Overexpression of the Colony-Stimulating Factor (CSF-1) and/or Its Receptor c-fms in Mammary Glands of Transgenic Mice Results in Hyperplasia and Tumor Formation

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ABSTRACT

A number of recent studies have suggested that the colony-stimulating factor (CSF-1) and its receptor c-fms may be involved in the development of mammary glands during lactation and breast cancer. To study the role of CSF-1 or its receptor in initiation of mammary tumorigenesis, we have generated two independent lines of transgenic mice that overexpress either CSF-1 or c-fms under the control of the mouse mammary tumor virus promoter. Mammary glands of the virgin CSF-1 transgenic mice show increased ductal branching, hyperplasia, dysplasia, and other neoplastic changes, which are indicative of increased cellular proliferation. Similar changes were also evident in the mammary glands of the c-fms transgenic mice. These changes became more prominent with age and resulted in mammary tumor formation. Moreover, secondary events like dimethylbenz(a)anthracene treatment accelerated mammary tumor formation in these mice. Although the expression of estrogen receptor α was not significantly changed in either of the transgenic mouse strains, progesterone receptor levels was higher in both transgenic lines as compared with the nontransgenic littermates. Expression of G1 cyclins was prominently increased in the mammary glands of both the CSF-1 and c-fms transgenic lines, suggesting increased cell cycle progression in these strains. In addition, the proliferation marker proliferating cell nuclear antigen (PCNA) and the mitogen-responsive transcription factor c-Jun were also increased as compared with the nontransgenic controls. These findings, along with the histological data, support the hypothesis that CSF-1 and its receptor are involved in the etiology of breast cancer.

INTRODUCTION

Breast cancer is the most prevalent type of cancer observed among nonsmoking women. The majority of primary breast cancers is hormone dependent and is associated with increased levels of the estrogen receptor (ER; Ref. 1). Several genes, including growth factors, proto-oncogenes, and tumor suppressors, have also been directly implicated in human breast cancer by repeated identification of mutant forms or overexpression in tumors obtained directly from patients (2, 3).

The macrophage colony-stimulating factor (CSF-1) is a proteoglycan hematopoietic factor that was first detected in macrophages and monocytes. The binding of CSF-1 to its receptor c-fms, which is a cell surface receptor belonging to a family of tyrosine kinase receptors, results in the dimerization and phosphorylation of c-fms, leading to macrophage proliferation via signal transduction pathways. Studies involving certain hematopoietic growth factors, such as CSF-1, have demonstrated that these substrates also affect normal and malignant cells of nonhematopoietic origin (4). High levels of CSF-1 were detected in pregnant human and mouse uterus as well as the placenta, suggesting a role for this growth factor in these tissues during pregnancy. High expression of CSF-1 has also been associated with a variety of gynecological cancers (5). For example, high expression of CSF-1 and c-fms was detected in ovarian adenocarcinoma.

CSF-1 is also involved in mammary development, as suggested by observations from mice homozygous for the osteopetrotic mutation (op/op), designated recently CSF1op/CSF1op (6). These mice completely lack active CSF-1 because of a frame shift in the CSF-1 open reading frame resulting in a severely truncated protein (6). Homozygous CSF1op/CSF1op mice exhibit lactation defect due to incomplete mammary gland ductal growth, precocious development of the lobular alveolar system, and, despite the expression of milk proteins, a failure to switch to the lactational state (6). Restoring normal circulating levels with externally supplied recombinant (human) CSF-1 enabled the correction of the lactational defect in these mice (5). These data suggest that CSF-1 has a role in the development of mammary glands during pregnancy. In addition, immunohistochemical studies detected high levels of CSF-1 in breast carcinoma patients (5). High CSF-1 levels were also found in the serum of patients with aggressive metastatic breast carcinomas (5). High levels of c-fms without the expression of CSF-1 have also been detected in ovarian, endometrial, and breast cancer cell lines (5). The above studies suggest the possible association of malignant phenotype and expression of CSF-1 and c-fms in the same tumor. These studies also indicate that autocrine and paracrine interactions of malignant phenotype and expression of CSF-1 and c-fms may participate in the biology of human neoplasms, including breast cancer.

Recent studies by Lin et al. (6) have shown that the absence of CSF-1 (CSF1−/−/CSF1−/−) in mammary cancer-susceptible polyoma middle T antigen (PyMT) mice retarded tumor progression and metastasis but did not affect primary tumor development. Conversely, overexpression of CSF-1 in the PyMT background resulted in the acceleration of malignant progression and metastasis.

