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M-CSF (Monocyte Colony Stimulating Factor) and M-CSF Receptor Expression by Breast Tumour Cells: M-CSF Mediated Recruitment of Tumour Infiltrating Monocytes?

Ruoping Tang, Frédéric Beuvon, Mario Ojeda, Véronique Mosseri, Pierre Pouillart, and Susy Scholl

Institut Curie, Service de Médecine Oncologique (R.T., M.O., P.P., S.S.), Laboratoire d'Anatomo-pathologie (F.B.), Service de Biostatistique (V.M.), 75231 Paris Cedex 05, France

Abstract Infiltrating immune cells in 30 primary human epithelial breast tumours were studied using specific anti-CD3 (T cells), anti-CD68 (macrophages), anti-CD57 (NK cells), and an anti-pan-B cell antibody (L26). The majority of tumour infiltrating inflammatory cells are T cells (40–50%) and monocytes/macrophages (15–35%).

The macrophage specific chemo-attractant and growth factor CSF-1 is detected by immunohistochemical techniques (IHC) at the level of invasive breast cancer cells in 46/50 tumours but not at the level of in-situ (pre-invasive) cancer. A mosaic staining pattern was usually observed, with a very high expression in areas of obvious stromal invasion (90% cells positive) and absent or trace staining in intraductal carcinoma. Macrophages and plasma cells are equally intensely positive. In-situ hybridisation experiments confirm the production of CSF-1 (mRNA) by tumour cells and show the same pattern of expression. Expression of the CSF-1 receptor protein (*fms*) was also observed by IHC in 41/48 invasive tumours, albeit at weaker intensities than in tumour infiltrating monocytes/macrophages. A concomitant expression of both CSF-1 and *fms* in in-situ carcinoma was never seen (n = 14). It is therefore proposed that the associated expression of CSF-1 and its receptor may be linked to the invasive potential of breast cancer, the monocytic infiltrate being an indication of the quantitative importance of CSF-1 production by the tumour.

Key words: breast cancer, tumour infiltrating lymphocytes, macrophage markers, M CSF-1/CSF-1, *fms*

The subversion of normal growth factor signaling pathways is critical to the neoplastic process. Advances in molecular biology have led to the identification of a number of genes that direct the expression of the neoplastic phenotype. These so called oncogenes encode growth factors and growth factor receptors or proteins regulating their expression. Aberrant expression of such genes can lead to uncontrolled cell proliferation and the acquisition of invasive and metastatic potential through a variety of mechanisms.

Macrophage colony stimulating factor (M-CSF), also called CSF-1, is a lineage specific hematopoietin that stimulates proliferation and survival of the mononuclear phagocyte series [1]. The purified growth factor is a 45–90 Kd homodimeric glycoprotein synthesized primarily by fibroblasts [2], but its synthesis can be induced in endothelial cells [3], monocytes, and macrophages in response to other cytokines [4,5]. More recently its expression in human placenta and uterus has been documented [6]. Both increased CSF-1 mRNA and protein concentrations have been shown in endometrical tissues at the onset of pregnancy. Immunohistological studies have shown that CSF-1 production in the pregnant mouse endometrium is localized to the glandular and endothelial cells [7]. Correlative evidence suggests a role for CSF-1 in placental development. CSF-1 deficient homozygous mutant females (*op/op*) when mated to homozygous mutant males were consistently infertile [7]. Homozygous females, when mated to heterozygous males were fertile, although at a lower rate, suggesting a compensatory mechanism for the absence of maternally produced CSF-1 [7]. The CSF-1 receptor (CSF-1R or protein *fms*) is encoded by the *c-fms* proto-oncogene [8] and is a member of a family of growth factor receptors

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Address reprint requests to Susy Scholl, Institut Curie, Service de Médecine Oncologique, 26 Rue d'Ulm 75231 Paris Cedex 5, France.

that exhibit ligand-induced tyrosine-specific protein kinase activity [9]. High levels of CSF-1 receptor expression have been observed in the intermediate trophoblast of the villous spouts during the first trimester of pregnancy. The synthesis of the *c-fms* proto-oncogene product was also detected in glandular epithelial cells and decidual cells of first trimester endometrium as well as in the extravillous trophoblastic layer of the chorion [10]. Expression of CSF-1 and *fms* have been reported in human breast carcinoma derived-cell lines and tumour specimens [11,12,13].

