MINIREVIEW

The Role of CSF-1 in Normal Physiology of Mammary Gland and Breast Cancer: An Update

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Colony stimulating factor (CSF-1) and its receptor (CSF-1R, product of c-*fms* proto-oncogene) were initially implicated as essential for normal monocyte development as well as for trophoblastic implantation. However, studies have demonstrated that CSF-1 and CSF-1R have additional roles in mammary gland development during pregnancy and lactation. This apparent role for CSF-1/CSF-1R in normal mammary gland development is very intriguing because this receptor/ligand pair has also been found to be important in the biology of breast cancer in which abnormal expression of CSF-1 and its receptor correlates with tumor cell invasiveness and adverse clinical prognosis. Recent findings also implicate tumor-produced CSF-1 in promotion of bone metastasis in breast cancer, and a certain membrane-associated form of CSF-1 appears to induce immunity against tumors. This review aims to summarize recent findings on the role of CSF-1 and its receptor in normal and neoplastic mammary development that may elucidate potential relationships of growth factor–induced biological changes in the breast during pregnancy and tumor progression. Exp Biol Med 229:1–11, 2004

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Macrophage colony stimulating factor (CSF-1) was first identified as a hematopoietic growth factor that stimulates the proliferation, differentiation and survival of monocytes, macrophages, and their bone marrow progenitors (1–3). CSF-1 is an extensively glycosylated proteoglycan synthesized in both soluble and membrane-bound forms (3). All known forms of CSF-1 bind to a unique cell surface receptor encoded by the cellular homologue of the retroviral oncogene v-*fms*, the c-*fms* proto-oncogene (4). The CSF-1 receptor (CSF-1R) is a member of a family of tyrosine kinase receptors, which includes the α and β forms of platelet-derived growth factor, the receptor for Steel factor (the product of c-kit proto-oncogene), and the receptor for basic fibroblast growth factor (5, 6). Activation of CSF-1R by its ligand triggers a series of rapid events including receptor dimerization, phosphorylation on at least six intracellular tyrosine residues, and association of the intracellular domain of the receptor with a variety of cytoplasmic effector proteins that activate multiple signal transduction pathways controlling cell proliferation and differentiation (7, 8). For example, several typical macrophage traits such as chemotaxis, phagocytosis, the synthesis and secretion of proteolytic enzymes (e.g., urokinase-type plasminogen activator, thromboplastin), and other cytokines (e.g., interleukin-1, TNFα, G-CSF, interferon) are induced by CSF-1 (9, 10).

Several years ago, additional roles for CSF-1 in nonhematopoietic systems were suggested by detection of high level synthesis of CSF-1 by the pregnant uterus coupled with high level expression of its receptor in the placental trophoblast (11, 12). Several independent studies have demonstrated that activation of trophoblastic CSF-1R

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by the locally high levels of CSF-1 produced by the endometrial epithelium is essential for normal embryonic implantation and placental development (13, 14).

Furthermore, several recent findings have implicated a role for CSF-1 and its receptor in the mammary gland during pregnancy (15, 16). In lactating breast epithelium, very high levels of CSF-1 and CSF-1R antigens are expressed (16) as opposed to the undetectable levels of CSF-1R and low levels of CSF-1 found in resting ductal epithelium (17). Studies with CSF-1 mutant mice revealed that during pregnancy there is a significant defect in the mammary gland ductal growth, precocious development of the lobuloalveolar system, and during lactation there is an inability to secrete milk proteins. Such potential roles for CSF-1 and CSF-1R in mammary gland development are intriguing, given their demonstrated biological actions and clinical significance in human breast carcinoma (17). This review will focus on recent attempts to understand the biological significance of the expression of CSF-1 and its receptor during normal and neoplastic mammary gland development.

Physiology of the Mammary Gland During Pregnancy and Lactation

The influence of sex steroid hormones, peptide hormones, and growth factors induces breast tissues to progress through a series of distinct developmental states during fetal life, infancy, childhood, puberty, pregnancy, and menopause (18, 19). Each developmental state is the end result of complex, stage-specific coordinated sequences of cell proliferation, migration, tissue remodeling, and differentiation of mammary epithelial and stromal elements. For example, the lactating mammary gland exhibits highly differentiated epithelial and stromal structures distinct from those seen at any other developmental stage (19, 20).

The first stage of lactogenic development is observed during early pregnancy and is characterized by the proliferation and growth of the cells of the distal portion of the ductal tree to form alveolar structures in response to elevated levels of estrogen and progesterone (20). As pregnancy proceeds, these alveolar lobules increase in size and number with new lobules developing from the lateral walls and termini of ducts. Concomitantly, stromal fat decreases, and the developing glandular structures become highly vascularized. Alveolar epithelial cells are cuboidal and contain large cytoplasmic lipid vesicles. This enhanced proliferation of lobuloalveolar structures requires estrogen, progesterone, prolactin, adrenal corticosteroid, insulin, and growth factors (20). From midpregnancy onward, the rate of mammary epithelial proliferation begins to decline, and the alveolar epithelium differentiates to assume a presecretory function (20). After parturition sudden decreases in the levels of circulating estrogens and progesterone and increases in levels of the lactogenic hormones prolactin, glucocorticoids and insulin, signal the onset of lactation (21). The alteration of hormonal milieu further stimulates the differentiation of the mammary gland to extrude secretory vacuoles which contain fats, a variety of milk proteins such as casein, α-lactalbumin, transferrin/lactoferrin, and many growth factors and other proteins whose functions remain unclear (e.g., whey acidic protein).