Although the studies by Lin et al. (6) strongly suggest that CSF-1 is involved in tumor progression and metastasis, they did not directly address whether the overexpression of CSF-1 or its receptor c-fms results in initiation of changes that could lead to tumor development. The aim of our study was to investigate the roles of CSF-1 and/or c-fms as initiating factors in mammary carcinogenesis. For this purpose, we have generated a CSF-1 transgenic mouse line and a c-fms transgenic mouse line that overexpress CSF-1 and c-fms, respectively, under the regulation of the mouse mammary tumor virus (MMTV) promoter. These mice exhibited hyperplastic developments of the mammary glands and decreased gland regression during involution, as well as severe dysplasia and spontaneous tumor development with age, which was accelerated with 7,12-dimethylbenz(a)anthracene (DMBA) treatment.

MATERIALS AND METHODS

Generation of Transgenic Mice. The CSF-1 transgene was constructed by inserting the human 1.6-kb CSF-1 cDNA (ATCC no. 53149) next to the
MAM-mammary tissue were prepared after fixation in 10% neutral buffered formalin. After subcloning the CSF-1 cDNA, the MAM-neo blue was modified as described in Tekmal et al. (7). The integrity of the final transgene construction (MMTV-CSF-1) was verified by restriction enzyme mapping and nucleotide sequence (data not shown). Before the microinjection, the transgene construction was freed of all of the bacterial sequences 5′ to the gene by digestion with PvuI, producing an 8.5-kb linear DNA fragment. The 8.5-kb linear DNA that was injected into the fertilized eggs of FVB strain mice consisted of the MMTV-LTR, CSF-1 cDNA, SV40 early-splicing region and polyadenylation sequences, and the neomycin gene with SV40 regulatory sequences. Tail DNA from weanling mice derived from the transgene-injected eggs was analyzed by Southern blot hybridization using CSF-1 cDNA as the probe. Similarly, the c-fms transgenic mice were generated by inserting the 3.7-kb c-fms cDNA next to the MMTV promoter in the modified MAM neo-blue vector (7). A 9-kb EcoRI fragment containing MMTV-c-fms was microinjected into fertilized ova of FVB mice. The mice were housed in a centralized animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care and United States Department of Agriculture and were maintained according to the recommendations established in the NIH guide for the Care and Use of Laboratory Animals.

**RNA Analysis.** Total RNA was isolated from mice mammary glands using the Tri-reagent according to the manufacturer’s protocol (Sigma, St. Louis, MO). Two hundred ng of the total RNA isolated from nontransgenic and CSF-1 transgenic mice was then used in reverse transcription (RT)-PCR to amplify human CSF-1-, endogenous mouse CSF-1-, and endogenous mouse c-fms-specific mRNAs. The human CSF-1-specific primer sequences used for the amplification were ATGACAGACAGTGGACTCGAG and TCACAACAATCAGTAGGTCAGG. The mouse CSF-1-specific primer sequences were CCGGATCTACTGCTTCTGCTGACTG and ATAGTGGCAGGATGTTGGGCGATCCCT. The mouse c-fms-specific primer sequences were GACCTGAGAAGGACAGACCTGACTG and GTGCACAGGTGGCATGTAATTGAGGGT. RNA amplification of CSF-1 and c-fms was carried out as described previously, and the PCR products were visualized by gel electrophoresis using 1% agarose gels. Real-time PCR was also performed on cDNA from RT reactions, using platinum Taq polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. SYBR green dye (1X; Fisher Scientific; Atlanta, GA) was added to the reaction mixture to detect amplicon synthesis in the SmartCycler real-time PCR thermal cycler (Cepheid, Sunnyvale, CA). For quantification, the cycle threshold number (Ct) exhibiting the maximum curve growth rate was determined by the Cepheid SmartCycler software. The relative gene expression of each sample, normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was calculated by the formula 2^(-∆∆Ct) (GAPDH - C target) gene.

**Western Blot Analysis.** After homogenizing and sonicating the mammary gland tissues in lysis buffer, the protein lysate was collected and used in Western blot analysis with specific antibodies. The conditions for Western hybridization were carried out as described previously (8). The antibodies used for Western analysis are: mouse-specific antibodies for anti-ERα (Ab-10; clone TE11.1D11), ERβ (PA1-310), progesterone receptor (PR; Ab-10), proliferating cell nuclear antigen (PCNA; Ab-1; clone PC10), and cyclin D1 (Ab-1; DCS-6) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse-specific antibodies, antiaactin (19–20) anticyclin E (M-20), were purchased from Santa Cruz Biotechnology. For the detection of tissue macrophages, rat antimouse (F4/80) antibody (1:200 dilution) from Serotec (Oxford, United Kingdom) was used. The samples were incubated overnight at 4°C in a humidified chamber. After three washings with 0.05 M Tris-HCl buffer, the slides were incubated with secondary antibody for 30 min at room temperature. The secondary antibodies used were biotinylated antirat IgG (H+L), biotinylated antigoat IgG (H+L), and biotinylated antirabbit IgG (H+L) and were obtained from Vector Labs. Slides were subjected to repeated washings, and sections were incubated with 1% Fast Red/naphthol solution for 5 min. Sections were washed again and coverslipped, and the staining pattern was observed using a light microscope.

