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Colony-Stimulating Factor-1 Receptor (c-*fms*)

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The macrophage colony-stimulating factor, CSF-1 (M-CSF), is a homodimeric glycoprotein required for the lineage-specific growth of cells of the mononuclear phagocyte series. Apart from its role in stimulating the proliferation of bone marrow-derived precursors of monocytes and macrophages, CSF-1 acts as a survival factor and primes mature macrophages to carry out differentiated functions. Each of the actions of CSF-1 are mediated through its binding to a single class of high-affinity receptors expressed on monocytes, macrophages, and their committed progenitors. The CSF-1 receptor (CSF-1R) is encoded by the c-*fms* proto-oncogene, and is one of a family of growth factor receptors that exhibits an intrinsic tyrosine-specific protein kinase activity. Transduction of c-*fms* sequences as a viral oncogene (v-*fms*) in the McDonough (SM) and HZ-5 strains of feline sarcoma virus has resulted in alterations in receptor coding sequences that affect its activity as a tyrosine kinase and provide persistent signals for cell growth in the absence of its ligand. The genetic alterations in the c-*fms* gene that unmask its latent transforming potential abrogate its lineage-specific activity and enable v-*fms* to transform a variety of cells that do not normally express CSF-1 receptors.

Key words: c-*fms* proto-oncogene, v-*fms* oncogene, macrophage colony-stimulating factor, (CSF-1, M-CSF), cell transformation, tyrosine kinases, leukemogenesis

Oncogenes of RNA tumor viruses arose through recombination between retroviruses and normal cellular proto-oncogene sequences that are generally assumed to play a vital role in regulating cell growth and differentiation [1]. Transduction of different oncogene sequences as cDNA copies in the genomes of retroviruses confers their ability to transform cells morphologically in tissue culture and to produce tumors in animals after a short latency period. Investigations of the genome structures of acutely transforming viruses and comparison of their viral oncogene sequences to their proto-oncogene counterparts has revealed that recombined oncogenes have frequently undergone structural alterations that contribute to their transforming potential. Moreover, the expression of oncogenes under the control of retroviral promoter/enhancer elements distorts their tissue-specific transcription and enables these genes to be expressed in cells that may not normally transcribe their respective proto-oncogene RNAs. Thus, the types of tumors induced by these viruses appear to reflect the susceptibility of host cells to viral infection

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and the tissue-specificity of viral transcriptional control signals, as well as the ancestral origins of the oncogenes themselves.

The v-*fms* oncogene of the Susan McDonough (SM) and HZ-5 strains of feline sarcoma virus (FeSV) was derived from c-*fms* proto-oncogene sequences of the domestic cat [2–4] which are now recognized to encode the receptor for the macrophage colony-stimulating factor, CSF-1 [5]. Although the CSF-1 receptor (CSF-1R) is normally restricted in its expression to hematopoietic cells of the mononuclear phagocyte series [6,7] and to placental trophoblasts during fetal development [8–10], the v-*fms* gene can transform fibroblasts, epithelial cells, and hematopoietic target cells that do not normally express the c-*fms* gene. Nucleotide sequencing analyses of the v-*fms* and c-*fms* genes, combined with their genetic manipulation and assays of their transforming potential in the context of retroviral vectors, have begun to pinpoint structural alterations in CSF-1R that alter its biological activity and unmask its latent transforming potential.

BIOSYNTHESIS AND BIOCHEMICAL PROPERTIES OF THE CSF-1 RECEPTOR

The receptor for CSF-1 is a member of a family of growth factor receptor genes —including the receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, and insulin growth factor-1 (IGF-1) [11–15]—which encode polypeptides with tyrosine-specific protein kinase activity. The CSF-1 receptor is synthesized on membrane-bound polyribosomes as an integral transmembrane glycoprotein, oriented with its aminoterminal ligand-binding domain (ca. 485 amino acids) in the cisternae of the rough endoplasmic reticulum (ER) and its carboxylterminal tyrosine kinase domain (ca. 435 amino acids) in the cytoplasm. The polypeptide acquires mannose-rich, asparagine(N)-linked oligosaccharide chains during its synthesis and is transported through the Golgi complex to the cell surface, undergoing concomitant modification of the oligosaccharides to complex carbohydrate chains. Post-translational processing of N-linked oligosaccharides is accompanied by an increase in the apparent molecular weight of CSF-1R on polyacrylamide gels containing sodium dodecyl sulfate, so that the intracellular and mature cell surface forms of the receptor can be readily distinguished, both by their sugar content and molecular mass [5,10,16,17].