A moderate/marked mononuclear cell reaction (MCR) has been associated with early local and distant relapse and shorter survival [14,15]. The mechanism by which this MCR could influence breast cancer prognosis is presently unknown, but the production and secretion of CSF-1 by breast tumor cells could result in a marked MCR at the tumor site.

In this work, we have characterized the infiltrating immune cells in human epithelial breast tumours. We have also studied the expression of both CSF-1 and its receptor (*fms*) in pre-invasive (in-situ) and invasive breast cancer. A potential role for CSF-1 as an autocrine or paracrine tumour cell growth factor coupled with a change to a "macrophage-like" stromally invasive phenotype is postulated.

MATERIALS AND METHODS

Immunohistochemistry

Immunohistochemical staining was carried out in paraffin embedded (formalin and Bouin fixed) blocks of a total of 37 patients with ductal infiltrating, 13 with lobular infiltrating, and 14 with in-situ breast adenocarcinoma. The samples were labelled by standard methods using biotinylated secondary antibodies, streptavidine peroxidase, and DAB-H2O2 (3,3'-diaminobenzidine-hydrogenperoxide) [16]. The primary antibodies used were, to CSF-1 receptor (*fms*), the monoclonal antibody 2E8 at a 1:500 dilution [17] and, to CSF-1, the rabbit polyclonal antibody 52P4 at a 1:1000 dilution (antibody 52p4 was raised to highly purified human urinary CSF-1) [18]. In the series of patients with ductal infiltrating cancer we characterized the immune cell tumour infiltrate using antibodies to T cells (anti-CD3 rabbit polyclonal antibody at a 1:100 dilution; Dakopatts), macrophages (anti-CD68 monoclonal antibody, Kp1, at a 1:100 dilution; Dakopatts), NK cells (anti-CD57 monoclonal antibody, Leu7, at a 1:100 dilution; Becton Dickinson), and an anti-pan-B cell antibody (monoclonal L26 at a 1:100 dilution; Dakopatts). All cases with more than 25% of the epithelial cells showing positive staining for CSF-1 were considered positive by immunohistochemical (IHC) assay. The evaluation of T cells, B cells, and monocytes was a percentage of the total immune cell infiltrate for each tumour section. The immunostaining for the *c-fms* proto-oncogene product was judged positive when distinct staining of tumour cells was observed and was visually compared with no staining on the surrounding endothelial and stromal cells, with the exception of monocytes and some plasmocytes. Positive and negative sections were included in subsequent batches to serve as internal controls. No attempt at quantitation was made. In most cases, relatively homogeneous staining of the tumour cells was observed. There was no enhancement of membrane staining as reported for immunochemistry staining of neu, but similarly to neu, the majority of tumour cells were positive rather than showing a mosaic staining pattern. All slides were evaluated by two independent investigators.

In Situ Hybridization (ISH)

ISH was carried out with digoxigenin-labelled (DIG) RNA probes [19]. The slides were dewaxed and rehydrated by sequential immersion in xylene and graded ethanols. Sections were digested with 1 μg/ml proteinase K for 15 min at 37°C and treated with triethanolamine and acetic anhydride for 10 min at room temperature. The slides were dehydrated in graded ethanol and air-dried.

The hybridisation mixtures consisted of 50% deionised formamide, 0.3 M NaCl, 10 mM Tris HCl (pH 8), 2 mM EDTA (pH 8); 10% dextran sulfate, 10 mM NaPO4 (pH 8), 1 × Denhardt, 50 ug/ml of denatured salmon sperm DNA, and 2 ng/ul of DIG-labelled RNA. Hybridisation was carried out at 42°C overnight. The coverslips were then removed and the sections were washed twice in 2 × SSC, 50% formamide at 50°C for 15 min, then treated with 5 ug/ml RNAse for 15 min at 37°C and rinsed again twice in 2 × SSC at 50°C for 5 min, followed by two washings in 0.2 × SSC for 15 min at 50°C. For detection of hybridisation signal, the binding of antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate) to hybridized DIG-labelled RNA was used. A subsequent enzyme-catalysed color reac-

tion with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) produces an insoluble blue precipitate, which visualizes hybrid molecules. This last reaction was terminated after 12 h following which the sections were counterstained and mounted (aquamount).