**Exposure of Transgenic Mice to DMBA.** MMTV-CSF-1 (n = 9) and MMTV-c-fms (n = 9) virgin female transgenic mice (56-day old), along with nontransgenic (FVB) virgin female mice (n = 11), were exposed to four doses of DMBA (1.0 mg/mouse/once a week). DMBA (1.0 mg; Sigma) in 100 μl of corn oil was delivered via orogastric tube. Four weeks after the last dose of DMBA exposure, the mice were palpated for tumors, and weekly observations continued until the termination of experiment. Mice were sacrificed shortly after tumor identification or at the termination of experiment when the animals reached the age of 12 months (about 10 months after the last dose of carcinogen exposure). Mammary gland, along with tumor (if tumor was present) along with non-tumor-containing mammary gland, was processed for microscopic observation, as described above.

**RESULTS**

**Generation of Transgenic Mice.** Transgenic mice overexpressing CSF-1 under the control of MMTV-LTR were generated by injecting the transgene construct shown in Fig. 1A into one-cell embryos. Two transgenic plasmids containing either human CSF-1 or mouse c-fms cDNA were used in microinjection to produce the corresponding transgenic lines: 1.5 kb of the MMTV-long terminal repeat (LTR); CSF-1 or c-fms (CSF-1 or c-fms) and MMTV-c-fms (CSF-1 or c-fms); SV40 splicing/polyadenylation sequences (SV40 Sequences). R restriction enzymes used in the linearization of the plasmids (PvuI for CSF-1 and EcoRI for c-fms). R. 10 μg of the genomic DNA of the CSF-1 (left) and of the c-fms (right) transgenic founders was analyzed by Southern blot analysis using 32P-labeled CSF-1 and c-fms cDNA (Lane 1), respectively. The typical restriction digestion pattern obtained by Southern blot analysis for the corresponding transgenes (left, CSF-1; right, c-fms). One copy and 10 copies (in 10 μg of total DNA) of the plasmid vector containing the CSF-1 cDNA or c-fms cDNA were loaded in Lanes 2 and 3, respectively.

**Fig. 1. A, structures of mouse mammary tumor virus–colony-stimulating factor (MMTV-CSF-1) and MMTV-c-fms transgenes and their expression in mammary glands.** Linearized plasmids containing either human CSF-1 or mouse c-fms cDNA were used in microinjection to produce the corresponding transgenic lines: 1.5 kb of the MMTV-long terminal repeat (LTR); CSF-1 or c-fms transgenic cDNA [Transgene-cDNA (CSF-1 or c-fms)]; SV40 splicing/polyadenylation sequences (SV40 Sequences). R. restriction enzymes used in the linearization of the plasmids (PvuI for CSF-1 and EcoRI for c-fms). R. 10 μg of the genomic DNA of the CSF-1 (left) and of the c-fms (right) transgenic founders was analyzed by Southern blot analysis using 32P-labeled CSF-1 and c-fms cDNA (Lane 1), respectively. The typical restriction digestion pattern obtained by Southern blot analysis for the corresponding transgenes (left, CSF-1; right, c-fms). One copy and 10 copies (in 10 μg of total DNA) of the plasmid vector containing the CSF-1 cDNA or c-fms cDNA were loaded in Lanes 2 and 3, respectively.
founder mice were generated, and both founder mice carried 10–15 copies of the transgene. One of the founder mice happened to be hemizygous and lost the transgene during the second generation. The other positive founder transgenic mouse, carrying ~15 copies of stably integrated CSF-1 transgene, as shown by Southern hybridization (Fig. 1B), was used to establish the CSF-1 transgenic line. To establish the c-fms transgenic mouse line, we used a positive founder mouse carrying ~10 copies of stably integrated c-fms transgene. Both CSF-1 and c-fms transgenic colonies are more than 3 years old and have undergone at least 10 generations of breeding. The transgene copy number and its expression are very stable throughout all generations. All of the studies were carried out with mice from the established colony that was generated using the founder mouse.