Fusion of secretory vesicles with the plasma membrane orients the receptor with its glycosylated ligand binding domain outside the cell and its tyrosine kinase domain at the inner surface of the membrane. Thus, antibodies directed to epitopes in the aminoterminal half of the polypeptide can react with receptor molecules on the surfaces of viable cells and can be used to purify receptor-bearing populations by fluorescence-activated cell sorting procedures [5,17]. In the absence of extracellular CSF-1, the receptors turn over with a half-life of 3–4 h, but binding of saturating quantities of CSF-1 to the receptor results in internalization of receptor-ligand complexes, loss of detectable CSF-1 binding sites at the cell surface, and rapid receptor degradation (downmodulation) [17,19–22]. In the absence of ligand, this is followed by a refractory period of several hours during which the receptor is reexpressed at the cell surface following de novo synthesis.

In purified membrane preparations incubated in the presence of [γ^{32}P]-ATP and manganese, binding of CSF-1 to its receptor activates the receptor kinase and leads to autophosphorylation of CSF-1R on tyrosine [5,21–24]. Similarly, exposure of murine macrophages to saturating quantities of CSF-1 results in phosphorylation of the receptor on tyrosine within 1 min and virtually complete receptor downmodulation within 15 min of stimulation [21,22]. Only the mature cell surface form of the receptor is phosphorylated

on tyrosine in response to the growth factor [22]. Although CSF-1 binding must induce a conformational change in the cell surface receptor that activates its intrinsic tyrosine kinase activity, the physical nature of the activating event remains unclear. For the EGF receptor, several lines of evidence suggest that the formation of receptor dimers may alter the affinity of the receptor for its ligand and induce the receptor kinase activity [25–30]. To date, CSF-1R has not been subjected to the same experimental scrutiny.

CSF-1R is also phosphorylated on serine residues, and phorbol esters that activate protein kinase C also induce rapid receptor degradation [20,31]. In macrophage cell lines chronically exposed to phorbol esters such as TPA, the activity of protein kinase C is itself down-regulated, and CSF-1 receptors eventually reappear at the cell surface. These receptors can still be down-modulated in response to CSF-1 (unpublished observations), suggesting that the biochemical mechanisms underlying ligand- and phorbol ester-induced receptor degradation differ from one another.

By Western blotting analysis using antibodies to phosphotyrosine, CSF-1 binding has been shown to induce phosphorylation of a series of cellular substrates [22] whose identity and physiological role, if any, in mediating signal transduction are unknown. CSF-1 induces immediate changes in macrophage membrane structure, including formation of filopodia and vesicles and enhanced phagocytic activity [32], and it increases the transcription of several cellular genes, including the *c-fos* and *c-myc* proto-oncogenes, within minutes to hours after stimulation [33,34]. In susceptible cells, these early events are followed by decreases in the rates of protein turnover within 2 h [35] and eventual mitogenesis [36]. In addition to its role in stimulating cell growth, CSF-1 augments the production of other macrophage cytokines, such as interleukin-1 and tumor necrosis factor; potentiates the release of plasminogen activator, thromboplastin, prostaglandins, and biocidal oxygen metabolites; stimulates killing of microorganisms; and promotes antibody-dependent lysis of tumor cells [37–41]. Thus, activation of the receptor kinase appears to initiate a cascade of intracellular events that ultimately affect the proliferation and functional activities of receptor-bearing cells.