CSF-1 in Human Milk

Human milk has been shown to contain large amounts of biologically active CSF-1 (22). Milk CSF-1 is an 80-kDa disulfide-linked homodimer similar to that of serum or urine CSF-1. Maximal milk CSF-1 concentrations can be observed on Day 2 postpartum with an average of 28,000 U/ml; however, 1 month after delivery, milk CSF-1 concentration has rapidly declined to less than 10,000 U/ml (22). The source of milk CSF-1 was investigated in lactating mammary glands by immunohistochemical staining, *in situ* hybridization, and CSF-1 promoter–driven reporter systems and found that CSF-1 messenger RNA (mRNA) and protein are expressed locally by the secretory epithelial cells of the mammary gland (16, 22–24). One report suggested that elevated concentrations of milk CSF-1 during lactation is the mere result of increased numbers of milk secreting epithelial cells because normal nonlactating epithelial cells synthesize significant amount of CSF-1 (22). However, our investigations summarized below clearly demonstrate that the levels of CSF-1 mRNA and protein are significantly upregulated in lactating epithelial cells *in vivo* and *in vitro* (16). A previous study investigated the effect of maternal CSF-1 on newborns and concluded that no significant difference was observed in the circulating levels of CSF-1 of breast-fed infants, compared with those observed in formula-fed babies. This result indicates that milk-borne CSF-1 may feed back negatively on endogenous growth factor levels or act locally in the gastrointestinal tract, or it is possible that milk CSF-1 may have its target of action in the mammary gland itself and not in the neonate (25).

Expression Pattern of CSF-1 and CSF-1R in Mammary Gland During Pregnancy and Lactation

Several immunohistochemical (IHC) and *in situ* studies have revealed that normal resting, nonlactating human breast tissue expresses a low but significant level of CSF-1 without expressing its receptor (17, 26). Such resting state CSF-1 expression has been localized to cells of both the mammary epithelium and the stroma. Levels of expression of CSF-1 and CSF-1R during lactation have also been studied immunohistochemically in paraffin sections from prelactating and lactating breast biopsies (16). All biopsies obtained from actively lactating women and half of the biopsies taken from women in the last trimester of pregnancy showed significant expression of CSF-1 and CSF-1R antigens in the epithelial cells of ducts and alveoli. The intensity of the observed IHC staining is comparable

with that observed when either breast carcinomas or syncytiotrophoblasts are stained for CSF-1R antigen and when breast carcinomas are stained for CSF-1 (26). Similar increases were observed in the levels of CSF-1 and its receptor in mouse mammary gland development during pregnancy. Both antigens were found to be highly elevated in the lactating epithelial cells of the postpartum murine mammary gland (unpublished observation).

Effects of Sex Steroid Hormones on the Expression of CSF-1 and CSF-1R During Pregnancy

The first real evidence for hormonal responsiveness of CSF-1 expression was derived from *in vivo* studies of the role of CSF-1 in mouse female reproductive organs, placenta, and uterus (27). These studies revealed that CSF-1 levels could be increased by estrogen up to 1000-fold in mouse uterus during pregnancy. In ovariectomized mice, concomitant administration of estradiol 17β and progesterone resulted in a dramatic increase in CSF-1 mRNA expression to levels comparable with those detected during pregnancy, suggesting that progesterone is also required for the observed upregulation of CSF-1 (11). CSF-1R expression shows distinctive patterns during mouse and human pregnancy with CSF-1R mRNA first detected in mouse oocytes prior to fertilization. In fertilized oocytes CSF-1R expression could be observed from the two- to four-cell stage until implantation (28). After implantation CSF-1R mRNA is detectable in the decidua (28). In human pregnancy CSF-1R expression has been observed in both placenta and uterus (29). In placenta the most intense expression was observed in syncytio- and invasive trophoblasts, which contain proliferating, invasive cells. To date the mechanisms that underlie the above-described hormonal regulation of CSF-1R expression in placenta and uterus remain unknown; however, it has been suggested that estradiol 17β progesterone, or both might regulate CSF-1R synthesis to synchronize the maternal preparation of the uterus with embryonic development (13). Analysis of CSF-1R promoter sequence suggested the presence of retinoic acid response elements (30) and both estrogen and progesterone receptor response elements (unpublished observations) in addition to glucocorticoid response elements (GREs) discussed below.