Characterization of CSF-1 and the c-fms Transgenic Mice. The expression of the CSF-1 transgene in mammary tissue from both nontransgenic and transgenic mice was analyzed using RT-PCR and Western blot analyses. The results in Fig. 2A show the overexpression of the CSF-1 transgene in the CSF-1 transgenic strain at the mRNA (Fig. 2A, left, top two panels) and protein (Fig. 2A, left, bottom two panels) levels. RT-PCR and Western analysis were also carried out to determine the effects of the human CSF-1 transgene overexpression in the mammary glands of transgenic animals on the expression of the endogenous mouse CSF-1 and CSF-1 receptor (c-fms). Mouse spleen tissue, a rich source of hematopoietic cells and CSF-1 expression was used as positive control (Fig. 2C). No detectable expression of the endogenous mouse CSF-1 was observed in the transgenic and non-

Fig. 2. Expression analyses of colony-stimulating factor (CSF-1) and its receptor c-fms in the mammary glands of CSF-1 and c-fms transgenic mice. A, expression analysis was carried out for the human CSF-1 transgene and endogenous mouse c-fms in the mammary glands of CSF-1 transgenic mice. Equal amounts of total RNA and proteins from mammary glands were analyzed by reverse transcription-PCR (RT-PCR; upper two panels) and Western blot analyses (bottom two panels), respectively, as described in the “Materials and Methods.” Lane 1, nontransgenic; Lane 2, CSF-1 transgenic. D, M, in thousands. Right panel, densitometric analyses were carried out on the RT-PCR and Western blot (West.) data. Normalized for the corresponding housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Actin], the data are presented as bar graphs. Hatched bars, nontransgenic (NT) control; solid black bars, transgenic line. B, expression analyses were carried out for the c-fms transgene at the RNA (upper two panels) and protein (bottom two panels) levels. Right panel, the densitometric data of the RT-PCR products and Western blot, which were normalized to the corresponding housekeeping genes GAPDH and actin, respectively, are presented as bar graphs. Hatched bars, nontransgenic (NT) control; solid black bars, transgenic line. C, RT-PCR analysis for the expression of endogenous CSF-1 (mouse) in both transgenic strains. Lane 1, CSF-1 transgenic; Lane 2, c-fms transgenic; Lane 3, nontransgenic. Lane 4, spleen positive control. H-CSF-1, human CSF-1; M-CSF-1, mouse-CSF-1. D, real-time PCR was also performed to quantify the expression of CSF-1 (curve growth, top panel) and c-fms (curve growth, bottom panel) in the CSF-1 and c-fms transgenic lines, respectively. The levels of expression were determined as a function of cycle threshold (Ct) value, as described in “Materials and Methods.” Insets in D, after normalizing to wild-type expression, the fold-difference is presented as bar graphs; hatched bars, nontransgenic control; solid black bars, transgenic line. D, top panel: •, GAPDH nontransgenic; ■, GAPDH transgenic; ▲, CSF-1 transgenic; ×, nontransgenic CSF-1. D, bottom panel: •, GAPDH nontransgenic; ■, GAPDH transgenic; ×, transgenic c-fms; ▲, c-fms nontransgenic.
transgenic mammary glands (Fig. 2C). However, a 3-fold increase was detected in the expression of endogenous mouse c-fms at the mRNA (Fig. 2A) and protein (Fig. 2A) levels in the mammary glands of CSF-1 transgenic animals as compared with that of nontransgenic animals, suggesting that CSF-1 induces the expression of its own receptor.

The overexpression of the c-fms gene was demonstrated in the c-fms transgenic line by RT-PCR and Western blotting (Fig. 2B). A 5-fold increase in c-fms expression was detected in the c-fms transgenic mice as compared with the nontransgenic control. No change was observed in the expression of endogenous CSF-1 gene in these mice (Fig. 2C).

Transgenic gene expression was also confirmed by real-time PCR of reverse-transcribed RNA from the mammary glands of CSF-1 and c-fms transgenic strains and the FVB wild-type. Fig. 2D shows about a 23-fold increase in the expression of CSF-1 in the CSF-1 strain as compared with control FVB (top panel). A 12-fold increase is observed in c-fms expression in the c-fms transgenic strain as compared with control (Fig. 2D).