Clinical Staging

Clinical staging of 37 patients with the ductal infiltrating (D.I.) and 13 with the lobular infiltrating (L.I.) types of tumour was as follows. D.I.: stage 1 (10), stage 2 (25), stage 3 (1), stage 4 (1); L.I.: stage 1 (3), stage 2 (10). Surgically confirmed nodal involvement was present in 46% of D.I. patients and 42% of L.I. patients. Most patients (72%) were post-menopausal. The mean age was 62.4 years. Histological classification according to Scarff Bloom and Richardson (SBR) showed (for the D.I. type) 21% of grade I, 37% of grade IIa, 29% of grade IIb, and 13% of grade III; 2 tumours were not graded. Fourteen patients with ductal in-situ carcinoma were also assessed.

RESULTS

Previous results have shown an association between the presence of oncogene (*neu, int2*) amplifications and a marked immune cell infiltrate in breast tumours [12]. These infiltrating immune cells have now been characterised by immunohistochemical methods in 30 primary breast adenocarcinomas. The relative quantity of positively staining cells was scored semiquantitatively by a visual score as a percentage of the total visible immune cell infiltrate for each section. Average percentages (Table I) show a high proportion of T cells (40–50%) and macrophages (15–35%), the latter being more frequently intratumoral than peritumoral (Fig. 1A), as well as being present at sites compatible with an invading tumour front (Fig. 1B). T cells (Fig. 1C) are quantitatively predominant but show higher proportions at the periphery of the tumour. B cells are present (>10% of total infiltrate) in less than half of the tumours analysed and typically arranged in a follicular fashion (Fig. 1D). Morphologically differentiated plasmocytes are rare and almost twice as likely to be intratumoral than peritumoral. Only 6/14 sections show few NK cells which remain consistently less than 10% of the total infiltrate.

Since macrophages commonly represent 15–35% of the infiltrate and CSF-1 is a potent chemoattractant for cells of the mono-myelocyte lineage, evidence was sought for local production of this macrophage-specific growth factor. Using the specific and well-characterized polyclonal antibodies 52P4 [18], we have been able to demonstrate the presence of CSF-1 not only at the level of macrophages, but also very strong immunostaining was observed on stromally invasive breast tumor cells (Fig. 2). Preincubation of the anti CSF-1 antibody with 5×10^6 units of recombinant human CSF-1 totally inhibits antibody binding to the tumour cells (data not shown). CSF-1 expression by breast tumour cells is mainly detected in cells which are infiltrating stroma (in invasive tumour cells in 92% of both ductal and lobular tumour types in this series, selected for a marked inflammatory cell infiltrate). In contrast, absence of, or very low specific binding of the anti-CSF-1 antibody to preinvasive (in situ) carcinoma was observed (Fig. 2; Table II), suggestive of a link between CSF-1 expression and invasive tumour behaviour.

In-situ hybridisation experiments were carried out using a common probe to the four published variants of CSF-1 mRNA (exon 1–8). Figure 3A,B show the staining patterns ob-

TABLE I. Characterization of Intratumoral and Peritumoral Immune Cell Infiltrates in Ductal Infiltrating Breast Cancer*

	n[a]	Antibody	PT[b]	PT range (%)	IT	IT range (%)	IT + PT
T cells	29	anti-CD3	53 ± 4.6	10–90	33 ± 5	0–100	43 ± 4
Macrophages	30	anti-CD68	16 ± 2.6	0–70	29 ± 4.5	0–80	23 ± 3
B cells	29	PANB	17 ± 4.6	0–70	8 ± 3.2	0–70	13 ± 3.7
Plasmocytes	27	Morphology	8 ± 2.5	0–50	14 ± 3	0–50	11 ± 2.5
NK cells	14	anti-CD57	4 ± 1.4	0–10	4 ± 1.4	0–10	4 ± 1.3

*For each type of inflammatory cell, the mean percentage of the total intratumoral (IT) and peritumoral infiltrate (PT) is indicated.

