormones. Such cytokines may intervene in the regulation and differentiation of breast cells.

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Address for offprints and correspondence: Alfonso Herruzo, Departamento de Obstetricia y Ginecología, Hospital Materno-Infantil, H.U. "Virgen de las Nieves", E-18014 Granada, Spain; *Tel:* 34-58-241202; *Fax:* 34-58-241513