Introduction of retrovirus vectors containing the human *c-fms* gene into mouse NIH/3T3 fibroblasts enables the cells to form colonies in semisolid medium in response to exogenous human recombinant CSF-1 [42]. Similarly, introduction of the murine *c-fms* gene into mouse fibroblasts confers their ability to express specific high-affinity binding sites for the murine growth factor [43]. Cotransfection of NIH/3T3 cells with vectors encoding the human *c-fms* and CSF-1 genes also induces cell transformation and tumorigenicity in nude mice by an autocrine mechanism [42]. In the latter cells, CSF-1R is persistently down-modulated, and neutralizing antibodies to CSF-1 do not efficiently revert the transformed phenotype [17]. This suggests that an intracellular interaction between CSF-1 and its receptor in autocrine transformed cells may potentially occur within the secretory compartment, thereby allowing ligand-receptor complexes to be rapidly directed to lysosomes. Together, these data provide formal genetic proof that *c-fms* encodes CSF-1R and indicate that the receptor gene specifies all the information necessary to initiate a biologic response, even in cells of heterologous species and in differentiated cell types that do not normally express the receptor [42].

STRUCTURAL AND FUNCTIONAL DIFFERENCES BETWEEN *c-fms* AND *v-fms*

The *v-fms* gene of the SM and HZ5 strains of FeSV was recombined into the open reading frame of the retroviral *gag* gene, so that the full genome length mRNAs of both viruses encode *gag-fms* fusion proteins of ca. 180 kDa ($gP180^{gag\text{-}fms}$) [3,4,44]. Recom-

bination between FeLV and c-*fms* occurred in a region of the proto-oncogene encoding the 5′ untranslated sequences of c-*fms* mRNA, prematurely opening the c-*fms* reading frame and preserving the intact ligand-binding domain of CSF-1R, including its signal peptide sequence [45,46]. During its synthesis, the viral polyprotein is cleaved by signal peptidase near the *gag-fms* junction [46]. This process generates an immature v-*fms*-coded glycoprotein ($gp120^{v\text{-}fms}$) that undergoes modification of its N-linked oligosaccharides in a manner analogous to CSF-1R to yield the mature cell surface form ($gp140^{v\text{-}fms}$) [47,48]. Unlike normal CSF-1R synthesis, processing of SM-FeSV $gp120^{v\text{-}fms}$ to $gp140^{v\text{-}fms}$ occurs very inefficiently, suggesting that the immature precursor has undergone some alteration that retards its intracellular transport from the endoplasmic reticulum [47]. Deletion of *gag* sequences from SM-FeSV does not affect the synthesis or trans_ rt of $gp120^{v\text{-}fms}$, because translation can begin at the normal c-*fms* initiator codon [46]. As expected, cells infected with SM-FeSV express CSF-1 binding sites, and chemical crosslinking established that CSF-1 was specifically bound to $gp140^{v\text{-}fms}$ molecules at the cell surface [23].

Expression of the v-*fms* gene product at the cell surface is required for cell transformation [49–52]. Unlike the normal CSF-1 receptor, $gp140^{v\text{-}fms}$ expressed in transformed cells is constitutively phosphorylated on tyrosine [22,53], and the viral receptor analog does not undergo rapid down-modulation in response to CSF-1 or phorbol esters [20]. Moreover, in membrane preparations, the v-*fms* gene product can be phosphorylated on tyrosine in the absence of its ligand, and addition of murine CSF-1 does not appear to up-regulate the receptor kinase activity [23]. However, stimulation of SM-FeSV-transformed cells with saturating concentrations of human CSF-1 can further up-regulate the receptor kinase activity [22], suggesting that its constitutive activity as an enzyme does not preclude a functional interaction with the homologous feline growth factor. The persistence of the v-*fms*-coded glycoprotein at the cell surface and its activity as a constitutive tyrosine kinase in the absence of its ligand contribute unregulated signals for cell growth. These properties must be due to particular genetic alterations in the v-*fms* gene that render its product transforming.