To examine the morphological features of the mammary glands, whole mounts of fat pads from transgenic and nontransgenic littermates were prepared (Fig. 3, A–F). As compared with wild-type mammary gland (Fig. 3A), increased ductal branching and lobulo-alveolar growth was observed in both age-matched (6–8 months) virgin CSF-1 (Fig. 3B) and c-fms (Fig. 3C) transgenic mice mammary glands. Quantitative analysis of ductal branch data indicate (Fig. 3, bottom panels) that, compared with age- and physiological-stage-matched wild-type FVB mice, the ductal branching is about 2–4-fold more in CSF-1 and c-fms transgenic females, respectively. The differences are statistically highly significant (P < 0.001). Although no mammary tumors were observed in young transgenic CSF-1 or c-fms females, severe glandular dysplasia, ductal hyperplasia, and palpable, as well as microscopic, mammary tumors (about 50%; n = 22) were observed in both CSF-1 and c-fms animals, ages >12 months (Fig. 4). Hyperplasia and dysplasia were evident in all of the observed animals. Both adenocarcinoma and papillary carcinoma was the main phenotype of these tumors. The size of the tumors was small (<1 cm³), weighing ~150–225 mg (wet weight) at the time of sacrifice. Even though mammary tumors (palpable and microscopic) were observed in aged animals, the long latency, low tumor burden (not more than one or two palpable tumors per animal; n = 9), small tumors, and lack of metastasis indicated that other secondary factors or events may play a role in the aggressive tumor formation with increased incidence and metastasis in these mice.

To test the pattern of CSF-1 and c-fms expression in transgenic mammary glands, we stained tissue sections with specific antibodies, as described in the “Materials and Methods” section. As expected, immunohistochemical data shown in Fig. 5, A–D, clearly agree with...
biochemical data presented in the Fig. 2. Both CSF-1 and c-fms expression was high and it was restricted to epithelial cells, suggesting that increased expression of growth factor or its receptor plays an important role in the initiation and progression of mammary cancer. Localization of CSF-1 expression by immunohistochemical staining in the luminal secretions confirmed the secretory nature of this growth factor protein and its potential paracrine effect on stromal and other cell populations.

Previous studies have shown that CSF-1 plays an important role in the infiltration and sustenance of tissue macrophages. To examine the presence of infiltrating tissue macrophages in and around mammary lesions, we stained tissue sections with macrophage-specific antibody. As shown in Fig. 5, compared with wild-type mammary tissue (Fig. 5G), there is significant increase in infiltrating tissue macrophages in CSF-1 transgenic mammary tissue and in mammary tumors (Fig. 5, F and H). These observations further confirm the critical role of CSF-1 in tissue macrophage infiltration and their sustenance.

DMBA Treatment Accelerates Mammary Tumorigenesis in CSF-1 and c-fms Transgenic Mice. The early presence of preneoplastic lesions (4–8 months) and development of mammary tumors with long latency in aged (>12 months) CSF-1 and c-fms mice suggest that additional biochemical changes are required for the development of mammary tumors from preneoplastic changes. To determine whether carcinogen treatment could accelerate tumor formation, 56-day-old transgenic mice along with control (FVB) animals were treated with DMBA as described in “Materials and Methods.” None of the control mice (n = 11) developed either palpable or microscopic tumors at the termination of the experiment, whereas all of the CSF-1 (n = 9) and c-fms (n = 9) transgenic female mice developed either palpable or microscopic tumors during the same period of time (when the animals reached 12 months of age). The median tumor incidence (tumor size, >1.0 cm$^3$) was 31 and 37 weeks, respectively, for CSF-1 and c-fms mice. Even though all of the transgenic mice developed tumors, none of them developed multiple palpable tumors. However, microscopic evidence of additional tumors

Fig. 4. Tumor formation in aged colony-stimulating factor (CSF-1) and c-fms transgenic mammary glands. Histological sections of palpable tumors in the mammary glands of aged (ages >12 months) virgin CSF-1 transgenic mice (A–C) and c-fms transgenic mice (D–F) were prepared. Microscopic tumors (arrows) were also observed in aged CSF-1 (G) and c-fms (H) animals. ×40.
in non-tumor-bearing mammary glands was quite evident in some mammary glands (data not shown). As seen in hyperplastic tissue, all tumors expressed CSF-1 and c-fms. Furthermore, the expression of both of the steroid receptors (ER and PR) was still present, suggesting that these tumors still maintain hormone responsiveness despite the overexpression of the growth factor and its receptor.

