JOURNAL OF CELLULAR PHYSIOLOGY 204:247–259 (2005)

Diverse Isoforms of Colony-Stimulating Factor-1 Have Different Effects on the Development of Stroma-Dependent Hematopoietic Cells

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Maintenance and differentiation of hematopoietic stem and progenitor cells are controlled by complex interactions with the stroma microenvironment. Stroma–cell interactions can be supported by locally expressed membrane-spanning cell-surface (cs) growth factors. CSF-1 is expressed by stroma as a soluble glycoprotein, as proteoglycan, or as a membrane-spanning cs glycoprotein. CSF-1 regulates the survival, proliferation, and differentiation of mononuclear phagocytes. Whereas the biological role of soluble CSF-1 is well characterized, the function of the membrane-spanning cell-surface CSF-1 (csCSF-1) remains unclear. To analyze the biological significance of csCSF-1 in vitro, we used an epithelial cell line to ectopically express the different CSF-1 isoforms. In co-cultures of CSF-1 transduced epithelial cells with primary, early hematopoietic progenitor cells we examined whether interaction between csCSF-1 and its receptor mediates cell proliferation, self-renewal, or differentiation. csCSF-1 induces long-lasting proliferation of stimulated cells and furthermore supports self-renewal. Ectopic secretion of soluble CSF-1 does not permit long-term growth of progenitor cells but induces differentiation of monocytes into macrophages. Previously, we showed that the soluble and cs isoforms of stroma-encoded SCF differently affect the development of hematopoietic cells. Cell-surface SCF (csSCF) promotes self-renewal of stimulated cells whereas soluble SCF causes clonal extinction. These results and those presented here for CSF-1 provide evidence for diverse functions of the isoforms of the ligands SCF and CSF-1 for two tyrosine kinase receptors of the subclass III both regulating hematopoiesis on stroma. J. Cell. Physiol. 204: 247–259, 2005. © 2005 Wiley-Liss, Inc.

Self-renewal, proliferation, and differentiation of hematopoietic progenitor cells are regulated by complex mechanisms that involve the bone marrow (bm) microenvironment. There is only limited understanding of how stromal cells maintain pluripotent hematopoietic stem cells while also inducing commitment and differentiation into different hematopoietic lineages (Dexter et al., 1977; Dorshkind, 1990). Stromal cells provide stimulating and inhibiting cytokines as well as cell-to-cell and cell-to-extracellular matrix (ECM) interactions for primitive hematopoietic cells (Dexter, 1979). An increasing number of cytokines have been shown to exist as integral membrane glycoproteins on stromal cells (Anklesaria et al., 1990; Lyman et al., 1995) or are bound to stromal cell-derived ECM molecules such as proteoglycans (Roberts et al., 1988). These proteins may participate in a novel mode of intercellular communication limited to adjacent cells (Massagué and Pandiella, 1993). Growth factors, which function both as soluble and cs proteins include members of the epidermal growth factor (EGF) family, such as stem cell factor (SCF) (Anderson et al., 1990; Huang et al., 1990), colony stimulating factor-1 (CSF-1, M-CSF) (Ladner et al., 1987; Ceretti et al., 1988), and flt3 ligand (Hannum et al., 1994; McClanahan et al., 1996).

Important insights about the function in vivo of the cs and soluble isoforms of a cytokine have been obtained by analysis of Sl^d (SCF) mutant mice. Sl^d encodes a biologically active secreted soluble SCF but lacks the membrane isoform due to an intragenic deletion of the transmembrane domain. Because the phenotype of Sl/Sl^d mice revealed very severe deficiencies like reduced stem cell numbers and anemia (McCulloch et al., 1965; Anklesaria et al., 1989), the membrane form of SCF appears to be critical for normal function. Accordingly, it has been demonstrated that the transgene expression of cell-surface SCF (csSCF) only in Sl^d mice compensates the anemic deficiencies caused by the Sl^d mutation (Kapur et al., 1998).

We have previously shown that the soluble and csSCF act functionally different on cord blood (CB) $CD34^+$ and other hematopoietic cells expressing the SCF receptor. Using mouse embryonic epithelial MMCE cells that do not express SCF and potentially interfering stroma encoded hematopoietic growth factors, we could show that only MMCE cells transduced with the csSCF could induce long-term growth of $CD34^+$ cells in vitro whereas those expressing ectopically the soluble SCF cDNA sustain short-term proliferation (Itoh et al., 1997; Friel et al., 2002). Thus, the long-term maintenance of hematopoietic precursors required the cell-to-cell contact and cs growth factors.

CSF-1 is a survival-, proliferation-, and differentiation-factor for macrophages (Guilbert and Stanley, 1980; Tushinski et al., 1982; Cecchini et al., 1994). CSF-1 and SCF are evolutionary related and show genetic

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: Os 31/16; Contract grant sponsor: Freie und Hansestadt Hamburg; Contract grant sponsor: Bundesministerium für Bildung und Forschung.

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Received 12 October 2004; Accepted 4 November 2004

DOI: 10.1002/jcp.20291

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and structural homologies: the gene structure, the sequence of the extracellular domains, the proteolytic maturation, and the tertiary folds of the proteins are very similar. Additionally, the receptors for CSF-1 and SCF appear to have diverged from an ancestor molecule of the PDGF receptor (Yarden et al., 1986, 1987).

The CSF-1 receptor (c-fms) is expressed on primitive multipotent hematopoietic cells (Bartelmez and Stanley, 1985), phagocyte progenitor cells (Tushinski et al., 1982), monoblasts, promonocytes, monocytes (Byrne et al., 1981), and tissue macrophages (Stanley et al., 1983). Several studies suggest that the CSF-1 receptor could also be involved in the regulation of more primitive cells: primitive hematopoietic cells express the CSF-1 receptor and CSF-1 synergize with IL-1, IL-3, IL-6, G-CSF, and GM-CSF to induce the formation of multipotential colonies (Broxmeyer et al., 1988; Bartelmez et al., 1989). Furthermore, the progenitor activity of day 12 spleen colony-forming cells can be blocked by neutralizing antibodies against the CSF-1 receptor (Gilmore and Shadduck, 1995). It was also shown that CSF-1 affects hematopoiesis in a very early stage in the AGM region although indirectly by enhancing growth factor and transcription factor expression (Minehata et al., 2002).

In this study, we analyzed whether csCSF-1 shows a qualitatively different biological effect on hematopoietic cells as compared to the soluble isoform. As a model of cell–cell interaction, we used the embryonic epithelial MMCE cells to express ectopically either wild-type, cs, or soluble CSF-1. In co-cultures with human and murine progenitor cells, we determined the proliferation-and differentiation capacity of hematopoietic precursors stimulated either by MMCE expressing cs- or soluble murine CSF-1. We also compared the effects of cs- and soluble CSF-1 with those of the corresponding isoforms of SCF.

MATERIALS AND METHODS

Cell lines

The murine MS5 stromal cell line was derived from irradiated normal long-term marrow culture (Itoh et al., 1989). MS5 cells were cultured in α-MEM with 20% horse serum. The murine embryonic epithelial cell line MMCE (Rapp et al., 1979) was maintained in MEM supplemented with 10% fetal calf serum. Growth of the human TF1 CD34$^+$ erythro-myeloid progenitor cell line in RPMI with 10% FCS is strictly dependent on GM-CSF (Kitamura et al., 1989). TF1 cells are blocked in differentiation. TF1 cells used in the experiments described here were adapted to permanent proliferation on MS5 stroma. MS5 stroma-dependent TF1 cells did not need GM-CSF but required stroma interaction for growth (unpublished). The murine Myl-D7 stem cell line of a lympho-erythro-myeloid phenotype was isolated from a long-term co-culture of spleen cells on MS5 stroma (Itoh et al., 1996). Myl-D7 cell growth is strictly (MS5) stroma-dependent in MEM supplemented with 10% FCS. The murine multipotential FDCP-mix cell line was derived from long-term cultures of murine bm (Spooncer et al., 1986) and was maintained in IMDM supplemented with 20% horse serum and IL-3. Source of IL-3 was conditioned medium (CM) from a murine IL-3 cDNA transfected cell line (BPV) obtained from F. Melchers at a concentration that stimulated optimal growth. Usually 10%–15% (v/v) of CM was added to cultures.

Cloning of CSF-1 isoforms and establishment of MMCE cell lines ectopically expressing different isoforms of CSF-1

Murine CSF-1 cDNAs encoding either the full-length transcript, the transcript for soluble- or cs- form only, or the full-length transcript in which the chondroitin-sulfate addition site has been eliminated by site-directed mutagenesis converting serine 276 to alanine 276 were cloned into a murine retroviral MESV-neo vector. Plasmids containing full-length- (3.9 kb) and partially spliced (2.1 kb) transcripts were kindly provided by R. Stanley (Albert Einstein University, New York, NY). CSF-1 vectors were transfected into GPE 86 cells by electroporation. Supernatants containing virus were used to infect MMCE cells. These were selected with 400 μg/ml G418 (GIBCO BRL Life Technologies GmbH, Karlsruhe, Germany). G418 resistant clones were isolated and analyzed for expression of the corresponding CSF-1 cDNA by RT-PCR as described earlier (Just et al., 1993). Three independently isolated clones of each infected MMCE cell line expressing quantitatively equal amounts of CSF-1 mRNA were chosen and used for co-culture experiments with hematopoietic cells. Expression of mRNA for the CSF-1 isoforms was examined by RT-PCR (data not shown).

Isolation and selection of cord blood CD34$^+$ cells

Umbilical CB samples were collected after birth. Informed consent according to approved institutional guidelines was obtained. Cells from different individuals were pooled. Low-density mononuclear cells (<1.077 g/ml) were isolated by centrifugation over Ficoll–Paque solution (Amersham Pharmacia Biotech, Uppsala, Sweden). CB CD34$^+$ cells were purified by immunomagnetic separation using the MACS CD34$^+$ direct isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. The purity of the enriched CD34$^+$ cells was 95%–99% when cells were passed twice on sorting columns. Purity of CD34$^+$ cells was measured by FACS analysis.