[a]n = number of patients.

[b]Mean percentage ± standard error.

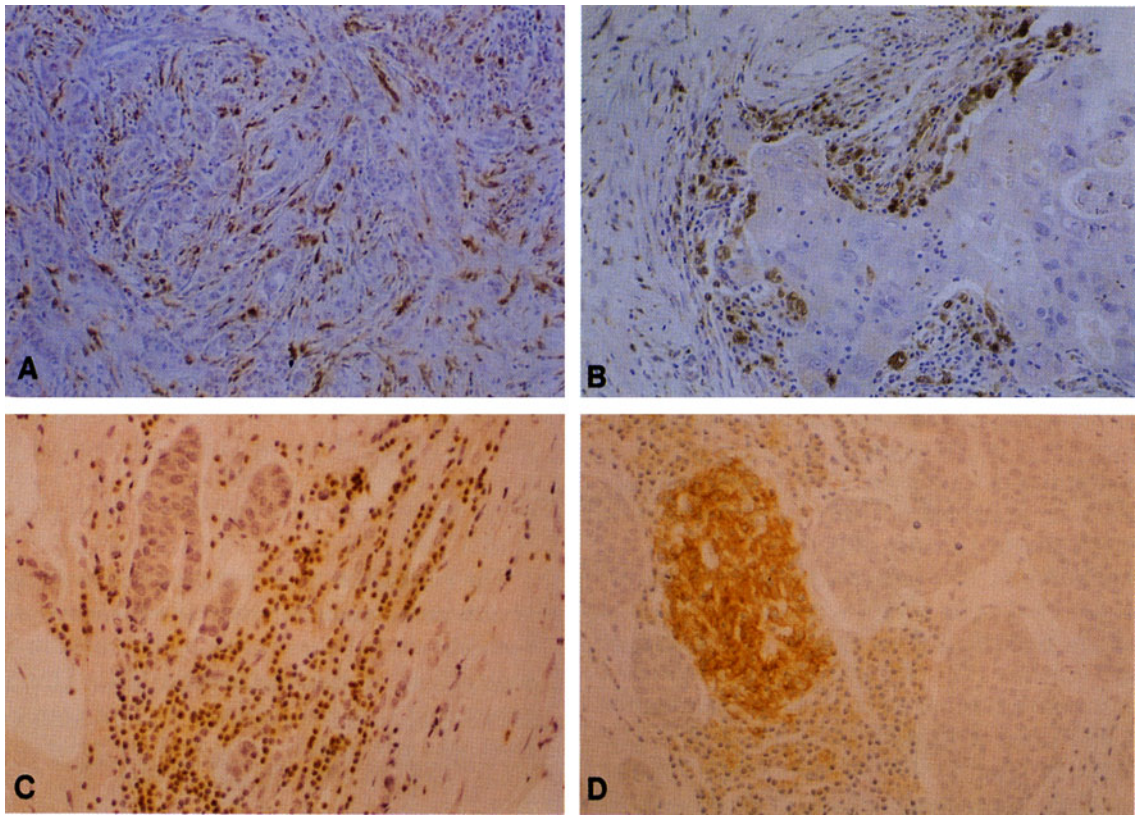

Fig. 1. Immunohistochemical (IHC) staining with primary antibodies—anti-macrophage antibody: anti-CD68 (Kp1; Dakopatts) (**A, B**), anti–T cells (anti-CD3; Dakopatts) (**C**), anti-B cell marker (L26; Dakopatts) (**D**)—was carried out as summaried in Methods. Elongated fibroblast-like monocytes (CD68 positive) are present in the stroma surrounding small tumour cell aggregates (A). In a different tumour, at higher magnification, large numbers of monocytes (brown staining) are shown in close contact with viable tumour cells (B, center). C shows CD3 positive T cells (small dark nuclei and brown cytoplasmic staining) in close contact with tumour cells. D shows a typical cluster of B cells. Photograph taken at the periphery of tumour.