Effects of Lactogenic Hormones on the Expression of CSF-1 and CSF-1R in Mammary Epithelial Cells

The dramatic increase in the expression of CSF-1 and CSF-1R antigens in lactating mammary epithelial cells strongly suggests that lactogenic hormones (prolactin, insulin, and glucocorticoids) might regulate the expression of both genes. The first evidence for such regulation derives from our own studies of the hormonal responsiveness of CSF-1R expression in neoplastic mammary epithelial cells lines (31). Levels of CSF-1R (c-*fms*) transcripts rapidly increase after dexamethasone (a potent synthetic glucocorticoid) treatment from barely detectable levels in untreated cells to levels 6-fold higher by 2 hrs and 25- to 50-fold higher by 8-24 hrs. A similar but much weaker effect was observed with progestins (most likely by cross-reactivity of high levels of progestins at the glucocorticoid receptor), but no effects were observed in these cell lines with prolactin, insulin, estrogens, androgens, and mineralocorticoids (25). Glucocorticoid-induced increases in the level of c-*fms* mRNA and protein could be blocked by pretreatment of breast carcinoma cells with the potent glucocorticoid competitive antagonist, mifepristone (RU486), a result that demonstrates that glucocorticoid-induced changes in c-*fms* transcript levels are mediated through ligand-activated glucocorticoid receptor (31).

Employing promoter/reporter constructs in which each of the known c-*fms* promoters was cloned upstream of firefly luciferase reporter gene, we demonstrated that most of the observed effects of glucocorticoids on c-*fms* transcript levels in mammary epithelial cells resulted from hormone stimulation of c-*fms* gene transcription (31). Computer analysis of the sequence of the *fms* promoters revealed that both contained several potential GREs. In each case, elimination of the GRE closest to the promoter (by *in situ* point mutagenesis or deletion) abolished all glucocorticoid stimulation of promoter activity in reporter gene constructs, demonstrating that the observed GRE elements found in the c-*fms* promoters are functional (31).

Further investigation of this promoter region showed that it contains an additional composite transcription factor–binding element that includes a consensus activator protein 1 (AP-1) sequence (TGAGTCA), an E-box (CAGGTG, binding site for basic-Helix-Loop-Helix domain-containing transcription factors) overlapping the AP-1 site and a potential GRE (GGTGAGTCATGATCA; Ref. 32). Comparing nuclear extracts from a breast carcinoma cell line that can be induced to express c-*fms* by glucocorticoids with one that does not respond showed significant difference in proteins that bind this sequence element. Mutation of the AP-1 site of the composite GRE significantly reduces the basal activity of the first promoter in cells not exposed to glucocorticoids and renders the promoter unresponsive to glucocorticoid stimulation (32). In summary, the ability to hormonally induce c-*fms* from the first promoter seems to require a well-regulated set of factors as might be expected for developmental and tissue-specific regulation.

The potential regulation of the expression of CSF-1 and c-*fms* genes by lactogenic hormones was studied *in vitro* on organ cultures of normal human breast exposed to lactogenic hormones: insulin, prolactin, and glucocorticoids. The organ cultures were prepared from slices of reduction mammoplasty biopsies obtained from normal-cycling females (16). Mammary epithelial organ cultures were maintained in defined media supplemented with certain cytokines and the three essential lactogenic hormones

(prolactin, glucocorticoids, and insulin) for 14 days. CSF-1 and c-*fms* expression were monitored by IHC studies on the paraffin sections of the organ culture samples. In agreement with previous *in vivo* IHC results on lactating breast specimens, significant increases in the of CSF-1 and c-*fms* genes were observed in the epithelial cells of mammary gland organ samples exposed to lactogenic hormones *in vitro* (16).

The increase in CSF-1R, but not in CSF-1, expression could be blocked by mifepristone (RU486), a glucocorticoid competitive antagonist, consistent with the effects of this agent on breast carcinoma cell lines (31). The latter result strongly suggests that glucocorticoids can regulate the expression of c-*fms* (CSF-1R) without affecting the expression of its ligand during lactogenic differentiation. This finding also indicates that insulin and prolactin might be essential for upregulation of CSF-1 expression during lactogenesis.

The role of prolactin and insulin was demonstrated by *in vitro* studies of CSF-1 expression in the normal mammary epithelial cell line (HC11) cultured in the presence of lactogenic hormones. The HC11 cell line was originally isolated from midpregnant mouse mammary gland tissue (33) and has been found to express significant levels of CSF-1 without expressing its receptor (34). HC11 cells retain important features of normal mammary epithelial cells as evidenced by their ability to differentiate and synthesize the milk protein β-casein after exposure to lactogenic hormones (33). These features make HC11 cells an ideal model for the study of gene expression during lactogenesis. Northern blot analysis demonstrated that exposure of HC11 cells to insulin or prolactin for 24 hrs could increase CSF-1 mRNA levels by 5- and 10-fold, respectively (16). When HC11 cells were treated with insulin and prolactin together for the same period of time, a significantly greater effect (~30-fold) was found on the induction of CSF-1 mRNA levels (16). Glucocorticoids had no effect on CSF-1 mRNA levels on their own, and they did not potentiate the effects of either of the two peptide hormones, insulin and prolactin, over those seen with each hormone alone (16). A recent report, however, suggested that glucocorticoids are able to stimulate the expression of the membrane form of CSF-1 at least in murine osteoblast cells (35).