Cell separation and enrichment of murine bone marrow lineage negative cells

Bone marrow from tibia and femur of 6 to 12 weeks old C57Bl mice was flushed out and pooled from 15 mice. Bone marrow cells were incubated with rat anti-mouse antibodies against specific markers for the lymphoid (B220, CD4, and CD8), erythroid (TER 119), and myeloid (Gr-1, Mac-1) lineages (all antibodies from Becton-Dickinson, Heidelberg, Germany). Selection of cells was started by addition of goat-anti-rat IgG-coupled immunomagnetic beads (Miltenyi Biotec). Lineage-positive cells were removed on a MACS column according to the manufacturer's protocol (Miltenyi Biotec). The lin$^-$ cell fraction was labeled with immunomagnetic conjugated Sca-1 antibodies (Miltenyi Biotec) to sort for Lin$^-$Sca-1$^+$ stem cells by passing them over an additional MACS column (Miltenyi Biotec).

Long-term growth assay

The limiting-dilution assay was used as a quantitative in vitro read out system to determine the absolute frequency of clonogenic hematopoietic cells. Cells were cloned by serial dilutions on different feeders. Clones (>10^2 cells) were transferred onto new feeders every week. The frequency of negative wells without clonogenic cells was determined and the cloning efficiencies on feeders were calculated due to the Poisson statistics. For co-cultures, 5×10^3/0.5ml irradiated MS5 cells or 1×10^3/0.5ml irradiated transduced MMCE cells (MS5 18,000 rad, MMCE 2,000 rad) were seeded into 48-well plates and grown overnight in medium. Representative results are shown in the figures.

CD34$^+$ and Lin$^-$Sca-1$^+$ cell proliferation assay

Co-cultures were established in 48-well plates with 2×10^4 CD34$^+$ or Lin$^-$Sca-1$^+$ cells per well. CD34$^+$ and Lin$^-$Sca-1$^+$ cells were plated on MS5 stromal or CSF-1 or SCF cDNA transduced MMCE cells in medium consisting of α-MEM supplemented with 25% FCS (CellSystems, St. Katharinen, Germany), glutamine 2 mM, Inositol 40 μM, folic acid 16 μM, α-monothioglycerol 100 μM, hydrocortison 1 μM, penicillin 1,000 U/ml, and streptomycin 100 U/ml. Every week cells were harvested by resuspension from single well and split for the colony-forming-unit (CFU) assay, FACS analyses and the transfer to new MS5, or MMCE cells expressing ectopically the same CSF-1, or SCF isoform as used before.

Colony-forming unit assay

To determine the number of clonogenic progenitors in the co-culture, 250–1,000 cells were plated in 1 ml Terry Fox methylcellulose supplemented with recombinant growth factors (CellSystems). CFU assays were performed every week of the cells in co-culture in duplicate. Phenotype and numbers of CFU-colonies (CFU-C) (>100 cells) [granulocytes/macrophages (CFU-G + M), erythroid (BFU-e), myeloid (CFU-GM), and multipotent (CFU-GEMM)] were determined visually by microscopy 14 days after seeding.

Growth inhibition assay

Cells (5×10^4) were plated with serial dilutions of anti-murine CSF-1 neutralizing antibody (R + D Systems, Weisbaden, Germany) onto MS5 stromal- or CSF-1- transduced MMCE cells. Maximum concentration of α-murine-CSF-1 was 5 μg/ml. Half-maximal inhibition of $CD34^+$ cells on CSF-1 expressing MMCE cells was obtained with 0.3 μg/ml α-CSF-1 antibody concentration. Murine neutralizing α-CSF-1 was added every 2 days to the co-cultures. At weekly intervals cells were removed from old feeders, counted, and transferred to new feeders. Potential non-specific inhibitory effects of the antibody preparation were excluded by adding isotype-matched IgG antibody to a control culture.

Quantitation of soluble CSF-1

CSF-1 content of conditioned media of CSF-1 cDNA transduced MMCE- or non-transduced MS5 stromal cells was determined by incorporation of ^{3}H-thymidine in quantitative short-term proliferation assays. As an indicator cell line, BAC1.2F5 cells (Morgan et al., 1987) were washed and incubated with serial dilutions of recombinant murine CSF-1 (R + D Systems). BAC1.2F5 cells (10^4/100 μl/well of a 96-well plate) were incubated for 48 h, pulsed with 0.5 mCi/well/250 μl of ^{3}H-thymidine (35 Ci/mmol) for 24 h, harvested, and incorporated radioactivity was measured by scintillation counting.

Flow cytometry analysis to detect csCSF-1 on MS5- and transduced MMCE- cells

Expression of csCSF-1 was measured by flow cytometry. Cells were suspended with 10% trypsin (Life Technologies, Karlsruhe, Germany) washed and adjusted to 10^6 cells/ml in PBS/2 mM EDTA/0.5% FCS. For labeling, cells were incubated with pre-titred goat-anti-mouse CSF-1 antibodies (1×10^6 cells) (R + D Systems) for 30 min at 4°C. Cells were washed and stained with anti-goat FITC-conjugated IgG antibodies (Sigma, München, Germany) at a concentration of 10^6 cells for 10 min at 4°C. FITC-conjugated isotype-matched goat IgGs were used as controls. After staining, cells were washed twice with PBS/2 mM EDTA/0.5% FCS and analyzed in a FACS Calibur cytometer using Cellquest software (Becton Dickinson).

Analysis of differentiation markers of co-cultured cells

Cell-surface expression of lineage-specific markers was analyzed by flow cytometry. Staining and analysis of cells was performed using the following antibodies: anti-huCD34-PE (0.5 μg/10^6 cells), anti-muCD34-FITC (0.75 μg/10^6 cells), anti-huCD115 (3 μg/10^6 cells, followed by staining with PE conjugated goat-anti-rat IgG-PE), muCD115-PE (7 μl of a working solution), anti-Thy-1-FITC (0.7 μg/10^6 cells), anti-muB220-PE (0.5 μg/10^6 cells), anti-muGr-1-FITC (0.75 μg/10^6 cells), anti-muCD11b-FITC (0.75 μg/10^6 cells), anti-muF4/80-PE (0.2 μg/10^6 cells), anti-muCD68 (10 μl of a 1/10 diluted working solution followed by a second staining step with 2 μl of goat-anti-rat PE), and anti-muSca-1-PE (0.5 μg/10^6 cells). PE- and FITC- conjugated isotype-matched mouse IgGs were used as controls (all antibodies from Becton Dickinson, Inc. with the exception of anti-F4/80 from Abcam, Cambridge, United Kingdom, anti-CD68 and anti-muCD115 from Serotec GmbH, Eching, Germany, and anti-huCD115 from Oncogene, Cambridge, MA).

Statistical Analysis

Data are presented as the mean ± standard error where applicable. Differences between groups were determined by two-sided Student's *t*-test analysis, assuming unequal variances. $P \leq 0.05$ was considered statistically significant.

RESULTS

Isoforms of CSF-1 cloned into retroviral expression vectors

The murine *CSF-1* gene consists of 10 exons. The first eight contain protein-coding sequences (Ladner et al., 1988). Only the N-terminal 152 amino acids (aa) of the full-length 520 aa CSF-1 precursor are required for in vitro biological activity (Kawasaki and Ladner, 1990). Differential proteolysis of this precursor within the secretory vesicle results in the secretion of the shorter glycoprotein and the longer proteoglycan isoforms. Alternative splicing of the coding exon 6 determines which isoform of CSF-1 is expressed. The mRNA produces cs precursors without proteolytic cleavage sites when a large part of exon 6 encoding them and the unique glycosaminoglycan addition site is spliced out. Thus, mouse CSF-1 exists at least in three biologically active isoforms: a membrane-bound cs glycoprotein, a secreted glycoprotein, and a secreted chondroitin-sulfate containing proteoglycan (Price et al., 1992). We have cloned the cDNAs for the full-length CSF-1 mRNA (wild-type CSF-1) as well as the cDNAs for the soluble- and cs isoforms in retroviral MESV-neo vectors (Fig. 1a). Additionally, a full-length cDNA in which the unique consensus sequence for the glycosaminoglycan addition in Ser-276 has been eliminated was also inserted in the retroviral MESV-neo vector (Ala-276 CSF-1) (Fig. 1a).

Characterization of CSF-1 transduced epithelial cell lines used to analyze the function of the CSF-1 isoforms

To express the different CSF-1 isoforms, we used the murine non-stromal embryonic epithelial cell line MMCE (Rapp et al., 1979) that does not produce CSF-1, SCF, or hematopoietic growth factors known to synergize with CSF-1 or SCF (Friel et al., 2002) as a co-culture system since all normal mouse stromal cell lines express the wild-type CSF-1 gene. A normal mouse stromal cell line (MS5) expressing the wild-type CSF-1- and SCF-gene was used as a control.

As shown in Figure 1, MS5 stromal cells expressed the csCSF-1 protein (37%). Comparable expression was also found in MMCE cells transduced with wild-type CSF-1 cDNA. MMCE cells ectopically expressing csCSF-1 cDNA presented similar amounts (44% as evidenced by FACS analysis) of cell-surface CSF-1 (csCSF-1) (Fig. 1b).