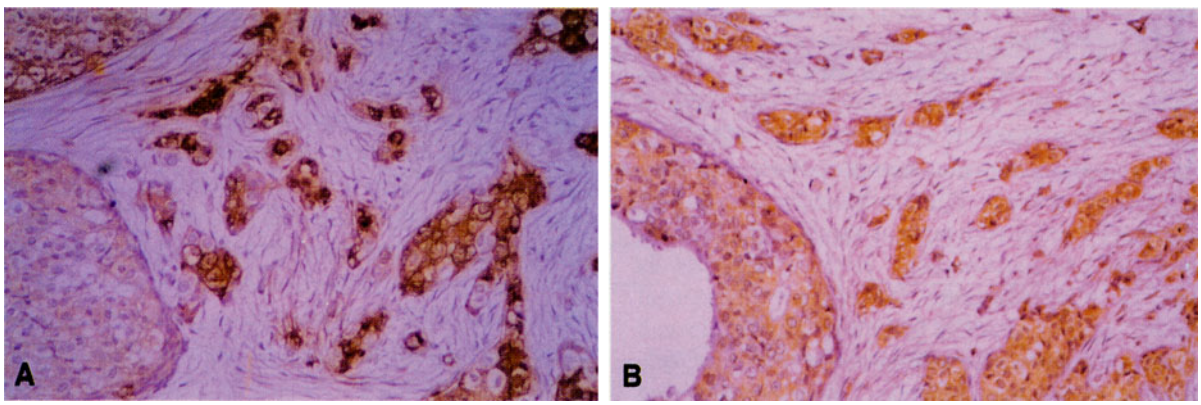

Fig. 2. **A:** Immunohistochemical staining with a specific anti-CSF-1 antibody (polyclonal 52P4; R. Stanley). The positive staining is associated with dissociated stromally invasive tumour cells. Intraductal (pre-invasive) tumour cells are not stained. **B:** IHC staining of a section of the same tumour with an antibody (2E8; L. Rohrschneider) directed against the tyrosine-kinase insert domain of the CSF-1 receptor. Both intraductal and invasive cells show positive staining for the CSF-1 receptor.

TABLE II. Expression of CSF-1 and *fms* in Ductal Infiltrating (DI) and Lobular Infiltrating (LI) Tumours, as well as in Carcinoma In Situ

	DI (%)	LI (%)	IS (%)
CSF-1	92 (34/37)	92 (12/13)	36 (5/14)
fms	82 (28/34)	93 (13/14)	21 (3/14)
Both	71 (22/31)	85 (11/13)	0 (0/14)

tained with antisense and sense probes, and clearly demonstrate a high intensity of staining at the invading tumour front. At higher magnifications (Fig. 3C,D) distinct granular cytoplasmic staining can be seen. CSF-1 therefore appears to be produced not only by hematopoietic cells but also by tumour cells.

Similarly *fms* (the receptor for CSF-1) expression, detected using a highly specific monoclonal antibody recognizing an epitope of the kinase insert domain (Fig. 4), was mainly associated with invasive tumour cells. Positive staining for *fms* was present in 82% of tumours of ductal infiltrating type, in 93% of tumours of lobular infiltrating type, and in 21% of in-situ carcinoma in this series. Normal glands showed positive staining in a few occasions.

Expression of both CSF-1 (>25% cells labelled) and *fms* (by IHC) is present in 22/31 of infiltrating ductal tumours and 11/13 infiltrating lobular tumours but absent in all (0/14) in-situ carcinomas (Table II).