Nucleotide sequence comparison and conceptual translation of the feline v-*fms* [44] and human c-*fms* genes [45] reveals scattered amino acid substitutions, many of which appear to be due to interspecies divergence. However, the carboxylterminal 40 amino acids of CSF-1R were replaced by 11 unrelated residues in the SM-FeSV v-*fms* product, resulting in the loss of a single tyrosine residue (tyr^{969}) four amino acids from the normal receptor C-terminus. A frameshift in this region of the v-*fms* gene of HZ5-FeSV results in a structurally different C-terminal modification that also deletes tyr^{969} [4]. Neither the full length c-*fms* gene nor C-terminally truncated or mutated analogs lacking tyr^{969} are active in transformation [42], whereas the v-*fms* gene transforms many different types of cells in the absence of ligand [2,3,20,54,55]. Replacement of the C-terminal region of the v-*fms* gene product with sequences from the normal receptor attenuates, but does not eliminate, the gene's transforming activity [42,56]. When tyr^{969} in these chimeric constructs was mutated to a phenylalanine residue, full transforming efficiency was restored, suggesting that tyr^{969} negatively regulates the receptor tyrosine kinase activity, possibly by acting as a phosphorylation site [42]. However, the mechanism of negative regulation remains unclear since CSF-1R does not contain detectable phosphotyrosine until it is stimulated by ligand [22]. In a cotransfection assay in which human c-*fms* was introduced into NIH/3T3 cells together with the CSF-1 gene, a mutant c-*fms* allele containing phe^{969} proved more efficient than the wild-type gene in inducing autocrine

transformation [42,57]. Thus, C-terminal truncation of the v-*fms* gene product accentuates its transforming activity but is not sufficient in itself to activate the gene's transforming potential. Other mutation(s) elsewhere in c-*fms* must therefore be necessary to render it transforming and ligand-independent in its activity.

We recently found that a chimeric gene specifying an aminoterminal portion of the extracellular domain of the human c-*fms* gene joined to the remainder of the feline v-*fms* oncogene encodes a functional CSF-1 receptor [69]. When expressed in NIH/3T3 cells, the chimeric gene product was efficiently transported to the cell surface, was phosphorylated on tyrosine only in response to human recombinant CSF-1, underwent ligand-induced downmodulation, and stimulated colony formation of the cells in semi-solid medium containing CSF-1. Since the chimeric gene product retained the complete v-*fms*-coded kinase domain, including the C-terminal truncation that eliminated tyr^{969} from the c-*fms* product, these data further emphasize the fact that the C-terminal alteration is not sufficient to activate the gene's oncogenic potential. Nor do other genetic alterations in the kinase domain of v-*fms* inherently abrogate its ability to undergo ligand-induced downmodulation. The putative "activating" mutations in v-*fms* might well be localized within the extracellular domain, inducing a receptor conformation that is enzymatically active. However, we cannot exclude the possibility that replacement of extracellular v-*fms* sequences with a functional human CSF-1 ligand-binding domain suppresses an activating mutation elsewhere in the v-*fms*-coded molecule. Clearly, phosphorylation of the receptor on tyrosine per se does not provide the signal for receptor degradation, since the v-*fms* gene product contains phosphotyrosine even in the absence of ligand. Moreover, the v-*fms* gene could contain several independent mutations, one of which affects kinase activity and another of which affects processing of $gp120^{v\text{-}fms}$ to $gp140^{v\text{-}fms}$ and down-modulation of the mature form of the glycoprotein. Further studies will be necessary to pinpoint more precisely putative mutation(s) that activate the latent transforming potential of c-*fms*.

