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Report

Circulating levels of the macrophage colony stimulating factor CSF-1 in primary and metastatic breast cancer patients. A pilot study

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Abstract

Earlier results [1], suggesting an autocrine tumor cell stimulation by CSF-1, are in agreement with data by Fildermann *et al.* [2], showing an enhanced motility and invasiveness in the CSF-1 receptor expressing BT20 breast cancer cell line upon stimulation with recombinant CSF-1. Tumor-cell secreted CSF-1 has also been shown to cause monocyte recruitment, but not cytotoxicity [3]. Down-regulation of monocyte class II antigen expression after exposure to high concentrations of CSF-1 [4] may decrease macrophage-mediated tumor cytotoxicity and favor tolerance. Raised CSF-1 serum levels may thus increase tumor metastatic behavior as well as cause immune suppression in advanced stage disease. We set out to evaluate serum CSF-1 levels in primary and metastatic breast cancer.

Serum samples from one hundred and eighteen primary breast cancer patients and seventy-five patients with metastatic disease were assayed by radio-immuno-assay (RIA) for circulating colony-stimulating factor 1. Mean serum levels were significantly higher in the metastatic population (9.7 ng/ml ± 0.8) as compared to the patients with primary tumors (4.2 ± 0.2) ($p = 0.0001$). Patients with early stage tumors (T0/T1/T2) had significantly lower levels than patients with tumors of larger size (T3/T4) ($p = 0.0001$).

Relapse and survival statistics were analyzed using Kaplan-Meier estimates. Samples from 118 primary breast cancer patients were available to study. The median follow up was 85 months (range: 1–108). An elevated CSF-1 concentration (> 6.6 ng/ml or > 550 Units/ml) was associated with a shorter disease free interval ($p = 0.03$). In a multivariate analysis, including T (clinical tumor size), N (clinical node status), histological grade, and hormone receptor status, CSF-1 remained significantly associated with a poorer outcome (relative risk of relapse: RR: 3.3 [1.3–8.5]), together with tumor size (RR: 2.8[1–8.2]) and clinically involved nodes (RR: 4.1[2.1–8]). These results were not modified following adjustment for type of treatment.

We conclude that raised circulating CSF-1 levels may be an indicator of early metastatic relapse.

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Introduction

CSF-1 (colony-stimulating factor-1) is produced by many different cell types (reviewed by Praloran [5]) and may be a common signal for monocyte mobilization during tissue morphogenesis. CSF-1 was originally distinguished from other colony-stimulating factors by its ability to promote survival, proliferation, and differentiation of macrophages from bone marrow progenitor cells [6, 7]. Subsequently it was shown to act by binding and activating a high affinity membrane tyrosine kinase receptor, the protein product of the oncogene c-*fms* [8–11]. A wide range of benign cells, and tumor cells have since been documented to synthesize CSF-1. These include endometrium, placental trophoblast, endothelial cells, fibroblasts, some T cells, interdigitating reticulum cells and tumors of various origins as well as tumor-derived cell lines [5]. Many tumors and tumor-derived cell lines express significant levels of the CSF-1 receptor protein as well, and a possible autocrine role of this growth factor in tumor progression has been suggested [12]. The normal steady-state circulating CSF-1 concentration is regulated by sinusoidally located macrophages which remove the growth factor from the circulation by CSF-1 receptor-mediated endocytosis and intracellularly destroy it [13]. *In vivo* animal studies, using ^{125}I CSF-1 as tracer, showed the half life of CSF-1 in the circulation to be approximately 10 minutes [13]. During pregnancy the very high uterine synthesis of CSF-1 may contribute to the slightly elevated circulating CSF-1 concentration [14, 15]. Modest elevations in the circulating CSF-1 concentration are evident in disease states such as myeloproliferative disorders [16, 17] and in ovarian cancer patients where they were correlated with poor outcome [18] as well as with disease activity [19, 20]. CSF-1 mRNA and protein were present at the level of the stromally invasive tumor cells in the majority of primary breast tumors in a population of 195 patients [1] and correlated with marked inflammatory cell infiltrates. In animal models, the transfer of CSF-1 in tumor cells induced a macrophage infiltration but not tumor suppression [3]. A predominant nuclear staining pattern in immunohistochemical assays using anti-CSF-1 antibodies was also associated with a poor prognosis [1] and we previously suggested that nuclear retention of CSF-1 may reflect CSF-1 turnover and function in tumor cells. Further studies are needed to establish the significance of CSF-1 in solid tumors. New approaches to therapy, and in particular high dose chemotherapy with growth factor rescue as well as cytokine gene modified tumor vaccines, will benefit from a better understanding of the pattern and biological effects of tumor produced cytokines.