Regulation of CSF-1 expression in many cell types appears to be a consequence of alteration of both transcript half-life and transcription rate (36, 37). Our own preliminary studies revealed that increases in CSF-1 mRNA levels by the peptide hormones prolactin and insulin are the consequence of both upregulation of the promoter activity of CSF-1 and prolongation of CSF-1 mRNA half-life by those lactogens (unpublished observation). These findings are fascinating because they appear to add CSF-1 to the growing list of so-called milk proteins whose expression is regulated by lactogenic hormones. Such an effect not only helps to account for the high levels of CSF-1 expression observed in lactating mammary epithelial cells *in vivo* and *in vitro* but also for the presence of significant levels of CSF-1 in milk. They also strongly suggest that the concerted action of the three lactogenic hormones (glucocorticoids, insulin, and prolactin) induces the expression of both CSF-1 and its receptor in mammary epithelial cells providing an autocrine loop for this growth factor/receptor pair during pregnancy and lactation.

The Role of Expression of CSF-1 and CSF-1R in Mammary Gland Development During Pregnancy

Several *in vivo* studies have investigated the potential role of CSF-1/CSF-1R during mammary gland development (15). Homozygous mutant (op^-/op^-) mice, whose specific genetic defect inactivated both copies of CSF-1 gene, are osteopetrotic consequent to a deficiency in osteoclast function; have severely reduced numbers of macrophages, impaired neuronal processing, and immune defects; and have reduced fertility and scant milk production after parturition. Investigation of the mammary gland development in op^-/op^- mothers revealed that the observed lactational defect is the result of incomplete mammary gland ductal growth, precocious development of the lobuloalveolar system, and inability to secrete milk proteins during lactation. Further examination of the op^-/op^- mice also revealed impaired branching morphogenesis during development (23).

To confirm that CSF-1 receptor is the only receptor for CSF-1, a recent study investigated the effect of null mutation introduced into CSF-1 receptor gene (38). Mice homozygous for this mutation ($Csf1r^-/Csf1r^-$) have an almost identical phenotype to the op^-/op^- mice, including failure of normal lactating mammary gland development (38).

Further studies showed that reintroduction of CSF-1 in the mammary epithelium of op^-/op^- mice using a tetracycline-binary transgenic system in which CSF-1 expression under the control of a mammary-specific promoter system (MMTV) demonstrated that local synthesis of CSF-1 in mammary epithelial cells recruits macrophages to that site and macrophage action are required throughout early mammary gland development (39).

What could be the key function(s) of macrophages during ductal outgrowth? Ductal outgrowth involves remodeling the mammary fat, and proteolytic enzymes play an important role in this process. Proteolytic enzymes such as certain metalloproteases and urokinase plasminogen activator (uPA) are known to be expressed by macrophages (40, 41). Furthermore, macrophages can activate cytokines like TGF-β1, which was shown to be an important factor of branching morphogenesis (42). Also, it was suggested that macrophages could remove apoptotic cells from the terminal buds during morphogenesis.

In summary, these recent findings clearly demonstrated that macrophages regulated by locally produced CSF-1 and circulating CSF-1 could potentiate multiple stages of mammary gland differentiation from branching morphogen-

esis during the establishment of the ductal tree to ductal development during pregnancy.

Expression of CSF-1 and CSF-1R in Breast and Other Epithelial Carcinomas

As described above, CSF-1R was originally identified as the oncogene (v-*fms*) transduced by a feline oncogenic retrovirus, which causes fibrosarcomas in cats (4). In earlier studies investigators have questioned whether abnormal expression of the cellular counterpart (c-*fms*) or mutation of c-*fms* gene can render normal human cells tumorigenic. Multiple studies have investigated the expression pattern of CSF-1 and CSF-1R in normal and neoplastic tissues and cell lines (17, 25, 43–52). Abnormal expression of CSF-1R, with or without CSF-1, has been documented in a wide variety of human carcinomas and carcinoma-derived cell lines including tumors of epithelial origin such as carcinomas of breast, ovary, endometrium, lung, kidney, pancreas, and prostate (17, 25, 43–52).

Previous IHC and *in situ* studies have demonstrated that 58% of all and 85% of invasive breast carcinomas expressed CSF-1R at levels comparable with those observed in trophoblast and macrophages, and, in these cases, CSF-1R expression was clearly localized in the neoplastic epithelial cells of the tumors as well as to stromal macrophages (25, 48). Furthermore, several earlier studies have reported that expression of CSF-1R in ovarian and endometrial carcinomas strongly correlates with such adverse prognostic features as high histologic grade and advanced clinical stage at presentation (50, 51). The prognostic value of CSF-1R expression in mammary epithelial tumors has been documented recently in a study of ipsilateral breast cancer recurrence (53). The activation state of CSF-1R was also studied on breast carcinoma specimens by IHC studies using an antibody that recognizes CSF-1R only after activation by its ligand or activating mutation(s). This study revealed that 52% of CSF-1R positive breast carcinoma express activated CSF-1Rs (54). Activation of CSF-1R by its ligand could occur in invasive tumor cells in which CSF-1R and CSF-1 are co-expressed (i.e., autocrine activation), or tumor cell CSF-1Rs could also be stimulated indirectly by CSF-1 synthesized by infiltrating monocytes or fibroblasts found in the tumor stroma (i.e., paracrine activation). Recent findings suggested that the effect of autocrine activation of CSF-1 receptor might predominate over paracrine effects, at least in ovarian carcinoma (55).