MS5 stromal cells secreted about 0.35 ng/ml CSF-1 (Table 1). No CSF-1 was found in supernatants of untransduced MMCE cells. CM from MMCE cells transduced with the soluble CSF-1 cDNA contained 4.8 ng/ml CSF-1. Supernatants from MMCE feeders expressing csCSF-1 cDNA contained very low amounts of soluble CSF-1 (about 0.15 ng/ml; Table 1).

csCSF-1, but not the secreted soluble isoform presented by artificial stroma promotes long-term expansion of human TF1 cells

Although SCF and CSF-1 show distinct hematopoietic activities, they are structurally and evolutionary related cytokines that interact with specific receptors belonging to the same subclass III of tyrosine kinase receptors (Stanley et al., 1997; Lyman and Jacobsen, 1998). Alternative splicing of the primary mRNA of both,

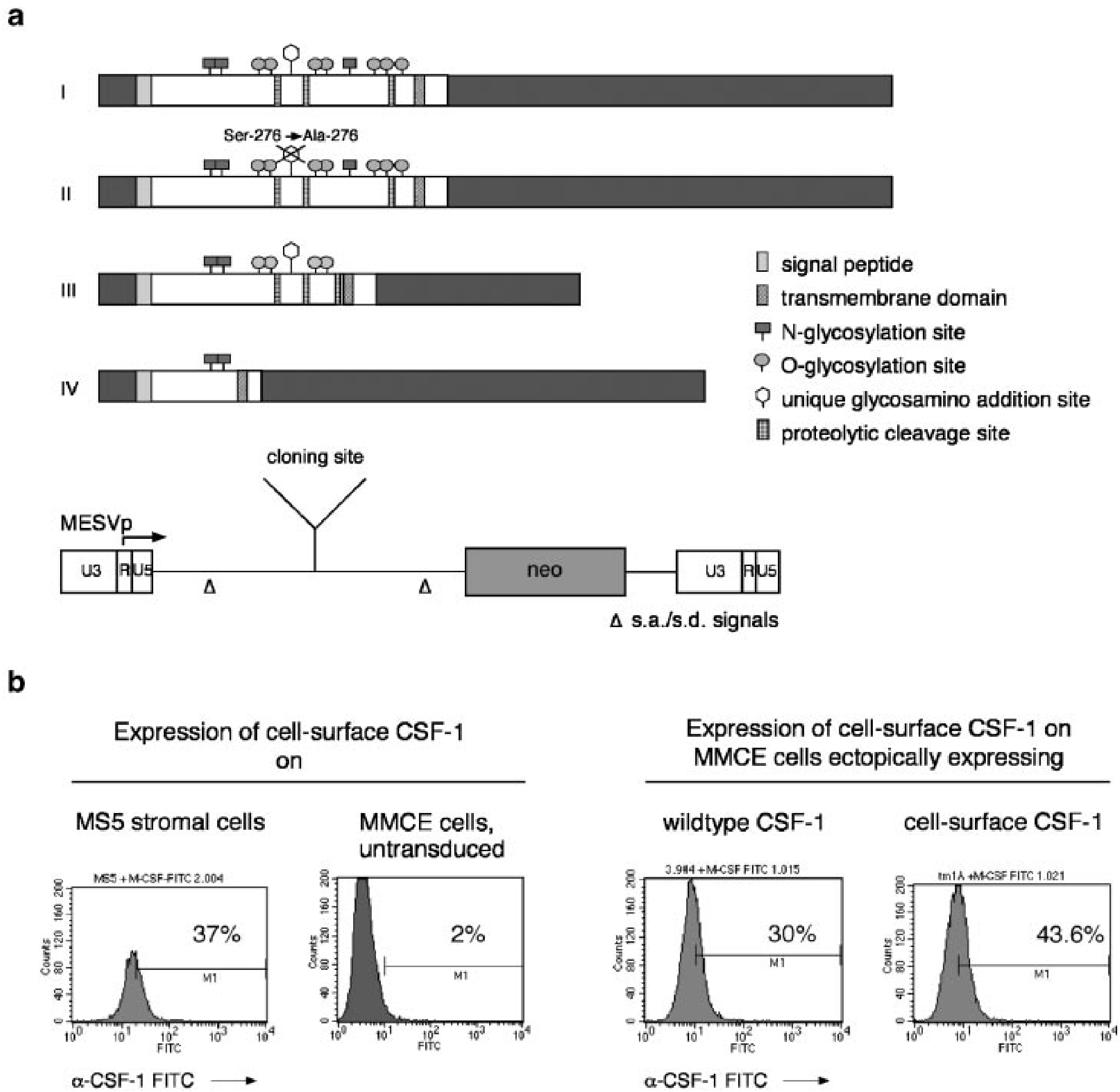


Fig. 1. **a**: Retroviral constructs used to express different CSF-1 isoforms. I: Full-length CSF-1 cDNA (wild-type CSF-1); (II) full-length CSF-1 cDNA with consensus sequence for the addition of chondroitin sulfate removed (Ala-276 CSF-1); (III) cDNA encoding the soluble isoform of CSF-1; (IV) cDNA encoding the cell-surface (cs) form of CSF-1. **b**: Presentation of cell-surface CSF-1 on MS5 stroma and CSF-1 transduced MMCE cell lines. Cells were stained for flow cytometry with goat-anti-mouse CSF-1 antibodies followed by FITC-conjugated anti-goat antibodies. Representative FACS analysis is shown. Forty-four-percent of MMCE cells transduced with the cs isoform of CSF-1 presented the protein on the cell-surface. Gate M1 was set to exclude background staining by isotype matched control antibodies.

CSF-1 and SCF, results in generation of cs and secreted forms (Ladner et al., 1987; Ceretti et al., 1988; Anderson et al., 1990; Huang et al., 1990). We therefore compared the biological effects of cs- and soluble- CSF-1 with those of the corresponding isoforms of SCF.

We first tested whether soluble and cs CSF-1 or SCF could equally transduce mitogenic signals to human hematopoietic precursor cells co-cultured on transduced MMCE feeders expressing the different CSF-1 or SCF isoforms.

Fig. 2. **a**: Cell-surface isoform of CSF-1 promotes long-term expansion of TF1 cells. Initially 1×10^3 cells were seeded on different feeders in 48-well plates. At weekly intervals cloning efficiency of cells was determined, and TF1 clones ($>5 \times 10^2$ cells) were serially transferred onto new feeders. Cloning efficiencies of TF1/MS5 co-cultures were set to 100% at each time point. The results are in percent of TF1/MS5 co-cultures. Each point is the mean ± SD of five independent experiments. Only the cell-surface (cs) isoforms of SCF or CSF-1 could induce long-term proliferation of TF1 cells. a: Comparison showed a significant difference ($P < 0.05$) after 3 weeks of co-culture; b: growth rates of cells stimulated by wild-type CSF-1 and Ala-276 CSF-1 differed not significantly ($P > 0.05$); c: csCSF-1 showed stronger growth enhancing properties than soluble CSF-1 ($P < 0.01$). **b**: Expression of the CSF-1 receptor on TF1 cells co-cultured on epithelial cells transduced with different CSF-1 isoform cDNA. For FACS analysis at day 14 of co-culture cells were labeled with rat-anti-human CSF-1 receptor (CD115) antibodies followed by PE-conjugated anti-rat antibodies. Expression of the CSF-1 receptor was upregulated by stimulation with either isoform of CSF-1. However, cells grown by soluble CSF-1 showed a twofold higher expression of the CSF-1 receptor than cells sustained by the cs form.

TABLE 1. Secretion of CSF-1 and SCF by MS5 stromal cells, epithelial MMCE cells untransduced, and CSF-1 and SCF transduced MMCE cells

Cell line	SCF in conditioned medium (CM) (ng/ml)[a]	CSF-1 in CM (ng/ml)[b]
MS5	0.08 ± 0.04	0.35 ± 0.07
MMCE untransduced	None	None
MMCE transduced by cDNA for:		
Cell-surface SCF	0.4 ± 0.14	None
Soluble SCF	4.1 ± 0.9	None
Cell-surface CSF-1	None	0.15 ± 0.02
Soluble CSF-1	None	4.8 ± 0.8

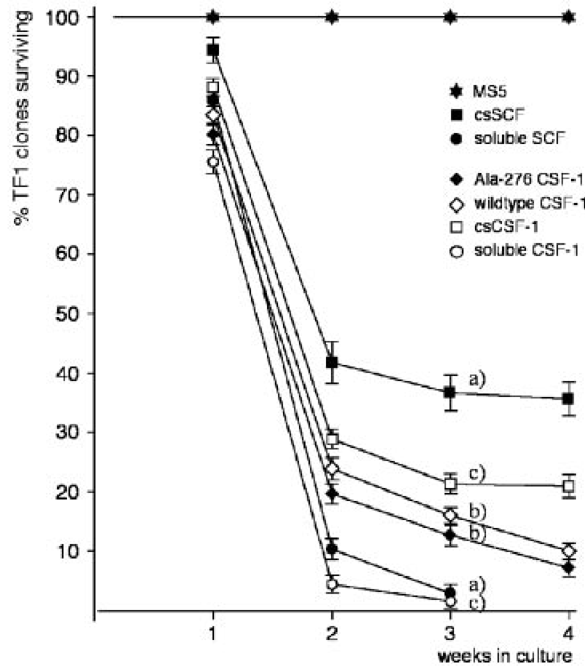
To harvest supernatants, 5×10^5 cells/ml were plated and incubated for 16 h. Supernatants were collected and concentrated fivefold.
[a]Concentrations of SCF of MS5 stromal- and SCF cDNA expressing MMCE cell lines were obtained from previous results (Friel et al., 2002).
[b]Amount of soluble CSF-1 in the conditioned medium (CM) were quantified in short-term 3H-thymidine proliferation assays (see "Materials and Methods"). Results are given as mean ± SD (three independent experiments). Parental MMCE did not produce CSF-1.

The cs forms of CSF-1 and SCF promoted long-term growth of TF1 cells at comparable cloning efficiencies (Fig. 2a). In contrast, both soluble CSF-1 and soluble SCF-1 supported proliferation of TF1 cells for only a short time (Fig. 2a) followed by clonal extinction. Soluble CSF-1 is known to induce low growth response of TF1 cells only but is unable to induce differentiation into macrophages (Kitamura et al., 1989).

TF1 cells stimulated by csCSF-1 showed a 10-fold higher clonability on day 21 than those exposed to soluble CSF-1. csCSF-1 thus promoted prolonged stimulation of TF1 cells and prevented clonal extinction. In contrast, wild-type- (encoding both proteoglycan and glycoprotein) or Ala-276- (encoding only glycoprotein) CSF-1 stimulated TF1 cells more than soluble glycoprotein CSF-1 but to a lesser extent than csCSF-1 (Fig. 2a).