TRANSFORMING POTENTIAL OF THE v-*fms* GENE

SM- and HZ5-FeSV were isolated from multicentric fibrosarcomas of domestic cats [4,58], and cell-free filtrates of the SM-FeSV-containing tumor cells induced fibroblast transformation [59] and fibrosarcomas when inoculated into kittens [58]. Although SM-FeSV transforms cultured fibroblast cell lines from cats, rats, and mice in vitro, it was not originally demonstrated to induce hematopoietic malignancies in vivo. Introduction of helper-free SM-FeSV into a CSF-1-dependent, SV4O-immortalized murine macrophage cell line abrogated their factor dependence and rendered the cells tumorigenic in nude mice [20]. Animals inoculated with these transformed macrophages developed high-grade histiocytic sarcomas metastatic to liver, lung, brain, pancreas, and bone, and cell lines reestablished from the tumors continued to express v-*fms*-coded kinase activity in culture. Because these cells did not express CSF-1 mRNA, their transformation was induced by a nonautocrine mechanism. Conceptually analogous results were obtained after introducing a murine retroviral vector containing the v-*fms* gene into interleukin-3 (IL-3)-dependent mouse FDC-P1 cells [54]. Again, cells expressing high levels of the v-*fms*-coded kinase became factor independent for growth in culture and tumorigenic in animals. In both cases, expression of the v-*fms* gene did not transmodulate the synthesis, expression, or affinity of normal CSF-1 or IL-3 receptors coexpressed in the target cells. This suggests that the v-*fms*-coded kinase provides persistent signals for cell proliferation

that short circuit the proximal signal transduction pathways engaged by the normal receptors. In addition, the fact that v-*fms* can transform both fibroblasts and immature myeloid cell lines indicated that its activity was not restricted to normal CSF-1 receptor-bearing cells.

To determine if v-*fms* would induce hematopoietic malignancies in an in vivo setting, normal murine bone marrow cells infected with helper-free SM-FeSV were used to reconstitute lethally irradiated mice [55]. Southern blotting analysis demonstrated integrated SM-FeSV proviruses in spleen cells from 50% of the engrafted recipients one month after transplantation, and 30–50% of the splenocytes from individual recipients were found to be derived by clonal expansion of single SM-FeSV-infected progenitor cells. Reinoculation of provirus-positive spleen cells into secondary lethally irradiated mice induced clonal erythroleukemias and B cell lymphomas in some of the animals. By contrast, other secondary recipients receiving similar grafts remained disease-free and were repopulated by uninfected stem cells. The provirus-positive cells present in the spleens of primary recipients therefore did not have an obligate proliferative advantage when transferred to naive animals, suggesting that the formation of clonal malignancies in a proportion of the secondary recipients must have involved further genetic events in vivo. As expected, once clonal malignancies were established, the tumor cells could be transplanted at 100% efficiency into tertiary recipients. Thus, expression of the v-*fms* gene in the spleen cells of primary recipients initiated their expansion as premalignant populations that ultimately gave rise to tumor cells through a multistep mechanism. The fact that v-*fms* induced tumors of multiple hematopoietic lineages involving cells that do not express CSF-1 receptors again suggests that the v-*fms*-coded kinase has lost the target cell specificity of the normal receptor and acts more promiscuously.

AUTOCRINE TRANSFORMATION BY CSF-1 AND c-*fms*

Cointroduction of the human c-*fms* and CSF-1 genes into NIH/3T3 fibroblasts transforms these cells by an autocrine mechanism and renders them tumorigenic in nude mice [42, and see above]. In recent experiments, we used retroviral vectors to introduce the human CSF-1 gene into a murine SV40-immortalized macrophage cell line that depends on CSF-1 for its proliferation and survival in vitro. Although murine CSF-1 does not stimulate human CSF-1 receptors, human CSF-1 is equally active on both murine and human cells. Whereas the infected cells were able to proliferate in the absence of exogenous CSF-1, they were not tumorigenic when inoculated into nude mice [24]. Control experiments confirmed that murine CSF-1 receptors were synthesized at high levels in these cells and underwent persistent down-modulation in response to the endogenously produced growth factor. Since fibroblasts could be fully transformed by an autocrine mechanism [17,42], and because the SV40-immortalized macrophage cell line was not resistant to transformation by v-*fms* [20, and see above], it remains unclear why the latter cells were not tumorigenic after introduction of the CSF-1 gene. Indeed, others have demonstrated that a mouse macrophage tumor induced by a c-*myc*-containing retrovirus expressed CSF-1 after a DNA rearrangement at the CSF-1 locus. Because the proliferation of these tumor cells was blocked by specific antiserum to the growth factor, CSF-1 gene rearrangement appeared to be a secondary event in tumor cell development [60].