Given that tumor cells synthesize CSF-1 in quantities high enough to be readily detected by IHC studies, it is not surprising that significant levels of apparently tumor-produced CSF-1 are found in the serum and ascites of patients with breast and other carcinomas (56–62). High circulating levels of CSF-1 have been reported to strongly correlate with active or recurrent disease in patients with ovarian and endometrial carcinoma (59–62), and with rapidly progressive metastatic disease in patients with breast carcinoma (56–58). An *in vitro* study also showed that the most invasive breast carcinoma cell lines co-expressed elevated levels of CSF-1 and uPA, a serine protease involved in extracellular matrix degradation (63). A recent finding showed that breast cancer cells could stimulate osteoclastogenesis and prolong osteoclast survival by expressing and secreting CSF-1 (64). This result was in a good agreement with another *in vivo* metastasis study that found an elevated level of CSF-1 in human breast carcinoma cell line that had invaded the bone, compared with cells that had invaded soft tissues (65). Results from both studies strongly suggested that CSF-1 might be an important factor in pathogenesis of osteolytic bone metastasis in breast cancer.

Furthermore, serum levels of CSF-1 in terminally ill breast cancer patients were reported to be increased to more than 10-fold normal levels, whereas IHC evidence of CSF-1 expression was found to be higher at the site of metastatic recurrence (56–58). Also, individual, stromally invasive breast carcinoma cells consistently expressed CSF-1, which was often not expressed in adjacent noninvasive, *in situ* carcinoma (56–58). The prognostic value of circulating CSF-1 has been evaluated in a pilot study with 118 breast cancer patients with primary and 75 patients with metastatic disease. Circulating CSF-1 levels were found significantly elevated in patients with metastatic disease and also were associated with shorter relapse interval (66).

In addition, expression of CSF-1 by breast carcinoma has itself been associated with genomic amplification of int-2 and ErbB2/neu proteins whose amplification, overexpression, or both were found to be indicators of poor prognosis in breast carcinoma (56, 67). CSF-1 expression in primary breast adenocarcinomas also correlates with infiltration of inflammatory cells and prognosis (68). This latter result strongly indicates that tumor-produced CSF-1 can recruit and activate large numbers of monocytes that in turn release trophic cytokines and other growth factors to enhance tumor cell growth. This hypothesis is also supported by another finding in which exogenous CSF-1 enabled macrophages to stimulate tumor growth in the CSF-1 deficient mouse (op^-/op^- mouse, see above) model system (69).

The above-mentioned *in vivo* findings are very intriguing, but unfortunately they do not explain the exact role of CSF-1 and its receptor in epithelial tumor development. To further characterize such a role, we also need *in vitro* systems (e.g., established epithelial cell lines) in which we can study the effect of the abnormal expression of CSF-1R with or without the expression of CSF-1.

Transformation Potential of c-*fms* Proto-Oncogene

A feline sarcoma virus containing the v-*fms* sequence was shown to induce fibrosarcomas when inoculated into cats (70). Analysis of the v-*fms* gene product demonstrated that it is constitutively active as a tyrosine kinase and provides tonic signals for cell growth in the absence of CSF-

1 (4, 6). The sequence changes necessary to convert the c-*fms* gene to an active oncogene were analyzed by comparison of v-*fms* and the feline cellular homologue c-*fms* gene (71–74). Construction of chimeric *fms* proteins along with site-directed and random chemical mutagenesis have demonstrated that multiple changes are necessary for the full activation of the oncogenic potential of the c-*fms* gene (73, 75). These include several mutations in the extracellular domain as well as truncations and point mutations in the carboxy-terminus of c-*fms* gene (71–75).

In addition, previous studies investigated a potential autocrine mechanism of transformation caused by overexpression or inappropriate expression of normal c-*fms* gene in nonmalignant cells synthesizing endogenous CSF-1. These studies revealed that overexpression of normal c-*fms* gene (cloned into a retroviral vector and transfected into CSF-1-expressing normal cells) can be sufficient to induce a fully transformed phenotype in fibroblast and epithelial cells (34, 76). This latter transformation mechanism provides an example of the importance of the abnormal (uncontrolled) coexpression of a growth factor/receptor pair in the initiation of a neoplastic phenotype in nonmalignant cells. However, we cannot rule out the possibility that both of the host cell lines used in these experiments might also contain uncharacterized mutations, which could account for some of the aspects of their transformed phenotypes.