DISCUSSION

Multiple markers of the myelo-monocytic lineage have previously been reported on breast epithelial cell lines [20]. Our data seem to conclusively localise the production of CSF-1, a macrophage-specific growth factor, at the level of the invasive tumour epithelial cell. CSF-1 is observed in other non-hematopoietic cell systems. In pregnancy, it is produced by the uterine secretory endometrium and reaches its highest levels at parturition [21]. High uterine levels may be a prerequisite for effective placental growth and phagocytic function [22]. Other cell types which produce one or more CSFs include fibroblasts, endothelial cells, T lymphocytes, and monocytes themselves [23]. The mechanism of induction and maintenance of production of this macrophage-specific cytokine by breast tumor cells is so far unknown. Known mechanisms for induction of CSF-1 production by specialised normal tissues operate via cytokines (IL3, GM-CSF, gamma interferon, TNF-alpha) or steroid hormones (progesterone/endometrial mucosa). CSF-1 can also be induced by several mitogens in NIH 3T3 cells [24]. Increased levels are due to transcriptional activation and last for several hours in contrast to other immediate early genes [24]. A tumour promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA) equally induces CSF-1 expression in human peripheral blood monocytes [4,23].

Our results confirm previous observations showing *c-fms* complementary transcripts in breast adenocarcinomas [13] as well as in human cell lines of breast [12], endometrial [25], and ovarian [25] origin. Moreover high levels of *c-fms* expression were predictive of aggressive clinical behaviour and poor outcome [25].

The regulation of *fms* expression in tumours of non-hematopoietic origin is not clear but has been shown to be inducible by micromolar concentrations of dexamethasone [13]. This glucocorticoid induced increase in *fms* transcript level was completely abolished by prior treatment with RU486 (Roussel-Uclaf, RU-38486, mifepristone), a potent antagonist of both glucocorticoids and progestins [13]. Whether CSF-1 is capable of inducing its own receptor and what stimulus (regulatory mechanism) initiates CSF-1 production and *fms* expression in glandular epithelial cells awaits further investigation.

Following the CSF-1 receptor activation in cells of the mono-myelocyte lineage, a cascade of biochemical responses is triggered that eventually culminates in DNA synthesis and mitogenesis [26]. Unregulated expression of either CSF-1 or an overactive receptor (overexpressed or rearranged/mutated) in myeloid progenitors can contribute to tumour formation and acquisition of an invasive phenotype [27,28].

We have shown a concomitant expression of *fms* and CSF-1 in invasive carcinoma. Coexpression of both genes was not detected in 14 cases of in-situ carcinoma. Based on these observations, we suggest that autocrine and/or paracrine interactions of CSF-1 with its receptor might cause a change in the biological behaviour of *fms* positive tumour cells, "switching on" an invasive phenotype. Monocytes and placental trophoblast have invasive potential and monocytes can be stimulated to grow and show phagocytic behaviour following stimulation of *fms* by its physiological ligand. As previously suggested, "Many of the adhesive interactions, hydrolytic enzyme