To determine whether the low response of TF1 cells to soluble CSF-1 was due to low CSF-1 receptor expression on the cs of co-cultured TF1 cells, expression of the CSF-1 receptor (CD115) was tested by FACS analysis. Induction of growth of TF1 cells (and all other cell types analyzed in this study) by either CSF-1 isoform was

a



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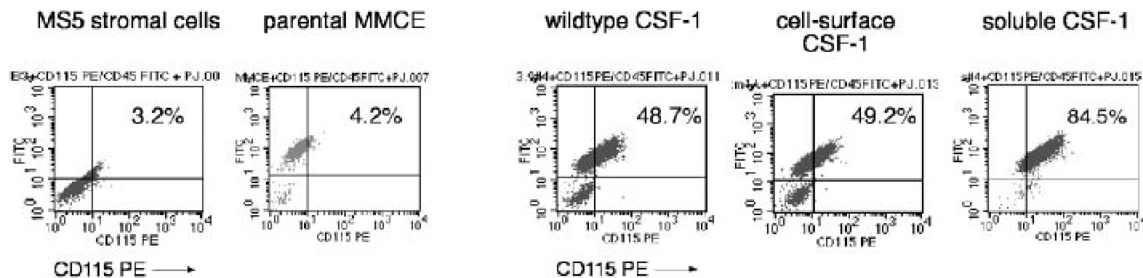

Fig. 2.

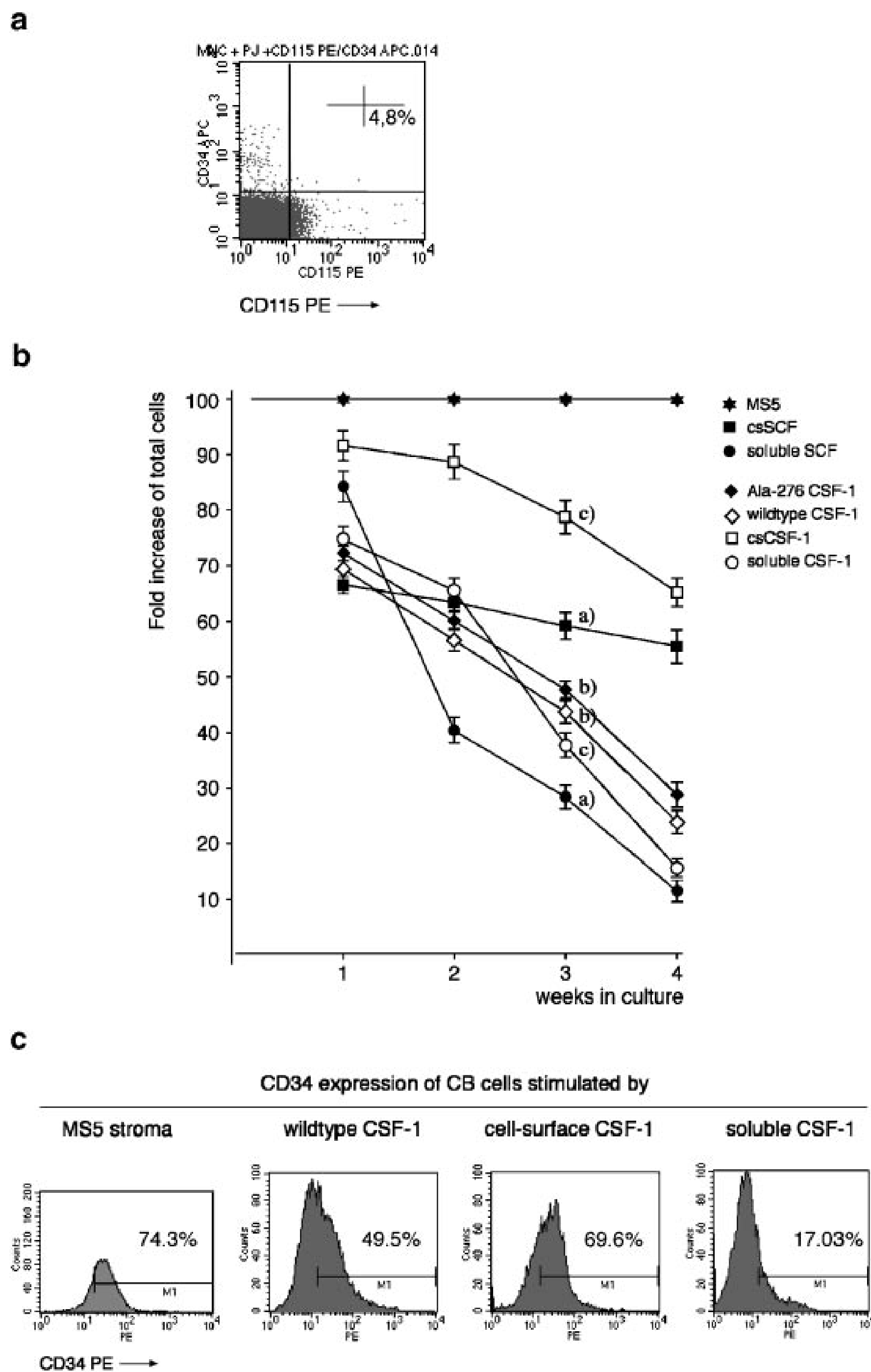

Fig. 3. **a**: Expression of the CSF-1 receptor on CB cells (for details see Fig. 2b). **b**: Human CB CD34$^+$ cells can be stimulated by different isoforms of murine CSF-1 as indicated. 2×10^4 cord blood CD34$^+$ cells were co-cultivated on feeders in 24-well plates as described in "Materials and Methods". Increase of total cells was weekly determined and cells were transferred to new feeders. The results are calculated as percent of CD34$^+$/MS5 control co-cultures. Amplification rates of CB CD34$^+$ cells on MS5 stroma were set to 100% at each time point. Each point represents the mean ± SD of five independent experiments. Cell-surface (cs) isoforms of SCF or CSF-1 induced longer-lasting self-renewal of primary CD34$^+$ cells. a: After 3 weeks in co-culture a highly significant difference between populations supported by csSCF and soluble SCF was found ($P < 0.01$); b: growth rates of cells stimulated by wild-type CSF-1 or Ala-276 CSF-1 were not different ($P > 0.05$); c: growth rate of population sustained by csCSF-1 was significantly different from cells stimulated by soluble CSF-1 ($P < 0.002$). **c**: Cell-surface CSF-1 supports early cells. Expression of the CD34 antigen of cord blood cells co-cultured for 14 days on MMCE cells expressing ectopically the different CSF-1 isoforms. Cells were stained with PE-conjugated anti-human CD34 antibodies. High expression of the CD34 antigen reflects the self-renewing potential of CB cells stimulated by csCSF-1. The effect of Ala-276 CSF-1 on sustaining early CD34$^+$ cells was comparable to the effect of wild-type CSF-1 (46.5% vs. 49.5%) and is therefore not shown.

correlated with an upregulation of the CSF-1 receptor (Fig. 2b). Exposure of TF1 cells to soluble CSF-1 resulted in an additional twofold increase in the CSF-1 receptor expression (Fig. 2b). Thus, clonal extinction of TF1 cells promoted by soluble CSF-1 could not be attributed to deficient CSF-1/CSF-1 receptor expression.

csCSF-1, but not secreted glycoprotein or proteoglycan murine CSF-1 stimulates self-renewal of CD34$^+$ cells

CD34$^+$ cells isolated from umbilical CB represent a quiescent subpopulation that initiate cell growth

following stimulation with cytokines (Mayani and Lansdorp, 1998). Five-percent of unstimulated CB mononuclear cells was CSF-1-receptor positive as measured by flow cytometry (Fig. 3a). To test which of the CSF-1 isoforms activate proliferation and which induce differentiation, $CD34^+$ cells were co-cultured on CSF-1 cDNA transduced MMCE cells. The stimulation potential of either CSF-1 isoform was compared to the corresponding SCF isoform. As a control $CD34^+$ cells were grown on MS5 stroma known to maintain hematopoietic progenitors for long-term (Issaad et al., 1993).

In the first week of co-culture all CSF-1- and SCF-isoforms promoted proliferation of $CD34^+$ cells with comparable rates (Fig. 3b). However, within 4 weeks the stimulatory activity of both wild-type- and Ala-276-CSF-1 declined dramatically. Obviously, the soluble isoforms provided only a limited expansion of CB $CD34^+$ cells.

The cs isoforms of CSF-1 and SCF in contrast induced high amplifications of $CD34^+$ cells on day 28 (Fig. 3b). Remarkably, the proliferation inducing signals of csCSF-1 or SCF remained constant and were similar throughout the culture (Fig. 3b).

The self-renewal inducing activity of the cs isoform and the differentiation promoting effect of the soluble form was indicated by CD34 antigen expression of the co-cultured cells. Cultures induced by csCSF-1 showed no decrease in the pool size of $CD34^+$ progenitors compared to $CD34^+$/MS5 stroma control cultures (Fig. 3c). The output of $CD34^+$ progenitors stimulated by wild-type- or Ala-276- CSF-1 was almost identical on day 14. In contrast, stimulation by soluble CSF-1 resulted in a marked (fourfold) reduction of $CD34^+$ progenitors (Fig. 3c). These results suggest that csCSF-1 can sustain self-renewal of early progenitors whereas soluble CSF-1 induces differentiation of stimulated cells.

The phenotype of clonogenic precursors was assessed by colony assays to compare the differentiation-inducing effects of either cs- or soluble- CSF-1. csCSF-1 sustained early cell subpopulations stronger than wild-type-, Ala-276 CSF-1, or soluble CSF-1 (four- to six-fold, respectively; Table 2) as evidenced by the higher proportion of colonies derived from early progenitor cells (BFU-e, CFU-GEMM). Stimulation of CB $CD34^+$ cells by soluble CSF-1 thus may lead to myeloid differentiation of committed progenitors. The cs isoform of SCF stimulated a higher proportion of CFU-GEMM than the corresponding CSF-1 isoform. This may be a consequence of the higher potential of SCF in supporting self-renewal of early progenitors (Table 2).

TABLE 2. Phenotype of clonogenic $CD34^+$ progenitors induced by the different CSF-1 isoforms

I.