Expression of CSF-1 and CSF-1R by Neoplastic Mammary Epithelial Cell Lines

In vitro expression of CSF-1 transcript and protein have been observed in mammary epithelial cells of benign (e.g., murine HC11 cells) and neoplastic (e.g., human SKBR3 cells) origin, whereas the expression of functional CSF-1R has been documented in several breast carcinoma cell lines (e.g., human BT20 and SKBR3 cells, Ref. 16, 17, 25, 34). Sequence analysis of CSF-1R mRNAs from these neoplastic cell lines indicated that their protein coding sequences do not significantly differ from those expressed by normal macrophages or placental trophoblast (25). Hence, activation of CSF-1R in breast carcinoma is probably not due to any known activating mutation(s) previously described in the c-*fms* gene sequence. For that reason, several years ago we and others began to study the effect of the activation of CSF-1R by its ligand on several cellular characteristics of normal and neoplastic mammary epithelial cells.

CSF-1/CSF-1R-Induced Cellular Invasiveness and Anchorage-Independent Growth in Mammary Epithelial Cells

Several reports have demonstrated that CSF-1R activation regulates normal cellular migration of macrophages through uPA mechanisms (3, 40, 41). The first indirect evidence for a similar involvement of CSF-1R in mammary epithelial cell invasion has come from a study that investigated migration through amniotic membrane by a CSF-1R positive breast carcinoma cell line (77). It was shown that dexamethasone (which increases CSF-1R levels, see above) and CSF-1 together could significantly enhance *in vitro* invasion and uPA production of these carcinoma cells (77). Also, it was reported that uPA mediates stimulation of invasion by CSF-1 in ovarian carcinoma cells *in vitro* (78). In addition, transfection of the wild type c-*fms* gene into a normal, noninvasive mammary epithelial cell line (HC11), which expresses CSF-1, resulted in a dramatic stimulation of the invasive phenotype and anchorage-independent growth of these cells (34). The stimulatory effect of c-*fms* expression on invasion by HC11 cells was shown to be efficiently blocked by specific inhibitors of the uPA/collagenase proteolytic cascade (34).

In the same study, the importance of the phosphorylation of specific tyrosine residues of CSF-1R in the autocrine activation of the CSF-1 receptor was also studied. Two major autophosphorylation sites were mutated (Tyr–>Phe 807 or Tyr–>Phe 721) and analyzed for their potential effect on CSF-1 receptor induced invasion and anchorage-independent growth in HC11 cells. Previous analyses of such phosphorylation site mutants showed that Tyr807 and Tyr721 play important roles in coupling the CSF-1 receptor to distinct intracellular signal transduction pathways (Fig. 1; Ref. 79–82). The Tyr807 site in particular was demonstrated to be crucial for CSF-1-dependent monocytic differentiation (81), whereas the Tyr721 site of the CSF-1 receptor is required for CSF-1 dependent mitogenesis in macrophages (82). Although mutations at Tyr807 reduced significantly the stimulatory effect of c-*fms* expression on the invasive ability of HC11 cells *in vitro* and *in vivo*, mutation at Tyr721 of c-*fms* had no effect on *in vitro* invasion (34). In contrast, mutations of Tyr721, but not of Tyr807, had a significant effect on c-*fms* induced anchorage-independent growth and the *in vivo* metastatic potential of the transfected cells (34).

These data clearly demonstrated that anchorage-independent growth and cellular invasiveness, two crucial steps in tumor development, could be independently regulated by separate sites of tyrosine-phosphorylation of CSF-1R. Because phosphorylation of these specific tyrosines activates completely separate secondary intracellular pathways (7, 8), these findings also indicate that anchorage-independent growth and invasiveness might be regulated independently by CSF-1R in mammary epithelial cells.

This HC11 system was recently utilized to identify genes that were differentially expressed in the parental HC11 cells and clonal cells transfected with wild-type CSF1R and to determine the effect of mutating phosphorylation sites 721 and 807 on gene expression levels, using a 4.6 K cDNA microarray system (83). The study found 52 genes that consistently revealed differential expression of close to 2-fold or more (with a confidence interval of 99%) between the HC11 cells transfected with wild type CSF-1 receptor and either parental HC11 line or HC11 clonal cells transfected with one of the Tyr–>Phe mutant CSF-1Rs.

Genes found to be differentially expressed include mitogen-activated protein kinase phosphatase 1, WDNM1 (also called extracellular proteinase inhibitor), trop 2 (also known as tumor-associated calcium signal transducer 2), procollagen type IV alpha, secretory leukoprotease inhibitor, tissue inhibitor of metalloproteinase 2, tubulin, alpha 4, and chaperonin 10. Many of the identified genes have not previously been shown to be associated with tumor invasion or metastasis. This study has also successfully linked certain genes to either the invasive or to the tumorigenetic phenotypes (83).