Material and methods

Patients

Serum samples from 118 primary breast cancer patients who had been treated either at Institut Curie or at Centre René Huguenin were available to study. The median follow up of the primary tumor population was 85 months (± 6.6; range 1–108). The median age was 56 and 57 years respectively (extremes: 35–85). Forty six percent of patients from Institut Curie (IC) were premenopausal against thirty nine percent from Centre René Huguenin (CRH). The histological type of most tumors was ductal invasive (104); 2 tumors were predominantly intraductal with microinvasion. Minor forms were: lobular invasive (3), medullary (4), mucinous (2), papillary (2), Paget's disease of the nipple (1). Tumor characteristics and their variations between centers are shown in Table 1. Patients from CRH had smaller tumors and were less frequently clinically node positive, and the tumors were more frequently poorly differentiated (grade III). Breast conserving treatment was more likely at IC than at CRH. Serum samples from 75 independent patients with metastatic relapse, prior or during treatment, were also available to study.

Serum samples

Frozen samples which had been previously drawn for routine marker (CEA or CA15.3) assay were stored at – 30° C until use. The RIA was performed in duplicate in a 2-step procedure as previously de-

scribed [21] with modifications described elsewhere [16, 17]. Recombinant human CSF-1 for iodination was obtained as a gift from Chiron Corp, Emeryville, CA, and an antiserum raised to partially purified human urinary CSF-1 [21] was used at a final dilution of 1 in 1000. Protein concentrations of standards and samples were kept constant by preparing solutions of blanks, standards, and antiserum in 10% normal rabbit serum and by diluting serum samples 1:10 in serum-free buffer prior to assay. The assay measures CSF-1 in the range of 28–900 pg/tube. Using different sets of reagents over a 2 year period, for 12 serum comparisons the inter- and intra-assay coefficients of variation were 18% and 14% respectively. In a study of 64 normal volunteers assayed as described in the same laboratory as used for the present study, the serum CSF-1 concentrations were normally distributed with a mean ± standard deviation of 4.46 ± 1.33 ng/ml and a range of 1.73–8.4 ng/ml. For the present study a total of 193 serum samples were assessed and results are expressed in ng/ml.

For all 118 primary tumor patients, one sample, collected at presentation or at the start of the primary treatment, was assayed. For the 75 patients with metastatic relapse, samples had been collected at different time points during the management of their recurrent disease. The first available sample was assayed.

Statistical methods

In earlier studies of American patients with ovarian cancer or leukemia, a cut-off of 500 to 550 U/ml (6.0 to 6.6 ng/ml) for CSF-1 measurements had been chosen in an attempt to discriminate between the presence and absence of disease and to maximally reduce false positive (3%) and false negative (9%) results [20, 17]. Previous studies with normal individuals had indicated that serum CSF-1 concentrations were normally distributed in the range of 1.7–7.1 ng/ml [16]. In the present study, the median value for the 118 patients for whom a serum measurement at diagnosis of the primary tumor was available, was 3.7 ng/ml (307 U/ml). Consequently, both the median value of the population as well as a previously defined cut-off [20] were used to test for outcome.

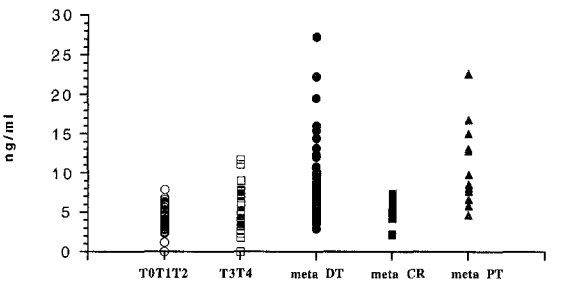

Fig. 1. Serum CSF-1 levels in primary and metastatic breast cancer patients. The mean serum CSF-1 concentration for all patients who had relapsed with metastatic disease was 9.7 ± 0.8 ng/ml, whereas the mean level for the newly diagnosed patients was 4.2 ± 0.2 ng/ml (p = 0.0001). The data for the metastatic population (meta) was classed into: prior to treatment (PT, n = 18), during treatment (DT, n = 56), and complete clinical responders (CR, n = 6) with mean CSF-1 levels of 10.9 ± 1.4; 9.9 ± 1 and 5.1 ± 0.7 ng/ml respectively. Smaller primary tumors (T0/T1/T2) were associated with lower CSF-1 levels (mean 3.8 ± 0.2) than more advanced primary tumors (T3/T4) (mean 5.3 ± 0.5). The differences between primary tumors of different stage, as well as between metastatic patients with or without active disease, were highly significant (p = 0.0001).