$CD34^+$ cells stimulated by:	Colonies/250 $CD34^+$cells seeded — Weeks in culture 1	2	3*	
MS5 stromal cells	152 ± 21.8	143 ± 38.2	122 ± 26.6	
Cell-surface SCF	132 ± 25.8	120 ± 21.6	102 ± 18.3	**
Soluble SCF	137 ± 25.8	62 ± 9.9	20 ± 4.4	
Wild-type CSF-1	125 ± 23.3	88 ± 18.4	33 ± 6.1	***
Ala-276 CSF-1	130 ± 27.1	92 ± 22.1	31 ± 5.2	
Cell-surface CSF-1	141 ± 38.6	127 ± 24.1	85 ± 17.8	****
Soluble CSF-1	128 ± 33.6	96 ± 19.1	39 ± 6.2	

II.

$CD34^+$ cells stimulated by:	CFU-G + M (in %) Weeks in culture 1	2	3		CFU-GM (in %) Weeks in culture 1	2	3		BFU-e (in %) Weeks in culture 1	2	3		CFU-GEMM (in %) Weeks in culture 1	2	3	
MS5 stromal cells	32 ± 6.1	35 ± 6.c9	38 ± 6.3		23 ± 3.7	27 ± 8.1	21 ± 3.5		37 ± 9	24 ± 5.6	27 ± 5.9		8 ± 3	14 ± 4.2	15 ± 3.7	
Cell-surface SCF	28 ± 4.1	24 ± 5.2	37 ± 7.1	(a)	23 ± 4.1	29 ± 7	26 ± 4.5	(d)	37 ± 7.5	29 ± 6.1	14 ± 3.1	(g)	12 ± 3.1	18 ± 3.7	24 ± 5.2	(j)
Soluble SCF	30 ± 5.2	34 ± 8.1	46 ± 8.7		25 ± 3.3	17 ± 3.5	19 ± 3.7		38 ± 9.6	37 ± 8.2	31 ± 5.5		7 ± 2	12 ± 2.8	4 ± 0.5	
Wild-type CSF-1	44 ± 7.8	51 ± 8.7	49 ± 8.8	(b)	21 ± 4.8	23 ± 2.7	23 ± 4.4	(e)	31 ± 8.2	19 ± 4.4	20 ± 3.2	(h)	4 ± 1.2	7 ± 1.4	8 ± 0.9	(k)
Ala-276 CSF-1	47 ± 9.1	49 ± 7.6	53 ± 5.8		22 ± 6.0	26 ± 4.3	24 ± 5.5		28 ± 6.9	19 ± 5.5	18 ± 4.6		3 ± 0.8	6 ± 2.1	5 ± 0.7	
Cell-surface CSF-1	30 ± 5.5	35 ± 5.8	36 ± 7.6	(c)	25 ± 4.5	29 ± 3.7	27 ± 4.9	(f)	37 ± 7.1	25 ± 6.1	25 ± 4.1	(i)	9 ± 1.9	11 ± 1.5	12 ± 2.3	(l)
Soluble CSF-1	43 ± 6.2	60 ± 11.5	59 ± 11.1		19 ± 3.6	19 ± 1.9	34 ± 6.9		35 ± 8.3	19 ± 4.4	5 ± 0.7		3 ± 0.4	2 ± 0.4	2 ± 0.3	

Two-hundred-fifty cord blood cells co-cultured on MMCE feeders expressing the different CSF-1 isoforms were weekly plated in methylcellulose supplemented with hematopoietic growth factors. On day 14 colonies were examined. CFU-values were calculated as percent of total CFU. Data are meaň SD of five experiments. *After 3 weeks in co-culture 500 cells were plated in methylcellulose. I: Cloning efficiency of $CD34^+$ cells stimulated by different SCF- or CSF-1- isoforms as indicated. II: Distribution of clonogenic $CD34^+$ progenitors stimulated by SCF- or CSF-1- isoforms to myeloid lineages.
Statistic calculations refer to mean values after 3 weeks in co-culture. I: Cloning efficiencies of cells stimulated by: **Cell-surface (cs) CSF-1 and soluble SCF yielded significant differences ($P < 0.02$); ***Wild-type CSF-1 or Ala-276 CSF-1 showed no gross difference ($P > 0.05$); ****csCSF-1 enhanced the outcome of clonogenic cells compared to soluble CSF-1 ($P < 0.02$). II: Hematopoietic activity of different SCF- or CSF-1- isoforms on clonogenic progenitors after 3 weeks stimulation; induction of G + M colonies, (a): csSCF and soluble SCF showed comparable effects ($P > 0.05$); (b): exposure to wild-type CSF-1 or Ala-267 CSF-1 yielded no differences ($P > 0.05$); (c): soluble CSF-1 stimulated more lineage-restricted cells than csCSF-1 ($P < 0.05$). Induction of GM colonies, (d): csSCF was more efficient than soluble SCF ($P < 0.05$); (e, f): differences between wild-type CSF-1 and Ala-276 CSF-1 as well as csCSF-1 and soluble CSF-1 were not apparent ($P > 0.05$). Induction of BFU-e colonies, (g): csSCF stimulated more erythroid colonies than soluble SCF ($P < 0.01$); (h): frequency of colonies stimulated by either wild-type CSF-1 or Ala-276 CSF-1 was comparable ($P > 0.05$); (i): csCSF-1 increased the output of erythroid progenitors when compared to soluble CSF-1 ($P < 0.01$). Induction of GEMM colonies, (j): csSCF enhanced the frequency of multi-potential cells in contrast to soluble SCF ($P < 0.02$); (k): wild-type CSF-1 showed a similar effect as Ala-276 CSF-1 ($P > 0.05$); (l): csCSF-1 induced higher expansion of multipotential cells than soluble CSF-1 ($P < 0.05$).

CSF-1 neutralization is associated with decreased proliferation of CD34$^+$ cells on CSF-1 producing MMCE cells

The forgoing experiments indicate that murine CSF-1 is active on human cells. While human CSF-1 is known to be active on murine cells, the converse has not been reported. Therefore, to confirm that murine CSF-1 can activate the human CSF-1 receptor, human CD34$^+$ cells were co-cultivated on MMCE feeders ectopically expressing one of the CSF-1 isoform cDNAs. Neutralizing antibodies against murine CSF-1 were added to the culture. CD34$^+$ cell numbers promoted by CSF-1 in either form showed a fourfold reduction in growth compared to the corresponding cultures in presence of IgG control antibodies (Fig. 4), confirming that expansion of human CD34$^+$ cells was indeed induced by murine CSF-1.

cs- and soluble- CSF-1 overexpressed by MMCE cells cause differential responses of Myl-D7 cells in co-cultures

We next analyzed the effects of the CSF-1 isoforms on the development of murine hematopoietic progenitor cells. Myl-D7, a strictly (MS5) stroma-dependent stem cell line (Itoh et al., 1996) was used in the experiments described here since it expresses among other multilineage markers high levels of CSF-1 receptors (at least 51%) (Fig. 6). Neither soluble nor wild-type CSF-1 could markedly stimulate Myl-D7 cells (Itoh et al., 1996).

A strong proliferation response of Myl-D7 cells was only obtained on feeders expressing csCSF-1 as is clearly shown in Figure 5. Although the proliferation stimulated by csCSF-1 declined slightly during the cultivation period, it was still fourfold stronger than that of wild-type- or soluble- CSF-1 at 3 weeks of culture (Fig. 5).

To characterize the discrepancy in the proliferation induced by soluble CSF-1 or csCSF-1, we examined the surface-marker phenotype of co-cultured Myl-D7 cells by FACS analyses and compared it to parental cells grown on MS5 (CSF-1$^+$SCF$^+$) stroma. Cells stimulated by csCSF-1 showed only a slight trend towards differentiation into the macrophage lineage as demonstrated in Figure 6. Only a small subfraction of cells expressed the mononuclear-phagocyte-specific marker F4-80 (Hume et al., 1984). In contrast, cells induced by wild-type- or soluble CSF-1 expressed 12-fold and 23-fold higher amounts of the F4-80 antigen. The multipotential phenotype of Myl-D7 cells, as indicated by the unchanged expression of the CD90 (Thy-1) and F4-80 antigens, was thus maintained in co-cultures sustained by csCSF-1. Soluble CSF-1, as expected, induced an apparent shift toward differentiation into the macrophage-lineage.

Stimulation by csCSF-1 supports the multipotential phenotype of FDCP-mix cells

Proliferation abrogation of Myl-D7 cells induced by soluble CSF-1 can be the result of either apoptosis or differentiation. Apoptosis and differentiation were not easy to distinguish because survival and development of Myl-D7 cells is dependent on stroma. Co-culturing of

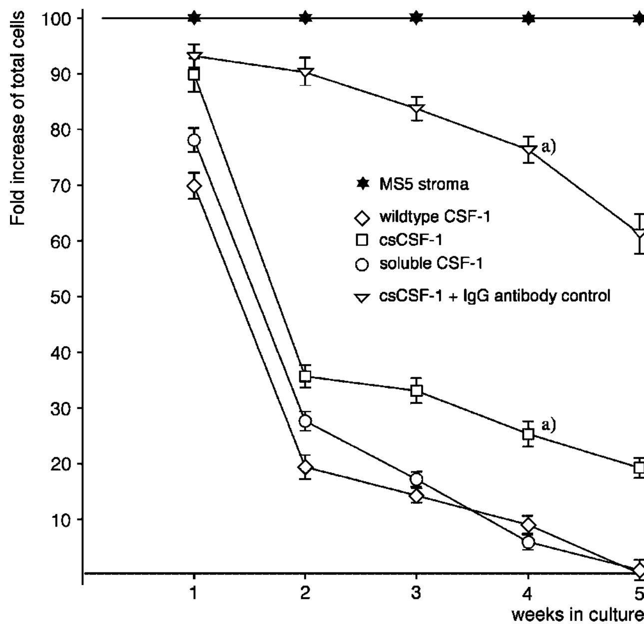

Fig. 4. Blocking of the human CSF-1/CSF-1 receptor complex by anti-mouse CSF-1 antibodies abrogates the proliferation of CD34$^+$ cells in co-culture. CD34$^+$ cells (5×10^4) were co-cultured in presence of 0.5 μg/ml anti-murine CSF-1 neutralizing antibody on MS5 stromal- and CSF-1 transduced MMCE- cells (see "Materials and Methods"). For an antibody control, CD34$^+$ cells were cultured in presence of an isotype-matched irrelevant antibody (0.5 μg/ml) on top of cell-surface (cs) CSF-1 cDNA transduced MMCE cells. Neutralizing CSF-1 antibody and IgG control antibody were added from day 7 on thereafter every 2 days. Increase of total cells was determined weekly. Cells were then transferred to new CSF-1 cDNA transduced MMCE- or MS5 cells. Results are in percent of CD34$^+$/MS5 control co-cultures. Proliferation rates of CB CD34$^+$ cells on MS5 stroma were set to 100% at each time point. Each point is the mean ± SD of five independent experiments. a: After 4 weeks in co-culture growth of cells on MMCE cells expressing ectopically csCSF-1 was profoundly lower in presence of anti-murine CSF-1 antibodies than in presence of IgG control antibodies ($P < 0.002$).