Potential Role of CSF-1/CSF-1R in Neoplastic Mammary Epithelial Cells

The parallels in the observed patterns of expression of CSF-1 and CSF-1R during lactogenic differentiation and tumor development become somewhat less surprising when one recognizes that many of the events that occur during normal mammary gland development have strong similarities to those observed during tumor progression. For example, normal resting ductal and alveolar epithelial elements proliferate and synthesize a variety of proteases (84) able to digest, remodel, and invade stromal elements to produce the extensively branched ductal and alveolar structures characteristic of the lactating breast. Although in normal mammary development these processes are strictly controlled not only by hormones (20) but also by a variety of growth factors and tyrosine kinases (85–87), adhesion molecules (88, 89), and transcription factors (90–92), it is not difficult to imagine that abnormal activation of this otherwise normal developmental program could yield a malignant phenotype in mammary epithelial cells. In fact, numerous experimental and epidemiological studies have suggested that specific details in the development of the mammary gland play a critical role in breast cancer risk (93). As such, even nonmalignant precursors of malignant cells possess the metabolic program to direct rapid proliferation and the secretion of proteolytic enzymes employed by neoplastic cells to invade the stroma and eventually disseminate (94).

For example, in normal mammary epithelial cells, activation of the CSF-1/CSF-1R induced signaling pathways dramatically stimulates cellular invasiveness through a uPA-dependent pathway *in vitro* (34, see above). This same pathway might be very crucial to normal mammary development because it has been shown that during lactogenesis ductal and alveolar epithelial elements express CSF-1/CSF-1R and synthesize a variety of proteolytic enzymes including uPA and other gelatin and casein-degrading proteinases (84, 94). In breast cancer, CSF-1/CSF-1R and uPA expression each appear to correlate with tumor invasiveness and an adverse clinical prognosis (58, 95). These parallel observations strongly suggest that one of the major functions for CSF-1/CSF-1R in normal and neoplastic cells might be the activation of uPA-dependent proteolytic pathways and the induction of cellular invasiveness (Fig. 2; Ref. 57). Also, recent evidence suggested that CSF-1 might not only act as an autocrine factor for breast cancer cells but also to recruit macrophages to the tumor in which they promote tumor progression.

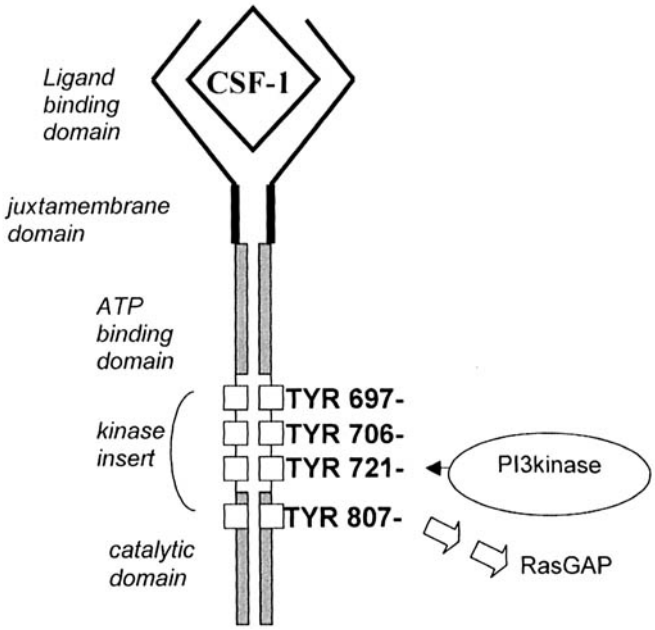

Figure 1. Activation of CSF-1 receptor by its ligand. Binding CSF-1 to its receptor triggers receptor dimerization, phosphorylation on at least six intracellular tyrosine residues (four of them located in the kinase insert domain as shown in the figure), and association of the intracellular domain of the receptor with a variety of cytoplasmic effector proteins. For example, phosphorylated tyrosine 721 binds to phosphatidylinositol 3-kinase (PI3 kinase) and activates the PI3 kinase pathway, and phosphorylated tyrosine 807 activates the RasGap signaling pathway.

The role of CSF-1 in metastatic process was further addressed with an elegant study, in which a mouse mammary cancer model was established by crossing a transgenic mouse susceptible to mammary cancer with mice containing a recessive null mutation in the CSF-1 gene, and tumor progression was followed up in these mice (96). Absence of CSF-1 had no effect on mammary tumor initiation and growth but significantly reduced primary tumor progression to malignancy as assessed by tumor morphology and lung metastasis. On the other hand, transgenic expression of CSF-1 in the wild type and null mutant mice led to a dramatic increase in tumor progression and pulmonary metastasis.