Expression of Steroid Receptors in the CSF-1 and c-fms Transgenic Mammary Glands. To investigate how the overexpression of CSF-1 in the mammary gland affects the expression of steroid receptors, we tested the expression of ERα, ERβ, and PR in the CSF-1 transgenic animals and compared them with that in the nontransgenic animals. Whereas the expression of ERα showed no significant difference between the transgenic and nontransgenic mice (Fig. 6A), the expression of both ERβ and PR was increased in the transgenic mice by ~3.0-fold and >10-fold, respectively (Fig. 6A). Even though the PR has two isoforms, only the major isoform (PRα) was detected. The observations were consistent with previous studies (10). As in the case of the CSF-1 transgenic line, no difference was detected in the expression of ERα in the c-fms line as compared with the nontransgenic control (Fig. 6B); however, neither was a difference detected in the expression of ERβ (Fig. 6B). The expression of PR was 2-fold higher in the c-fms transgenic mice than in the nontransgenic mice (Fig. 7B).

Expression of Cell Cycle Genes in the CSF-1 and c-fms Transgenic Mammary Glands. CSF-1 is known to be involved in the activation of G1 cyclins (11). To investigate whether G1 cyclins are induced in the mammary glands of CSF-1 and c-fms transgenic animals, we tested the expression of genes involved in cell cycle

Fig. 5. Immunohistochemical localization of colony-stimulating factor (CSF-1) and c-fms transgene expression and infiltrating macrophages in wild-type and transgenic mice. A and B, no immunostaining in the absence of primary anti-CSF-1 or anti-c-fms antibodies in transgenic mammary tissues, respectively. C, cytoplasmic and periplasmic staining of CSF-1 in transgenic mammary tissue. The staining of luminal contents in CSF-1 transgenic mammary tissue is notable. D, positive staining for c-fms in transgenic mammary tissue. E, no immunostaining in the absence of primary antibody specific for macrophages in CSF-1 mammary tissue. F, infiltrating macrophages are more in number and stained well in c-fms tumor. G, staining of scattered macrophages around the edges of lobule in the mammary tissue section of nontransgenic mice. H, a large number of infiltrating macrophages are present in the surrounding tumor cells of CSF-1-overexpressing mammary tumor. ×100.
In the progression of breast cancer and the role of CSF-1 infiltration of tissue macrophages (13) and their sustenance, the presence of infiltrating macrophages are higher in and around preneoplastic lesions and mammary tumors in CSF-1 transgenic mice. These observations further suggest that, in addition to initiation of mammary hyperplastic and dysplastic changes and tumor formation in transgenic mice, CSF-1 may also help in the infiltration and sustenance of tissue macrophages.

The appearance of spontaneous mammary tumors with long latency (only in aged mice) and low tumor burden in CSF-1 and c-fms mice suggests that additional events accumulating with age may need to take place to promote mammary tumor development in these mice. We, therefore, tested whether treatment with carcinogen would accelerate tumor development. Transgenic mice (CSF-1 and c-fms) treated with DMBA developed palpable mammary tumors by age 8–9 months (6–7 months after treatment). The data suggest that the mutagenic effects of DMBA advanced the mammary preneoplastic changes, brought on by CSF-1 or c-fms overexpression, to tumor development. It could also be argued that initiating events took place during aging or carcinogen treatment, and the presence of increased CSF-1 or c-fms levels acted as promoter for tumor development; however, this may not be as likely as the first possibility, because the onset of preneoplastic changes due to CSF-1 or c-fms overexpression occurred in young mice (6–8 months) and without DMBA treatment. The expression of CSF-1 and c-fms and the presence of steroid receptors (ER and PR) in tumors, like our other transgenic mammary cancer model (14) suggests that CSF-1 and c-fms mammary tumors are still hormone responsive despite growth-factor activation and makes this model the more interesting one for further investigation. Furthermore, the data reported by Lin et al. (12) showed that, in CSF1+/CSF1" mice, the lack of active CSF-1 in the PyMT background resulted in delay in tumor progression and metastasis but had no effect on primary tumor development. The data strongly suggest that CSF-1 is not involved in primary tumor development but, rather, in progression and metastasis. It is worth noting that these studies addressed mammary tumor progression due to CSF-1 in PyMT mice, which are prone to mammary tumor development. Similarly, the data from our CSF-1 and c-fms transgenic models suggest that CSF-1 signaling alone may result only in extensive preneoplastic changes and delayed spontaneous tumor development, and that other alterations may be necessary for full development of these tumors.