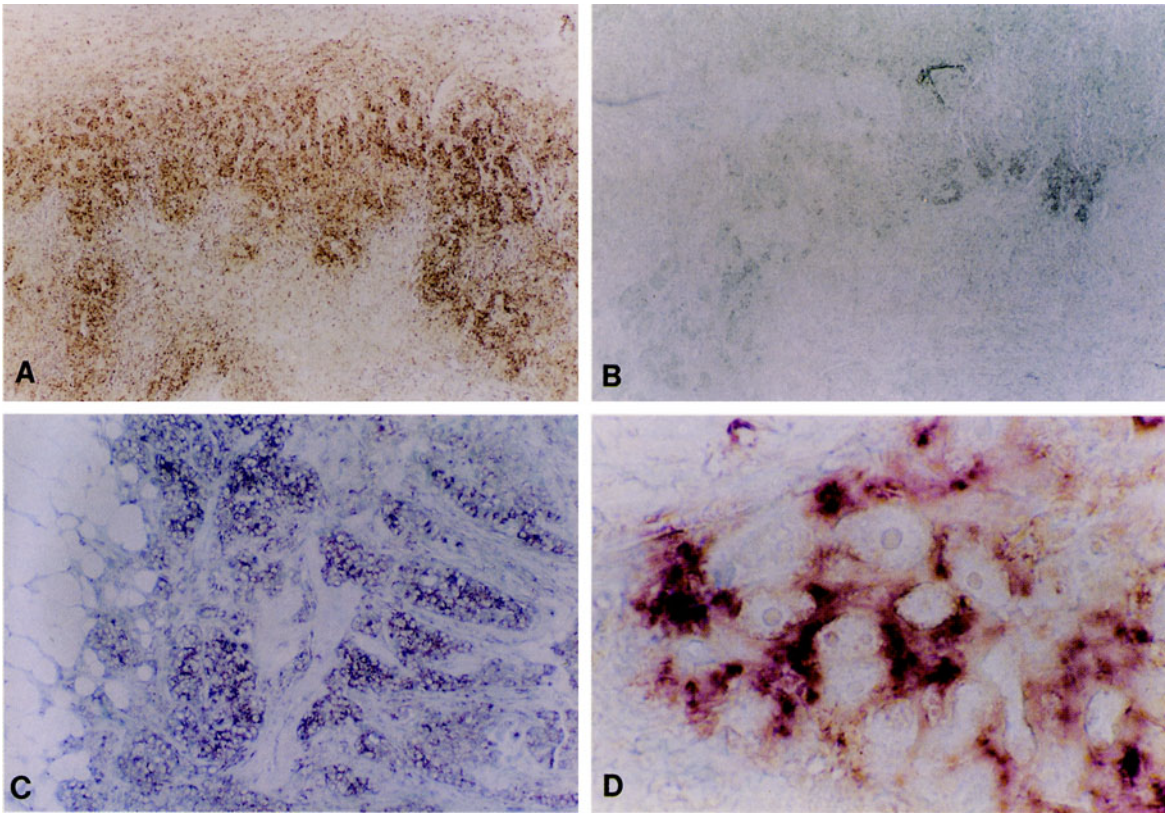

Fig. 3. In situ hybridization with human CSF-1 probes (exon 1–8); anti-sense (**A**) and a negative control using a sense probe (**B**) at low magnification. In a separate tumour photographed at higher magnifications, intracytoplasmic granular staining can be seen (**C, D**). D: 100-fold magnification, oil immersion.

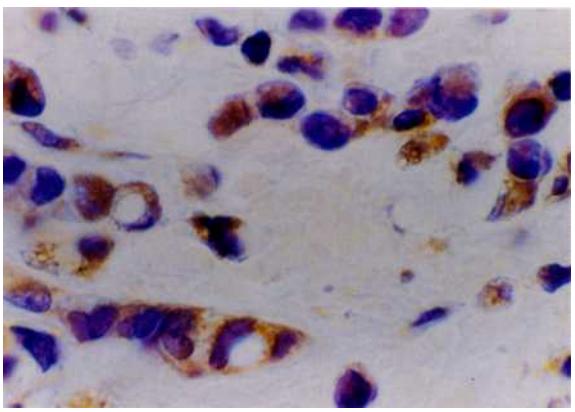

Fig. 4. Immunohistochemical staining of a lobular type tumour section showing positive staining for the CSF-1 receptor. The primary antibody was a monoclonal antibody directed against a tyrosine kinase insert epitope unique to *fms* (2E8; L. Rohrschneider).

activities and chemotactic and proliferative responses of metastatic tumour cells may mimic the equivalent functions of monocytes and macrophages as they migrate across blood vessels to gain access to sites of inflammation" [29].

CONCLUSIONS

In a previous report we have shown that the amplifications of *neu* and *int2* in breast tumour epithelial cells are strongly associated with a marked immune cell infiltrate. In this work, we show that these tumours contain a high proportion of macrophages (15–35%).

Our results concur with those of Kacinski et al. [13] and clearly document that a macrophage growth factor (CSF-1) can be produced by breast tumour cells. Here we present evidence which links the presence of macrophage phenotypic features with stromal invasion by the tumour. We postulate that the autocrine and paracrine interactions between CSF-1 and *fms* play a key role in tumour invasiveness. In fact, many of the attributes of metastatic tumour cells mimic equivalent functions of monocytes and macrophages.

Ongoing experiments address the mechanism of CSF-1 and *fms* induction in these neoplastic cells as well as their potential role in the initiation of metastases.

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