Potential Role of Membrane CSF-1 in Breast Cancer-Specific Immunity

It has been widely shown that breast tumors contain marked leukocytic infiltration, which significantly correlates with poor prognosis (97). The majority of these cells are macrophages, and CSF-1 is the main chemoattractant for these cells. Certainly one can ask why these macrophages do not have cancer-killing activity. Why are breast cancer cells

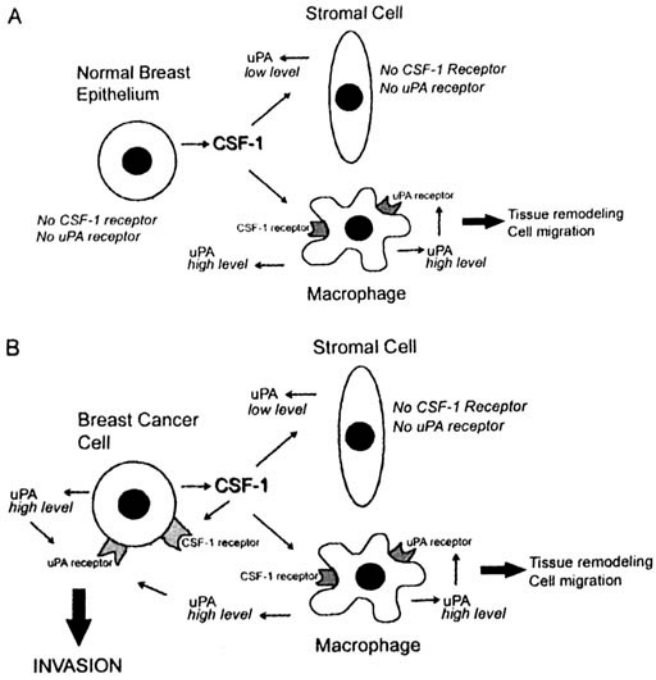

Figure 2. Autocrine and paracrine interaction between epithelium and adjacent stroma in normal breast (A) and breast cancer (B). Normal breast epithelium secretes significant amounts of CSF-1, which can trigger high levels of uPA in CSF-1 and uPA receptor-positive macrophages, which leads to enhanced macrophage migration and tissue remodeling. Normal breast epithelium does not express receptor for CSF-1 and uPA; therefore, it is not sensitive to CSF-1 or uPA stimuli from normal stromal fibroblast and/or macrophages. However, breast cancer cells express significant amounts of CSF1 and uPA receptor, which make breast cancer cells responsive to internal and external stimuli or CSF-1 and uPA and could explain their significant invasive phenotype.

immune to the immune response and what factors are important in the immunity?

A recent fascinating observation suggested that a membrane form of CSF-1 (mCSF-1) introduced into a highly malignant rat breast cancer cell line (MADB106) can induce breast cancer–specific immunity (98). When the authors transduced the mCSF-1 into MADB106 cells and then subcutaneously inoculated the cells into normal rats, the animals not only rejected the mCSF-1-transfected cells but more interestingly showed rechallenge resistance to unmodified parental cells but not to a glioma cell line.

The biological function of the membrane isoform of CSF-1 was also studied in T9 glioma cells in which the expression of mCSF-1 but not the secreted form of CSF-1 induced macrophage-mediated killing of these transfected cells (99). However, when the mCSF-1-transfected cells were injected into rats, no tumor growth occurred. Taken together, these findings strongly indicated that the membrane form of CSF-1 could induce organ-specific immunity. Agents modulating the expression of the membrane form of CSF-1 such as dexamethasone (see above) are already being investigated for their potential effect on breast cancer immunity. An interesting hypothesis was proposed recently by Dan *et al.* (100), which suggested that if we were able to convert breast cancer cells from making the sCSF-1 form to making the mCSF-1 form, it may be possible to stimulate the infiltrating macrophages into becoming cytotoxic macrophages, which would eliminate the tumor.

Summary

CSF-1 and CSF-1R appear to be important to the physiology of normal and neoplastic mammary gland (summarized in Table 1). Levels of CSF-1 and CSF-1R expression are upregulated by hormones implicated in normal mammary gland development. Results from CSF-1-deficient mice strongly demonstrated that this ligand/receptor pair is important in multiple stages of mammary gland differentiation from ductal and alveolar development to milk protein secretion. In breast cancer, levels of CSF-1 and CSF-1R expression were shown to correlate with tumor cell invasiveness and adverse clinical prognosis. In summary, these findings clearly demonstrate that a greater understanding of the role of growth factors and their receptors in normal mammary gland development has obvious relevance to the field of breast cancer, and it is quite possible that hormonal or pharmacologic therapies that interfere with the expression of CSF-1 and its receptor and/or the intracellular signal transduction pathways they regulate could be useful adjuncts to the treatment of human breast cancer.

Table 1. Comparison of the Expression and Potential Function of CSF-1 and CSF-1R in Normal Breast Epithelium and Breast Cancer

Factor	Normal breast epithelium	Breast cancer
CSF-1 expression	Detectable level	Detectable level
CSF-1R expression	No expression in nonpregnant breast; high level expression during pregnancy	High level expression in 58% of all and 85% of invasive breast carcinoma
Function of CSF-1/CSF-1R	Ductal and alveolar development; milk production; breast remodeling during pregnancy and lactation	Anchorage-independent growth tumor progression; invasion and metastasis; macrophage recruitment; breast cancer immunity

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