Human monocytes can synthesize CSF-1 after treatment with other cytokines, including γ-interferon and granulocyte-macrophage (GM)-CSF [61,62]. Thus, CSF-1

may normally function as an autocrine or paracrine growth factor to potentiate the activity of monocytes during an inflammatory response. The number of CSF-1 receptors per cell increases significantly as cells of the mononuclear phagocyte lineage differentiate, and mature monocytes and macrophages express the highest numbers of receptors per cell [19]. Whereas the mature cells require CSF-1 primarily as a survival and priming factor, earlier progenitors exhibit a proliferative response. If the pleiotropic effects of CSF-1 are mediated through its interaction with a single class of high-affinity receptors, it may prove that different physiologic substrates for the CSF-1R kinase are expressed as precursors of mononuclear phagocytes differentiate. Monocytes and macrophages might be relatively refractory to the proliferative effects of CSF-1 and, therefore, might not be readily converted to a tumorigenic phenotype in response to autocrine signals. Alternatively, these cells might synthesize negative regulatory cytokines, such as transforming growth factor β [63], which limit their tumorigenic potential in vivo.

POSSIBLE ROLES OF *fms* GENES IN HUMAN DISEASE

At least three different mechanisms involving *fms* expression could contribute to tumor formation: First, in cells in which c-*fms* is normally expressed, genetic alterations in the proto-oncogene analogous to those observed in v-*fms* might lead to persistent ligand-independent signals for cell proliferation. Second, unscheduled expression of the c-*fms* gene could render cells inappropriately responsive to CSF-1, leading to their clonal expansion, and increasing their probability of undergoing further genetic changes that contribute to malignancy. Third, inappropriate expression of CSF-1 in cells of the mononuclear phagocyte lineage might stimulate their growth through an autocrine mechanism.

In mice, the Friend murine leukemia virus (F-MuLV) can produce clonal myelogenous leukemias after insertional mutagenesis at the c-*fms* locus [64]. Integration of F-MuLV upstream of the CSF-1R coding region (and probably downstream of the normal c-*fms* promoter) induces the transcription of c-*fms* mRNA in immature myeloid cells, leading to the synthesis of unaltered receptor molecules. It has been postulated that these cells are rendered CSF-1 responsive and may be inappropriately expanded in the bone marrow microenvironment, ultimately becoming leukemic as part of a multistep process [65,66]. Although immature myeloid cells containing clonally integrated F-MuLV proviruses at the c-*fms* locus are initially factor-dependent and nontransplantable, factor-independent leukemic cell lines can eventually be established that cause myelogenous leukemias when passaged in vivo. At least some of the leukemic cell lines have been found to synthesize CSF-1 [65,66], whereas others have lost the unrearranged c-*fms* allele [64]. This suggests that secondary events occurring subsequent to F-MuLV insertion might involve either the CSF-1 or c-*fms* genes themselves. In certain circumstances, rearrangement at the CSF-1 locus in mononuclear phagocytes can serve as a second event that contributes to a tumorigenic, factor-independent phenotype [60, and see above].

We have recently used antibodies to human CSF-lR to screen cases of acute myelogenous leukemia (AML) for c-*fms* expression [18]. Approximately 40% of human AMLs express apparently normal CSF-1 receptors that undergo downmodulation in response to CSF-1 or phorbol esters. Although these receptors should be expressed as a differentiation-specific phenotypic marker of malignant cells of the mononuclear phagocyte series, there was no strict correlation between receptor expression and other morphological or immunophenotypic parameters characteristic of monocytic differentiation.

We would therefore predict that the expression of c-*fms* in those AMLs that lack monocytic characteristics might either be due to rearrangements upstream of CSF-1R coding sequences or, alternatively, to the aberrant expression of *trans*-acting factors that regulate tissue-specific expression of the c-*fms* promoter. Although the human c-*fms* gene has been molecularly cloned [67,68], and its nucleotide sequence has been recently determined (A. Hampe, personal communication), the promoter has not been identified and may map at a considerable distance from sequences encoding CSF-1R. The detailed molecular characterization of this region will be necessary in order to elucidate the precise mechanisms by which CSF-1 receptor expression is regulated during normal myelopoiesis and in myeloid malignancies.

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