Comparisons between percentages were by chi-square test, comparison of means by analysis of variance, and confirmed by a non parametric Mann-Whitney test in case of small sample size (n < 30). BMDP programs were run on a VAX 6000 computer. Survival curves were drawn using Kaplan-Meier [22] estimates and comparison of survival distributions was made by log rank test [23]. Survival estimates were calculated according to above or below median (>< 3.7 ng/ml) or raised (> 6.6 ng/ml) CSF-1 levels at diagnosis of the primary tumor (n = 118). Due to the paucity of late events, survival curves are presented only up to 84 months, but statistical tests take into account the total number of events up to 108 months. No local or metastatic recurrences were seen after 76 months of follow up. The prognostic relevance of CSF-1 was assessed in a proportional hazards model as described by Cox [24], taking into account tumor stage, grade, ER and PR status, and type of treatment. Categorical variables, such as CSF-1 levels, tumor stage, grade, and age were modeled by sets of binary variables, in order to avoid any assumption concerning the relative risks

of relapse between the various subgroups. Missing values for ER and PR status were coded as a separate variable.

Results

Serum levels (ng/ml) were measured by RIA and the values of the different groups are indicated in Fig. 1. Smaller tumors (T0/T1/T2) were associated with lower CSF-1 levels (mean 3.8 ± 0.15) than more advanced tumors (T3T4) (mean 5.3 ± 0.5). The differences between primary tumors of different stage, as well as between metastatic patients with or without active disease, were highly significant ($p = 0.0001$). The group of metastatic patients with documented complete response (meta CR) following chemotherapy had significantly lower CSF-1 levels than patients with active disease (Fig. 1).

An influence of CSF-1 on outcome was tested in the primary tumor population. Samples were available from two centers as indicated in Table 1 and differences regarding tumor size, grade, and type of treatment are indicated. Patients from IC were more frequently of larger size, but less frequently of

Table 1. Tumor characteristics

Characteristic	Combined, n = 118	IC, n = 35 (29%)	CRH, n = 83 (71%)	p
Clinical tumor size				0.02 (T1 vs T2 vs T3 vs T4)
T0	2 (2)	0	2 (2.5)	
T1	25 (21)	3 (8.5)	22 (26.5)	
T2	58 (49)	16 (46)	42 (51)	
T3	18 (15)	9 (26)	9 (11)	
T4	12 (10)	6 (17)	6 (7)	
Tx	3 (3)	1 (2.5)	2 (2)	
Clinical node status				0.07 (N0/N1a vs N1b vs N2)
N0/N1a	73 (62)	17 (49)	56 (67)	
N1b	35 (29)	13 (37)	22 (26.5)	
N2	7 (6)	4 (11)	3 (4)	
Nx	3 (3)	1 (3)	2 (2.5)	
Bloom-Richardson grading				0.001 (I vs II vs I II)
NC	16 (13)	6 (17)	10 (12)	
I	13 (II)	8 (23)	5 (6)	
II	61 (52)	20 (57)	41 (49)	
III	28 (24)	1 (3)	27 (33)	
ER status				ns (– vs +)
–	23 (19.5)	4 (11)	19 (23)	
+	59 (50)	15 (43)	44 (53)	
unknown	36 (30.5)	16 (46)	20 (24)	
PR status				ns (– vs +)
–	37 (31)	10 (29)	27 (33)	
+	49 (42)	14 (40)	35 (42)	
unknown	32 (27)	11 (31)	21 (25)	
Treatment				0.0001 (BCT vs Mast)
Breast conserving	59 (50)	31 (88)	28 (34)	
lumpectomy	40	14	26	
no surgery	19	17	2	
Mastectomy	59 (50)	4 (12)	55 (66)	
Adjuvant				
chemotherapy	49 (41.5)	14 (40)	35 (42)	
hormonetherapy	52 (44)	12 (34)	40 (48)	

BCT = breast conserving treatment; Mast = mastectomy; ER = estrogen receptor; PR = progesterone receptor; NC = not classified; IC = Institut Curie; CRH = Centre René Huguenin.