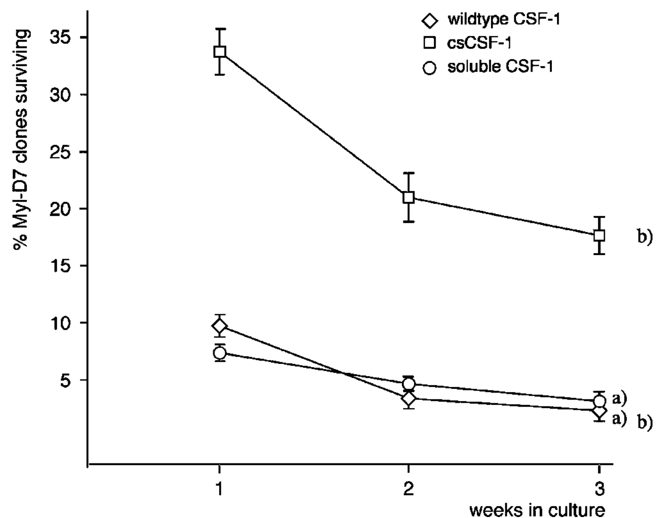

Fig. 5. Proliferation of the murine Myl-D7 stem cell line is efficiently stimulated only by cell-surface CSF-1. Myl-D7 cells (2×10^3) were cultured on MMCE cells expressing the different CSF-1 isoforms in 48-well plates ("Materials and Methods"). Cloning efficiencies were determined weekly and Myl-D7 clones (>10^2 cells) were transferred onto new MMCE cells. Values of Myl-D7/MS5 co-cultures were set to 100% at each time point (data not shown). Results are express as percentage of control Myl-D7/MS5 cultures and are mean values ± SD (four experiments). After 3 weeks in co-culture, (a) no difference was found in the proliferation rates of cells stimulated by either wild-type- or soluble- CSF-1 ($P > 0.05$); (b) mean values for populations stimulated by cell-surface- or soluble- CSF-1 were significantly different ($P < 0.001$).

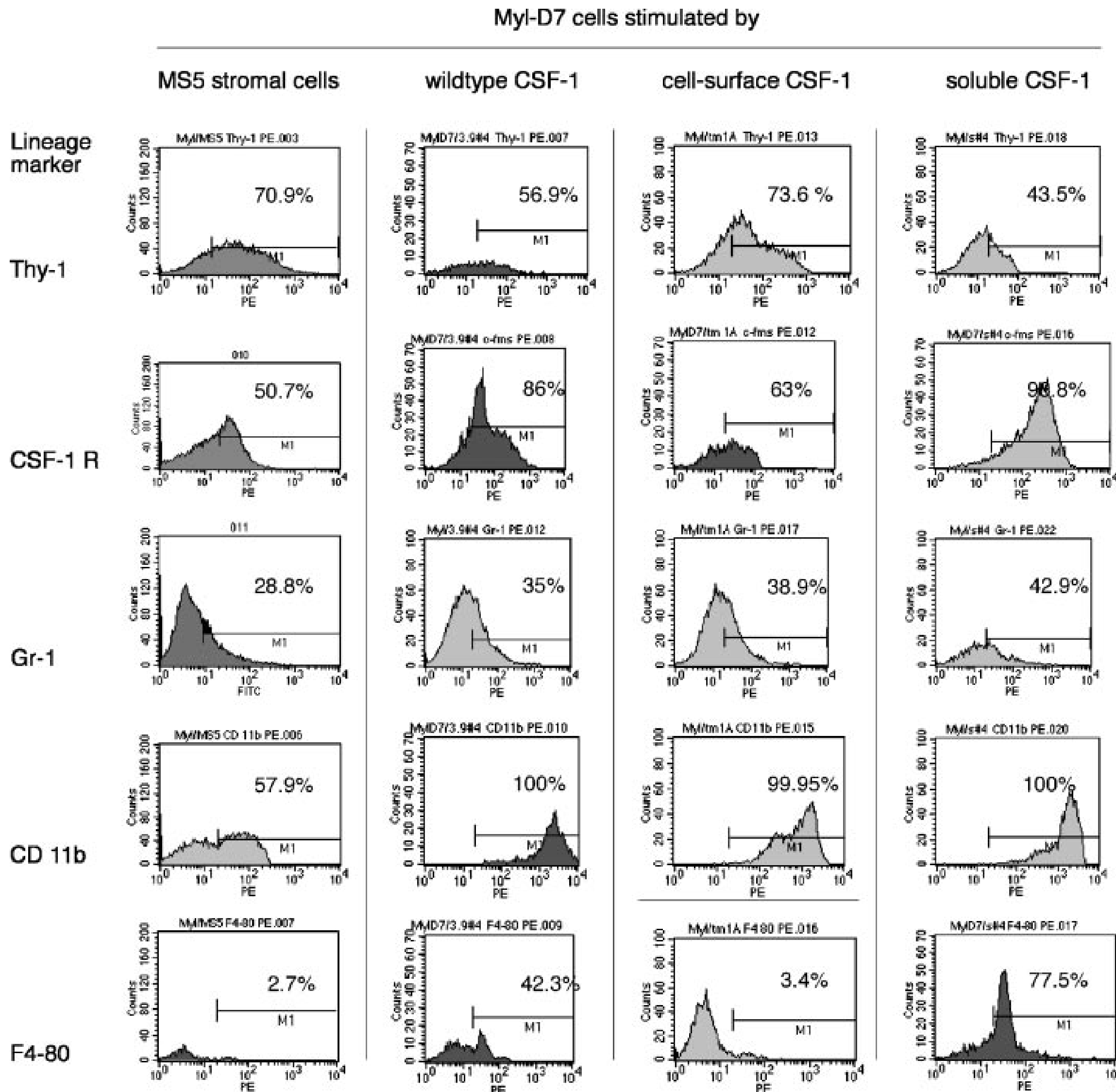

Fig. 6. Cell-surface CSF-1 maintained the multipotential phenotype of Myl-D7 cells. After 1 week in culture cells were stained with antibodies against lineage markers as indicated and against CSF-1 (CD115) receptor. For CSF-1 receptor staining cells were labeled with rat-anti-mouse c-fms antibody and stained with PE-conjugated goat-anti-rat antibodies. All data are representatives of three separate experiments. Marker positive cells were defined by a previously set gate with PE (or FITC) expression greater as the isotype control. The number of differentiated cells was significantly lower in Myl-D7 cells stimulated by csCSF-1 than in cells supported by the soluble form.

Myl-D7 cells on MMCE feeders can lead to spontaneous differentiation. We envisaged that separation between apoptosis and differentiation might thus be easier when studied in FDCP-mix cells, which grow in a stroma-independent but factor-dependent manner. The multipotent FDCP-mix cells show self-renewal when cultured in presence of high concentrations of IL-3 and can be induced to differentiate into cells of the granulocytic- or phagocytic lineage by soluble growth factors (Spooncer et al., 1986; Heyworth et al., 1990; Just et al., 1991).

FDCP-mix cells were thus cultured on MMCE feeders expressing either CSF-1 isoform. The phenotype of the co-cultured cells was determined by flow cytometry. csCSF-1 stimulated FDCP-mix cells showed a similar phenotype as parental cells supported by IL-3. This was not only seen by the unaltered expression of the macrophage lineage-specific markers CD68, CD11b, and Gr-1 but also by coexpression of the CD34- and B220-antigens which are typical for the multipotential phenotype (Fig. 7). The only exception was a marked upregulation of the macrophage-specific antigen F4-80, possibly caused by presence of low amounts of soluble CSF-1 (cf. Table 1) that could have been proteolytically processed from a fraction of the cs protein.

Soluble- as well as wild-type- CSF-1, as expected, induced differentiation into the granulocyte-macrophage direction: almost all FDCP-mix cells presented the macrophage specific markers CD68 and F4-80 (Fig. 7) although to a lesser extent the antigens Gr-1 and CD11b (Fig. 7). Addition of recombinant CSF-1 (4 ng/ml) to parental FDCP-mix cells grown without IL-3 indicated that the upregulation (1.5- and 9-fold, respectively) of the macrophage specific markers CD68 and F4-80 resulted from the CSF-1 exposure. Spontanous differentiation in FDCP-mix cells thus is unlikely (Fig. 7).