DISCUSSION

Recent evidence suggests that CSF-1 plays an important role in the development of functionally lactating mammary glands (6). In addition, several recent studies suggest that CSF-1 may play a role in aggressive breast cancer and the invasive potential of breast cancer leading to metastasis (6). To investigate the role of CSF-1 and c-fms in mammary gland development and breast cancer initiation, we have generated a transgenic mouse strain carrying the human CSF-1 cDNA and another carrying the mouse c-fms cDNA, each under the control of the MMTV promoter. Overexpression of CSF-1 and c-fms (both RNA and protein) and localization of CSF-1 or c-fms protein in ductal mammary epithelial cells of hyperplastic/dysplastic lesions suggests the importance this growth factor and its receptor in the initiation of mammary preneoplastic/neoplastic changes. The mammary glands of these mice exhibit increased ductal branching, hyperplastic growth, and no reduction in mammary growth during involution. The presence of preneoplastic lesions in virgin CSF-1 or c-fms transgenic mouse lines suggests that the overexpression of CSF-1 or c-fms may lead to initiating preneoplastic changes in mammary glands.

Consistent with the known role of CSF-1 (5, 6, 12) and its receptor

regulation. Western blot analyses showed an increase in the expression of the cell cycle proteins, cyclins D1 (7-fold) and E (3-fold) in the CSF-1 transgenic line as compared with the nontransgenic control (Fig. 7A). An increase in protein expression was also observed in the cellular proliferation marker, PCNA (13-fold) and the transcription factor c-jun (3-fold) in the CSF-1 transgenic mammary glands as compared with the nontransgenic gland (Fig. 7A), which is consistent with the observed increase in cellular proliferation in the mammary glands of the CSF-1 transgenic mice. A 2-fold and 3-fold increases were detected in the levels of cyclins D1 and E, respectively, in the c-fms transgenic line as compared with the nontransgenic control (Fig. 7B). The PCNA levels are also increased (4.6-fold) in the mammary glands of the c-fms transgenic line as compared with the nontransgenic control (Fig. 7B).

**Fig. 7. Expression analysis of cell cycle factors in colony-stimulating factor (CSF-1) and c-fms transgenic mice.** Levels of cell cycle factors were examined in CSF-1 (A) and c-fms (B) transgenic mammary glands. An equal amount of protein from nontransgenic (Lane 1) and CSF-1 transgenic (Lane 2) mammary glands was used in Western blot hybridization with protein-specific antibodies, as described in the “Materials and Methods.” kDa, M, in thousands. In A and B, right panels, the densitometric data, which were normalized for the invariant housekeeping gene Actin, are presented as bar graphs. Hatched bars, nontransgenic (NT) control; solid black bars, transgenic line. ER, estrogen receptor; PR, progesterone receptor.

**Fig. 6. Expression analysis of steroid receptors in colony-stimulating factor (CSF-1) and c-fms mice.** Steroid receptors levels were examined in CSF-1 (A) and c-fms (B) transgenic mammary glands. An equal amount of protein from nontransgenic (Lane 1) and CSF-1 transgenic (Lane 2) mammary glands was used in Western blot hybridization with protein-specific antibodies, as described in the “Materials and Methods.” kDa, M, in thousands. In A and B, right panels, the densitometric data, which were normalized for the invariant housekeeping gene Actin, are presented as bar graphs. Hatched bars, nontransgenic (NT) control; solid black bars, transgenic line. ER, estrogen receptor; PR, progesterone receptor.
ations are required for increased tumor incidence with decreased latency and metastatic potential. Combined, the data suggest that through its mitogenic activity, CSF-1 could act on initiating hyperplastic and dysplastic changes, and, although it may not be involved in promoting tumor development, it does play an important role in progression and metastasis.

CSF-1 mammary-specific expression of the human CSF-1 transgene in virgin CSF-1 transgenic mice (6–8 months) was confirmed by RT-PCR and Western blot analysis. Whereas there was no detectable expression of endogenous CSF-1 between the nontransgenic and CSF-1 transgenic mice, at least a 3-fold increase was observed in the expression of the endogenous c-fms in the transgenic versus nontransgenic mammary glands. This suggests that CSF-1 can induce the expression of its receptor, which is required for CSF-1 function. In addition, a similar increase in mRNA and protein levels in the CSF-1 transgenic mice suggests that the regulation of c-fms by CSF-1 is probably occurring at the transcriptional level.

The increase in endogenous c-fms expression in CSF-1 mammary glands may be due to an increase in the infiltration of c-fms-positive macrophages. However, the observed increase in endogenous c-fms expression may not be attributed solely to macrophage infiltration because mouse (endogenous) CSF-1 expression, also occurring in macrophages, was not changed in the CSF-1 transgenic mammary glands. Consistent with the known role of CSF-1 (5, 6, 12) and its receptor in the progression of breast cancer and the role of CSF-1 in the filtration of tissue macrophages (13) and their sustenance, the presence of infiltrating macrophages is greater in and around preneoplastic lesions and mammary tumors in CSF-1 transgenic mice than in non-transgenic mice. These observations further suggest that, in addition to the initiation of mammary hyperplastic and dysplastic changes and tumor formation in transgenic mice, CSF-1 may also help in the infiltration and sustenance of tissue macrophages. The lack of demonstrable expression of CSF-1 in macrophages in early lesions, and the increase in expression of CSF-1 only macrophages in tumors (Fig. 5), it is possible that at this early stage of initiation, there may not be significant macrophage infiltration; therefore, they may have little contribution to the increase in c-fms expression in the CSF-1 transgenic mammary glands. Thus, it is likely that the increase in c-fms expression is due to an autocrine feedback loop in CSF-1-overexpressing mammary cells.