Table 2. Serum CSF-1 levels and patient characteristics

	Serum CSF-1 concentration (ng/ml)		
	< 3.7	3.7–6.6	> 6.6
Age (p = 0.03)			
< 55	35* (61)**	21 (43)	3 (25)
> 55	22 (38)	28 (57)	9 (75)
Clinical tumor size (p = < 0.0001)			
T0–T2	47 (85)	36 (75)	2 (20)
T3–T4	8 (15)	12 (25)	10 (80)
Type of treatment (p = 0.007)			
Breast conserving	24 (42)	24 (49)	11 (92)
Mastectomy	33 (58)	25 (51)	1 (8)

Clinical nodal status as well as ER and PR status, tumor grade, number of involved nodes, and adjuvant chemo- or hormonetherapy were not significantly associated with CSF-1 levels.

* Number of patients; ** Percentage of patients within particular CSF-1 group.

grade III. Treatment management was more often conservative at IC.

Associations between CSF-1 levels and patients characteristics are shown in Table 2. Cut off points for CSF-1 had been chosen according to the median value of the entire population (3.7 ng/ml) as well as to a previously established cut-off between normal and cancer patients (6.6 ng/ml) [20]. Only 10% of the primary breast cancer population had elevated CSF-1 levels according to this upper cut-off point. Postmenopausal patients (> 55) were associated more frequently with higher CSF-1 values (p = 0.03). When patients > 55 were subdivided in groups of increasing age, there was no linear relationship between age and CSF-1 values, but rather an increased incidence of elevated values between ages 55 and 65. These data need to be viewed with caution due to the small sample size of elevated values in this series. A strong direct correlation was apparent between tumor size and serum levels of CSF-1 (p = 0.0001). Finally, patients with high CSF-1 levels were on average treated more conservatively, reflecting the different selection and different treatment attitudes of each center.

Endpoints were local recurrence, distant recurrence, disease-free interval, and survival. There was

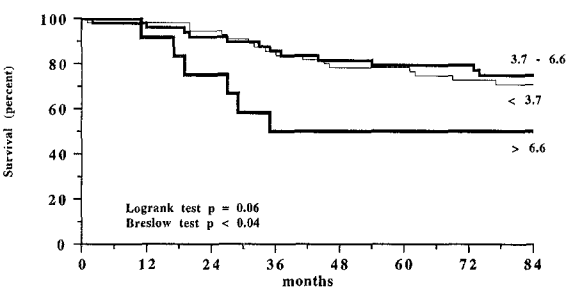

Fig. 2. Survival according to CSF-1 levels at diagnosis. Influence of CSF-1 levels at diagnosis on subsequent outcome was tested in 118 patients, grouped according to serum concentration. Median follow up from diagnosis was 85 months (± 6.6). Number of patients at risk for low, intermediate, and high levels of CSF-1 were: at start: 34, 17, 16; at 36 months: 26, 16, 10; and at 72 months; 22, 14 and 8.

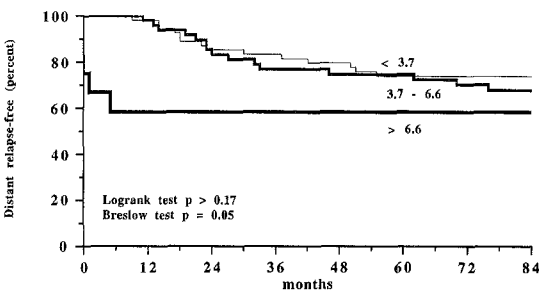

Fig. 3. Metastatic relapse according to CSF-1 levels at diagnosis. Metastatic disease includes both clinically overt and occult metastatic disease in the newly diagnosed patients. For patients with levels > 6.6 ng/ml, compared to patients with values < 6.6 ng/ml, the p value of the log rank test was 0.075 and the p value of the Breslow test < 0.02. Number of patients at risk for low, intermediate, and high levels of CSF-1 were: at start: 34, 17, 16; at 36 months: 13, 14, 9; and at 72 months: 18, 11, 7.