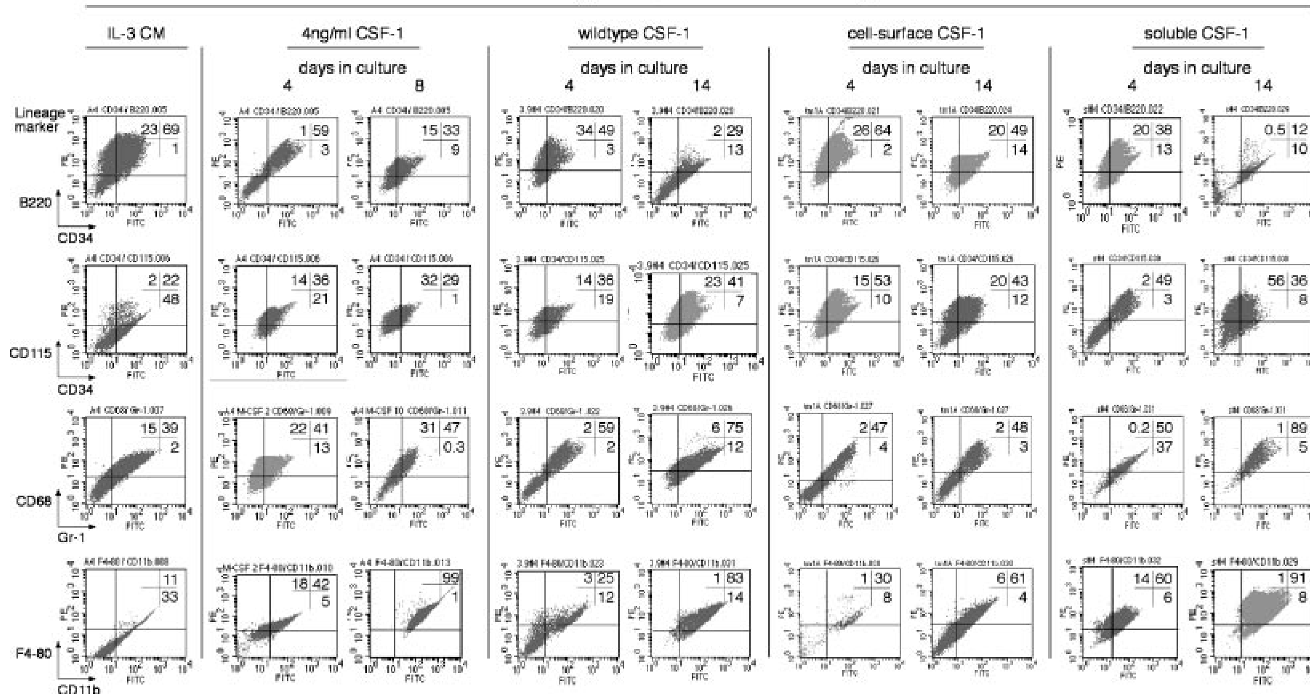

Fig. 7. FDCP-mix cells exposed to cell-surface CSF-1 showed a similar phenotype as FDCP-mix cells stimulated by IL-3. FDCP-mix cells were cultured on epithelial feeders expressing wild-type-, cell-surface-, or soluble CSF-1. Cells were harvested at day 4 and 14 and stained with a panel of macrophage-lineage specific antibodies as indicated (for gate-setting see note to Fig. 6). For comparison, parental FDCP-mix cells were either cultured in medium supplemented with 15% IL-3 conditioned medium (CM) (normal culture condition; see "Materials and Methods") or in medium with addition of 4 ng/ml recombinant CSF-1 but without IL-3 CM. As shown by FACS analysis, in FDCP-mix cells stimulated by csCSF-1 the undifferentiated status was predominant. In populations sustained by soluble- or wild type-CSF-1 the reversed situation was shown.

A strong sustained mitogenic signal is predominantly induced by csCSF-1 in $Lin^{-}Sca\text{-}1^{+}$ primitive quiescent stem cells

We have shown that csCSF-1 supports survival and proliferation of hematopoietic progenitors. In an attempt to determine whether csCSF-1 could stimulate a subpopulation of very primitive cells, we co-cultured lineage marker negative mouse bm cells ($Lin^{-}Sca\text{-}1^{+}$) (Fig. 8a) on CSF-1 cDNA transduced MMCE- and on MS5 stromal cells (Fig. 8b). From 2 weeks on a continuous decrease in the proliferation of hematopoietic cells supported by wild-type CSF-1, 276-Ala CSF-1, or soluble CSF-1 was seen. The better growth response of cells exposed to csCSF-1 was again seen by higher (3-fold and 10-fold) levels of proliferating cells over 4 weeks of co-cultivation in contrast to soluble CSF-1 (Fig. 8b).

The number of colonies obtained in the CFC assay of $Lin^{-}Sca\text{-}1^{+}$ cells that had been stimulated with each of the CSF-1 isoforms corresponded to the resultant amplification (cf. Table 3, Fig. 8). The cloning potential of cells after 3 weeks in culture maintained by csCSF-1 was about fourfold higher than in cultures stimulated by soluble-, wild-type-, or Ala-276- CSF-1. The qualitative differences between these CSF-1 forms in sustaining primitive hematopoietic progenitors (Table 3) were again obvious. The number of colonies produced by early progenitors (CFU-GEMM) in the population stimulated by csCSF-1 was 4-fold higher than in parallel cultures promoted by wild-type- or Ala-276 CSF-1 and even 13-fold higher than in cultures sustained by soluble CSF-1 (Table 3). Conversely, soluble CSF-1 apparently induced differentiation into mature cells. Following 2 weeks co-culturing, 87% of all colonies were derived from macrophage precursors, whereas only 30% of the colonies induced by csCSF-1 was of that type.

DISCUSSION

Although structurally related, CSF-1 supports the survival, proliferation, and differentiation of macrophages (Pollard and Stanley, 1996) whereas SCF is a co-stimulatory factor with minor lineage specifity synergizing with various cytokines to stimulate growth of multipotential progenitors (McNiece et al., 1991; Ramsfjell et al., 1997).

We implied in a previous study that the cs- and soluble isoforms of SCF are partly antagonistic in stimulating hematopoietic precursor cells. Membrane-bound SCF induced a strong proliferation signal whereas soluble SCF caused clonal extinction following a short proliferation inducing period (Itoh et al., 1997; Friel et al., 2002). To analyze the potential roles of the CSF-1 isoforms, we used the murine epithelial cell line MMCE to express different CSF-1 cDNAs. In this system, we documented that murine CSF-1 can activate the CSF-1 receptor on human cells as demonstrated by the inhibition of proliferation when CSF-1 neutralizing antibodies were added to cultures. We have no information on the relative amounts of murine and human CSF-1 needed to stimulate human cells, but it is likely that higher concentrations are necessary and that these are achieved by the continuous production of the CSF-1 isoforms by transduced MMCE cells. Consistent with our previous results with SCF, the data presented here indicate that binding of the cs isoform of CSF-1 to the CSF-1 receptor leads to long-term high proliferation rates of human and murine hematopoietic progenitor cells. In contrast, activation of the CSF-1 receptor by

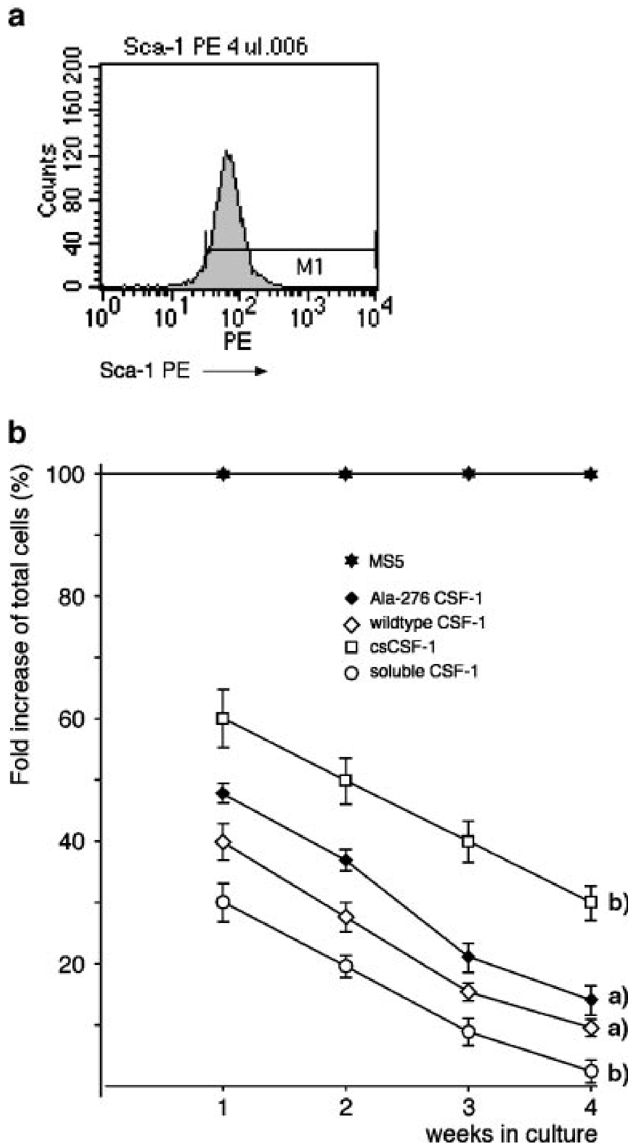

Fig. 8. Expansion of murine lineage negative bone marrow cells co-cultured in presence of different CSF-1 isoforms. Lineage-negative cells were sorted by labeling bone marrow cells with antibodies against lineage-specific markers (see "Materials and Methods") conjugated to microbeads or coupled to microbeads through streptavidin–biotin compounds. Lineage-positive cells were sorted out (>90%; data not shown) by magnetic cell separation. **a**: Lineage-negative cell population used for co-cultivation experiments was enriched for early progenitors (>90% Sca^+). **b**: Cell-surface CSF-1 can stimulate very early cells. $Lin^-Sca\text{-}1^+$ cells were co-cultivated on MMCE cells ectopically expressing different CSF-1 isoforms as indicated ("Materials and Methods"). Increase of $Lin^-Sca\text{-}1^+$ cells on MS5 stromal cells was set to 100% at each time point. Proliferation of $Lin^-Sca\text{-}1^+$ cells on MMCE cells was shown as percent of $Lin^-Sca\text{-}1^+$/MS5 controls. Each point represents the mean ± SD (three separate experiments). After 3 weeks in co-culture, (a) the proliferation rates of populations stimulated by either wild-type CSF-1 or Ala-276 CSF-1 differed not significantly ($P > 0.05$); (b) growth rate of cells stimulated by soluble CSF-1 was profoundly lower than that of cells stimulated by csCSF-1 ($P < 0.05$).

soluble CSF-1 resulted in short-term proliferation and differentiation of hematopoietic progenitors.