Signaling by the CSF-1/c-fms system results in accelerating cellular proliferation. Consistently, stimulation by CSF-1 results in the induction of G1 cyclins that promote cell cycle progression from the G1 to S phase. Our expression data of factors involved in cell cycle and cellular proliferation is in agreement with the enhanced cellular proliferation in the mammary glands of CSF-1 transgenic mice and suggests that CSF-1 via c-fms induce the expression of these factors in the mammary glands of the transgenic mice. We have observed an increase in the expression of both cyclin D1 and cyclin E (which promote cell cycle advance from the G1 phase into S phase), as well as of PCNA (a marker for cellular proliferation), in the transgenic mammary glands as compared with the nontransgenic gland. We have also observed, in the CSF-1 transgenic mice, an increase in c-jun, which is an early transcription factor activated during the induction of cellular growth. Combined, the expression profile of these factors is indicative of increased cellular proliferation in the mammary glands of the CSF-1 and c-fms transgenic mice.

The expression of ERα was not affected in the CSF-1 transgenic mammary gland; however, the expression of ERβ, and more drastically PR (≥10-fold), was increased in the virgin transgenic gland as compared with the nontransgenic gland. The presence of steroid receptors in these mice and increased mammary growth in parous animals suggests that the mammary tumors are still hormone responsive despite growth factor activation. The ongoing studies of CSF-1/c-fms double transgenic mice with MMTV-aromatase mice (7, 8, 14) should shed more light on the synergistic role of steroid hormones and growth factor interaction in promoting mammary tumor growth. Increased ductal branching and the consistent overexpression of (15) the major isoform of progesterone receptor (PRA) (10) in CSF-1 transgenic mice suggest that this growth factor, in some way, influences PR expression by growth factor cross-talk through membrane-mediated signal transduction pathways. Furthermore, the ability of CSF-1 and other members of this family (16) to bind and activate unique tyrosine kinase receptors [leading to proliferation of early progenitor or stem cells and induction of PR in ductal or lobulo-alveolar progenitors depending on the type of mitogenic stimulus (17)] suggests that the CSF-1/c-fms action that led to increased lateral branching and lobulo-alveolar growth might have contributed to increased PR expression. More studies are needed to address the role of CSF-1/c-fms-mediated mechanism in up-regulation of PR. The ongoing studies with PR knockout mice with CSF-1/c-fms double transgenic mice should shed more light on these observations. These findings suggest that CSF-1 may also regulate gene expression by inducing the expression of the steroid receptors ERβ and PR by nongenomic pathways, because there is no known direct interaction of CSF-1 with steroid receptors; this, in turn, could have additional mitogenic effects on the cellular component of mammary ducts. Interestingly, although no difference was observed in ERβ expression, PR expression was increased by 2-fold in the c-fms transgenic mice as compared with nontransgenic control. Thus, the difference in ERβ and PR expression, as compared with control, is more pronounced in the CSF-1 transgenic strain than in the c-fms strain. One possible explanation is that in the CSF-1 strain, both CSF-1 and endogenous c-fms are elevated, whereas only c-fms expression is elevated in the c-fms transgenic strain; therefore, the CSF-1 signaling pathway may be activated at a higher extent in the CSF-1 transgenic mice, resulting in a more pronounced induction of ERβ and PR. This is consistent with the induction profile of the expression of downstream genes, such as cyclin D and PCNA, in the CSF-1 strain as compared with the c-fms strain.

We report the development of mouse transgenic models expressing CSF-1 and c-fms in the mammary glands. These models provide the opportunity to examine directly the effects of CSF-1 and its receptor in mammary gland development and tumorigenesis. The CSF-1/c-fms transgenic models provide in vivo tools to examine the effects of CSF-1 signaling on the mammary gland during pregnancy and lactation as well as on the regulatory pathways involved in breast cancer. We have shown that increased expression of CSF-1 and/or its receptor results in the development of preneoplastic changes that could lead to tumor formation in aged transgenic mice.

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