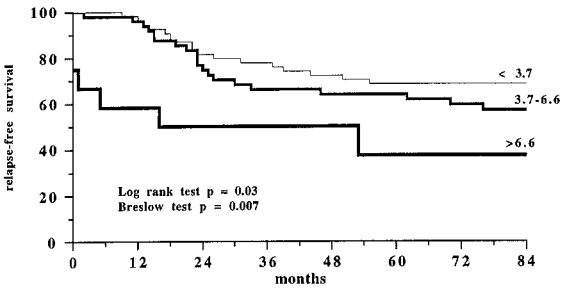

Fig. 4. Disease-free interval according to CSF-1 levels at diagnosis. For patients with levels > 6.6 ng/ml, compared to patients with values < 6.6 ng/ml, the p value of the log rank test was < 0.02. Number of patients at risk for low, intermediate, and high levels of CSF-1 were: at start: 34, 17, 16; at 36 months: 20, 11, 9; and at 72 months: 15, 9, 6.

no significant survival difference between the three classes of CSF-1 levels (Fig. 2), except for the initial slope of the curves (Breslow test $p < 0.04$). There was a significant decrease in the initial slope of the survival curve in the group with serum CSF-1 levels > 6.6 ng/ml, compared with either of the other groups ($p = 0.03$). Fig. 3 shows the association between early metastatic relapse and raised CSF-1 levels; elevated CSF-1 is associated with both clinically overt and occult metastatic disease in newly diagnosed patients. Due to the limited number of patients in this series, we focused on disease-free interval (Fig. 4) since 44 events (metastatic, local, or regional relapses) had occurred at the time of analysis. Factors involved with outcome are shown in Table 3. Large clinical tumor size ($p = 0.0009$), positive clinical node status ($p = 0.0001$), raised CSF-1 levels ($p = 0.03$), and negative progesterone receptor status ($p = 0.05$) were significantly associated with relapse. According to a forward stepwise Cox regression model, a combination of 3 factors was selected as increasing the risk of relapse: a positive clinical node status (RR: 4.1), CSF-1 levels above to 6.6 ng/ml (RR: 3.3), and a T2/T3 or T4 tumor (RR: 2.8).

Discussion

The production of CSF-1 has now been reported in a wide range of cells and tumors of non-hematopoietic origin [1, 12, 18, 25–29]. Previous work not only documents the presence of CSF-1 protein and transcripts in solid tumors, but also the presence of elevated plasma CSF-1 levels in patients with active and recurrent neoplastic disease [19] and its association with a poor outcome [18]. The CSF-1 receptor has also been shown to be expressed in the same tumor types [1, 29] and tumor derived cell lines, suggesting a cellular response to the growth factor via an autocrine mechanism that could support tumor growth. We have previously shown that CSF-1 protein and transcripts are present in invasive [28] but not in pre-invasive (*in-situ*) breast carcinoma cells [27].

A number of essential biological functions of monocytes-macrophages, including migration [30], production of proteolytic enzymes [31], and down regulation of their MHC class II antigen expression [4] are inducible by CSF-1. The expression of CSF-1

Table 3. Univariate analysis of disease-free interval

Variable	n	O/E*	p (trend)
Age			0.06
< 40	13	1.05	
41–55	46	0.74	
56–65	27	1.83	
> 65	32	0.82	
Clinical tumour size			0.0009
T0/T1	27	0.31	
T2	58	0.97	
T3	18	1.93	
T4	12	2.35	
Clinical node status			< 0.0001
N0/N1a	73	0.54	
N1b/N2	42	2.15	
CSF-1 levels (ng/ml)			0.03
< 3.7	57	0.76	
3.7–6.6	49	1.08	
> 6.6	12	2.35	
ER status			n s
+	59	0.9	
–	23	1.42	
PR status			0.05
+	49	0.73	
–	37	1.42	
Bloom-Richardson grading			n s
n c	16	0.46	
I	13	0.72	
II	61	0.94	
III	28	1.57	

* O/E = number of observed events/number of expected events.

and its receptor in both monocytes and metastatic tumor cells could partly explain the biological basis for phenotypic parallels between the two cell types. Monocytes, like metastatic tumor cells, can invade stroma, travel to distant sites, and adhere to parenchyma via specific homing receptors.

We set out here to evaluate circulating CSF-1 levels at different time points during the course of breast cancer. Despite some overlap in values for individual patients (Fig. 1) and despite the non-specificity of this marker for cancer of the breast, we did see a highly significant link with disease activity and tumor progression. In particular, patients with the highest values in the metastatic group were in a phase of rapid progression and died within a few months of this CSF-1 assay. Similarly, patients with very high values prior to treatment had the largest tumor burden. Few patients in this series had a well documented complete response at the time of serum storage, but the patients with a documented remission had significantly lower CSF-1 serum levels as compared to any other metastatic group. Although our results need to be confirmed in a large prospective series, the consistent variation in CSF-1 levels with disease stage/progression led us to believe that the observed patternis highly unlikely to be due to statistical error in the presence of small sample size.