Cell-surface and soluble isoforms of CSF-1 not only differ in growth induction but also in their influence on the development of stimulated cells. Activation of the CSF-1 receptor by csCSF-1 resulted in maintenance of the progenitors characterized by the unaltered expression of either the CD34 antigen in human- or the B220-, CD34-, and Thy-1 markers in murine- (Myl-D7- and FDCP-mix) cells. Only a small subpopulation of Myl-D7- or FDCP-mix cultures was induced to differentiation as indicated by the marginal upregulation of the macrophage-specific markers F4-80 and CD11b (FDCP-mix cells) or CD11b and Gr-1 (Myl-D7 cells). The appearance of differentiated cells in these cultures was most likely caused by presence of small amounts of soluble CSF-1 which have been derived from the cs protein by proteolytic cleavage by a putative alternative cleavage site. csCSF-1 is able to stimulate self-renewal of human $CD34^+$ progenitors as well as murine lineage-negative bm cells. In both cultures, BFU-e- and CFU-GEMM levels were substantially higher in csCSF-1 stimulated cultures compared to those cultured with soluble-, wild-type-, or Ala-276 CSF-1.

Our results that CSF-1 can directly affect early hematopoietic progenitors agrees with earlier reports showing that blockage of the CSF-1 receptor resulted in inhibition of the multipotential progenitor (day 12 CFU-S) activity (Gilmore and Shadduck, 1995) and that CSF-1 can synergize with IL-3 in stimulating high proliferative colony-forming cells (Bartelmez et al., 1989).

The growth inducing effects of the cs forms of CSF-1 and SCF were comparable. However, aspects of the phenotype of stimulated cells show the qualitative difference between both. csSCF stimulated more the primitive colony-forming precursors (BFU-e, CFU-GEMM) of human $CD34^+$ cells than the corresponding isoform of CSF-1, demonstrating the stronger effect of csSCF in inducing self-renewal of early progenitors. The results suggest that the cs isoform of CSF-1 can replace the cs isoform of SCF in supporting long-term proliferation but not for stimulation of self-renewal as shown by FACS analysis and colony assays.

Growth abrogation of human- and murine hematopoietic- cells caused by soluble-, wild-type-, or Ala-276 CSF-1 could be, in contrast to the effects of the csCSF-1, a consequence of the induction of differentiation into the monocytic lineage. The lack in supporting progenitor subpopulations was not unexpected since macrophage precursors are known to be the major targets of soluble CSF-1 (Stanley et al., 1983).

SCF is active only in cooperation with other cytokines (Lemoli et al., 1993; Ramsfjell et al., 1997). Therefore growth abrogation induced by soluble SCF was not accompanied by significant differentiation of stimulated cells. Instead SCF leads to clonal extinction (Itoh et al., 1997; Friel et al., 2002).

Wild-type CSF-1 showed similar effects in promoting growth and differentiation of human and murine hematopoietic cells as the soluble isoform suggesting that full-length CSF-1 mRNA in epithelial MMCE cells was primarily translated into secreted CSF-1 molecules. Alternative splicing of the full-length primary transcript to that coding for the secreted form of CSF-1 is found dominantly in many cell types like fibroblasts or endothelial cells (Ladner et al., 1987, 1988; Uemura et al., 1993) but in bm stromal- or liver- cells (Pogue-Geile et al., 1995) csCSF-1 is predominantly expressed.

The soluble CSF-1 isoform in which the site for the addition of the chondroitin sulfate chain has been altered (Ala-276 CSF-1) showed similar physiological effects as wild type CSF-1. Secretion of the proteoglycan CSF-1 peptide has been suggested to be associated with binding to the ECM (Price et al., 1992) possibly by

TABLE 3. Clonogenic potential and distribution of lineage negative progenitors was differently stimulated by various CSF-1 isoforms

I.

$Sca^{+}Lin^{-}$ cells stimulated by:	Colonies/500 cells seeded			
	Weeks in culture			
	1	2	3*	
MS5 stromal cells	178 ± 24.9	94 ± 12.2	68 ± 8.1	
Wild-type CSF-1	71 ± 7.9	26 ± 3.2	14 ± 2	**
Ala-276 CSF-1	82 ± 9.8	33 ± 6.3	23 ± 3.8	**
Cell-surface CSF-1	96 ± 12.4	49 ± 7.1	31 ± 4.9	***
Soluble CSF-1	46 ± 5.5	23 ± 3.2	9 ± 1.8	***

II.

$Sca^{+}Lin^{-}$ cells stimulated by:	CFU-G+M (in %)				CFU-GM (in %)				BFU-e (in %)				CFU-GEMM (in %)			
	Weeks in culture				Weeks in culture				Weeks in culture				Weeks in culture			
	1	2	3		1	2	3		1	2	3		1	2	3	
MS5 stromal cells	9 ± 1.2	16 ± 7.1	13.9 ± 1.8		25.8 ± 3.4	45.7 ± 6.4	35.3 ± 5		11.2 ± 1.3	17.4 ± 2.4	28.1 ± 3.7		10.1 ± 1.4	27.7 ± 4.4	22 ± 3.5	
Wild-type CSF-1	53 ± 5.8	57 ± 6.9	84 ± 11.3	(a)	47 ± 7.1	20.1 ± 2.2	6.2 ± 0.9	(c)	0	17 ± 3.1	5.9 ± 0.9	(e)	0	6.8 ± 1	2.8 ± 0.4	(f)
Ala-276 CSF-1	43.6 ± 4.3	52.8 ± 7.8	76.5 ± 9.4	(a)	44.9 ± 8.2	36 ± 6.3	14.2 ± 2.1	(c)	6.8 ± 2.3	9.1 ± 1.9	5.3 ± 2.1	(e)	4.7 ± 0.9	3.1 ± 0.4	4 ± 0.6	(f)
Cell-surface CSF-1	27.2 ± 3.5	32.6 ± 4.6	36.2 ± 4.7	(b)	46.7 ± 6.1	37.2 ± 5.5	42.6 ± 5.1	(d)	11.9 ± 1.5	11.6 ± 1.7	8.1 ± 1.5		14.1 ± 2.3	18.6 ± 3.2	13 ± 2.1	
Soluble CSF-1	56 ± 7.3	87 ± 12.2	95 ± 13.3	(b)	29 ± 4.1	5.3 ± 0.8	2.2 ± 0.4	(d)	7.8 ± 1.5	0	0		6.9 ± 1.1	0	0	

Five-hundred lineage-negative Sca-1^{+} cells stimulated by either wild-type-, cell-surface (cs)-, or soluble- CSF-1 were plated in methylcellulose. Fourteen-day later colonies were examined. CFU-values are calculated as percent of total CFU. Results are mean ± SD of three experiments. *After 3 weeks in culture, 1,000 cells were plated in methycellulose. I: Cloning efficiency of $Lin^{-}Sca^{+}$ cells stimulated by different CSF-1 isoforms as indicated. II: Distribution of clonogenic progenitors to myeloid lineages stimulated by different CSF-1 isoforms.
Stastic calculations refer to mean values obtained from 3 weeks co-cultures. I: Comparison of the cloning efficiencies of cells stimulated by: **wild-type CSF-1 and Ala-276 CSF-1 as well as ***cell-surface (cs) CSF-1 and soluble CSF-1 showed significant differences (both $P < 0.02$). II: Comparison of the hematopoietic activity of different CSF-1 isoforms on clonogenic progenitors after 3 weeks of stimulation; induction of G + M colonies, (a): no difference in the potential of wild-type CSF-1 or Ala-276 CSF-1 ($P > 0.05$); (b): soluble CSF-1 stimulated more lineage-restricted colonies than csCSF-1 ($P < 0.01$). Induction of GM colonies, (c): Ala-276 CSF-1 induced more colonies than wild-type CSF-1 ($P < 0.02$); (d): csCSF-1 increased the frequency of bipotent colonies compared to soluble CSF-1 ($P < 0.01$). Induction of BFU-e and GEMM colonies, (e, f): wild-type CSF-1 or Ala-276 CSF-1 stimulated erythroid- or multipotential cells equally (both $P > 0.05$).

generating a local reservoir of CSF-1 molecules for cells. The fact that Ala-276 CSF-1 showed comparable functions as wild-type CSF-1 suggests that in our co-culture system addition of the proteoglycan chain has no influence on the biological activity of the CSF-1 peptide.

Differential effects of the isoforms of a particular factor could be the consequence of receptor-mediated signaling. Proliferation leading to self-renewal could depend on the signaling intensity through the factor/receptor complex (Zandstra et al., 2000). In this context, cs factors mediating cell–cell interactions may substitute for high local concentrations of a soluble factor resulting in delayed internalization of the factor/receptor complex that normally takes place immediately after soluble factor/receptor interaction. Indeed, it was reported, that csSCF is more persistent in inducing phosphorylation of the SCF receptor than soluble SCF (Miyazawa et al., 1995). The molecular mechanisms by which the CSF-1/CSF-1 receptor complex causes different biological effects are not known. We infer that csCSF-1 in analogy to SCF may induce a strong receptor activation thereby favoring self-renewal of early cells whereas soluble CSF-1 provides only a short receptor activation resulting in monocytic differentiation of CSF-1 receptor presenting cells.

In summary, our study shows that CSF-1 can generate different developmental effects depending on which splice variant is expressed by stromal cells. Splicing of CSF-1 mRNA precursor leads to csCSF-1 proteins that may support the self-renewal of early hematopoietic precursors. Alternative splicing of the CSF-1 mRNA produces soluble CSF-1 proteins that induce, coupled with a short proliferation period, differentiation of committed hematopoietic cells to mature F4-80^{+} macrophages. Thus, the cs isoform of a differentiation-inducing soluble factor can perform functions like an early acting cytokine whereas soluble CSF-1 is more needed for terminal differentiation.

ACKNOWLEDGMENTS

The Heinrich-Pette-Institut is financially supported by the Freie und Hansestadt Hamburg and the Bundesministerium für Bildung und Forschung. We thank Dr. R. Stanley (Albert Einstein College of Medicine, New York, NY) for critical reading of the manuscript and for providing us with CSF-1 cDNAs.

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