A second goal was to define the use of CSF-1 as a serum marker in patients with early disease, in an attempt to predict which patients might benefit from further adjuvant treatment. Serum samples from two different centers were assayed, and despite differences in patient characteristics between centers, there were common trends in our results including the statistical correlation between raised CSF-1 levels and larger size tumors. The immediately post-menopausal group (age 55–65) had a higher incidence of elevated CSF-1 values, corresponding to the age group with a peak incidence of breast cancer. Since our study population is small, these results need to be viewed with caution and will be reanalyzed in an ongoing large prospective trial.

Since many different clinical situations may influence CSF-1 serum levels, the choice of a cut-off value to discriminate effectively between 'normal' and cancer patients appeared complex. Following guidelines in a recent publication by Simon and Altman [32] we opted to use both a cut-off point reported from another study [6.6 ng/ml or 550 U/ml] [17] as well as a second cut-off point at the median value [3.7 ng/ml or 307 U/ml] of the present population. The upper cut-off value agreed with the upper limit of normal plasma CSF-1 levels observed in an earlier study of 64 normal volunteers [17]. By computing the statistical significance level for all possible cut-off points in the present study, all values above 6.0 ng/ml were significant, and these data will be reassessed in an ongoing prospective study.

Univariate analysis, screening for factors influencing outcome in the newly diagnosed patient group (n = 118), revealed CSF-1 levels above 6.6 ng/ml to be significantly associated with relapse, together with the classical parameters (T, N, PR status). Since CSF-1 levels were significantly correlated with tumor size, we carried out a multivariate analysis which confirmed a risk associated with elevated

Table 4. Multivariate analysis of disease-free interval (44 events)

Step	Variable	RR [95% CI]	p
1	N0/N1a	1	
	N1b/N2	4.1 [2.1–8.0]	< 0.0001
2	CSF-1 < 6.6 ng/ml	1	
	CSF-1 > 6.6	3.3 [1.3–8.5]	< 0.008*
3	T0/T1	1	
	T2–T4	2.8 [1–8.2]	< 0.03

RR = relative risk; CI = confidence interval

Results for T, N, and CSF-1 are not modified following adjustment for types of treatment.

* CSF-1 p value at last step was 0.015.

CSF-1 values. The predictive value of CSF-1 for metastatic relapse (Fig. 3) did not appear to extend beyond the first year following the CSF-1 assay, and repeated yearly evaluations of CSF-1 appear indicated to follow the disease activity. CSF-1 measurements may thus be valid for the early detection of clinically occult metastatic disease.

Many biotherapy trials are attempting to modulate immune response at the tumor site. So far, few studies have addressed the biological relevance of CSF-1 production by primary or metastatic tumor cells. Our own results, showing a correlation between tumors with high percentages of CSF-1-expressing tumor cells and marked monocyte infiltrates [1], have been confirmed by animal studies [3]. In particular, the transfer of the gene coding for CSF-1 into tumor cells not only induced a macrophage infiltration, but these macrophages appeared tolerant since they did not elicit tumor suppression. Ongoing developments into immunotherapy trials of cancer, frequently coupled with immunoreactive cytokines, warrant further studies geared to a better understanding of the biological effects of cytokines that are abundant at active tumor sites.

Drawing comparisons with the well documented effects of CSF-1 on monocyte survival, it is tempting to speculate that if CSF-1 did influence the survival of receptor-bearing metastatic tumor cells, we could understand the occurrence of clinically detectable metastases 10 or 15 years following the primary treatment. If CSF-1 did influence the invasive properties of receptor-bearing tumor cells, a rise in CSF-1 might influence tumor progression (discussed in: [33]) and be associated with a poor prognosis. Lastly, if CSF-1 did influence the metastatic homing of receptor-bearing tumor cells to specific sites of monocytic development, we would observe metastases arising in organs rich in specialized monocytes, such as bone, liver, dermis, lung, and pleural and peritoneal cavities, findings which happen to concur with observations in clinical practice. Interestingly, recent studies in the mouse suggest that locally produced CSF-1 may be responsible for the development and maintenance of macrophages at many of these sites [34, 35].

In conclusion, we see evidence of increasing production and secretion of CSF-1 as breast tumors invade and metastasize. Future studies will attempt to evaluate the role of CSF-1 on primary tumor cell viability in tissue culture as well as its potential in inducing immune tolerance, as suggested by the tremendous rise in CSF-1 levels in the pregnant uterus in the presence of a fetus which can be considered as an allograft, together with the mild immunosuppression commonly observed in end-stage breast tumor patients.

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