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Regulation of low density lipoprotein receptor activity in Chinese hamster ovary cells transfected with the c-*fms* gene

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Abstract Chinese hamster ovary (CHO) cells were transfected with the human c-*fms* gene, which encodes the receptor for macrophage colony-stimulating factor, to examine the effects on the low density lipoprotein (LDL) receptor activity. Degradation of [^{125}I]LDL was significantly reduced in the transfected CHO cells as compared to non-transfected cells when incubated in lipoprotein-deficient serum. Quantitative analysis of LDL receptor mRNA using a competitive PCR method demonstrated that LDL receptor suppression occurred at transcription. These findings suggest that expression of the c-*fms* gene is involved in the regulation of LDL receptors on macrophages.

Key words: c-*fms*; Low density lipoprotein receptor; Macrophage colony-stimulating factor (M-CSF); Gene transfection

1. Introduction

The c-*fms* proto-oncogene encodes the macrophage colony-stimulating factor (M-CSF) receptor, which plays a critical role in the proliferation and differentiation of cells of the monocyte–macrophage lineage [1,2]. Binding of M-CSF to its receptors activates the intrinsic tyrosine kinase activity of the receptors. Rapid phosphorylation at tyrosine residues ensues, initiating a cascade of metabolic and gene regulatory events involved in cell growth and differentiation [3,4]. In the initial stages of atherosclerosis, monocytes migrate from the circulating blood to the subendothelial space, where they mature and differentiate into macrophages and endocytose lipoprotein cholesterol through lipoprotein receptors [5–8]. LDL receptor activity is reduced and scavenger receptor activity is induced during maturation and differentiation of monocytes to macrophages in vitro [9–11]. Since expression of c-*fms* generally coincides with differentiation to the monocyte–macrophage lineage, lipoprotein receptor activities may be related to c-*fms* gene expression in monocytes and macrophages. However, the influences of c-*fms* expression on lipoprotein receptor activities has not been well studied. In the present study, we transfected the human c-*fms* gene into CHO cells to examine its effects on LDL receptor activity.

2. Materials and methods

2.1. Materials

CHO-K cells were purchased from American Type Culture Collection. Ham's F-12 medium (HAM), RPMI 1640 medium, newborn calf serum (NCS) and fetal calf serum (FCS) were purchased from Gibco. Geneticine (G-418) was purchased from Sigma Chemical Co. (St. Louis, MO). Na^{125}I and [^{3}H]thymidine were purchased from ICN Biomedicals Inc. [α-^{32}P]dCTP and [^{125}I]protein A were purchased from Amersham International plc. Recombinant human M-CSF was obtained from Morinaga Milk Industry Co. (Tokyo, Japan). pSM c-*fms* containing the human c-*fms* cDNA and anti-c-*fms* polyclonal antibody were kindly provided by Charles J. Sherr. The human c-*fms* cDNA was inserted into a plasmid derived from the DNA provirus of the Susan McDonough strain of feline sarcoma virus (SM-FeSV) [12]. All reagents were of analytical grade.

2.2. Cells and lipoproteins

CHO-K cells were maintained in HAM containing 10% NCS in a humidified 5% CO_2 incubator at 37°C. Mouse peritoneal macrophages were prepared according to the method of Cohn and Morse [13], and human monocyte-derived macrophages were cultured as described [14]. After 4 days of intraperitoneal injection of 1 ml of 2.5% thioglycolate medium I (Wako Pure Chemical Industries Ltd., Japan), peritoneal macrophages were harvested from C57BL6/J male mice in phosphate-buffered saline (PBS) containing 40 U/ml heparin. The cells were cultured with serum-free RPMI medium on 12-well plastic dishes at a final concentration of 2×10^6 cells/well and the cells were used 24 h after seeding.

LDL was prepared from human plasma containing 0.1% EDTA, 0.02% sodium azide and 0.5 mg/ml benzamidine at a density of 1.019–1.063 g/dl by sequential ultracentrifugation [15]. The lipoprotein-deficient serum (LPDS) was also isolated at a density >1.21. LDL was acetylated by repeated additions of acetic anhydride as described [7]. LDL and acetyl-LDL were radioiodinated with Na^{125}I using the iodine monochloride method [16].

2.3. Transfection of human c-fms cDNA

The human c-*fms* cDNA (3.0 kb) was isolated after digestion with *Bam*HI from pSMc-*fms*, a pBR325-based plasmid containing the human c-*fms* cDNA [12]. The c-*fms* cDNA fragment was inserted into the *Bam*HI site of the pBluescript II vector with a ligase, and then excised with *Hin*dIII and *Xba*I. Finally the c-*fms* cDNA was ligated orthodronomically into the *Hin*dIII–*Xba*I site of the Rc/CMV vector. Rc/CMV vector containing human c-*fms* cDNA was transfected into CHO-K cells by the calcium phosphate precipitation method using a transfection kit (CellPhect; Pharmacia LKB Biotechnology Inc). After 2 days of transfection, the cells were subcultured on a 100-mm dish in 10 ml HAM containing 10% NCS and 600 μg/ml G 418. After 3 weeks of culture with G418, G418-resistant clones were individually subcultured and screened for c-*fms* expression by Northern blot analysis as described below.

2.4. Northern blotting and Western blotting

RNA was isolated from cells by the acid guanidium thiocyanate/phenol/chloroform (AGPC) method. Total RNA (20 μg) was analyzed by gel-electrophoresis of 1% agarose, 2.2 M formaldehyde gels and transferred to nitrocellulose filters. The filter was hybridized with c-*fms* probe labeled with [α-^{32}P]dCTP by the random primer DNA labeling kit (Boehringer Mannheim Biochemica).

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Cells were dissolved in 100 μl of lysis buffer containing 10 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, and 1 mM PMSF, at pH 7.5. The protein (100 μg) was subjected to 7% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. Thereafter, the nitrocellulose membrane was incubated with anti-c-*fms* polyclonal antibody and then incubated with [^{125}I]protein A.

2.5. Degradation of lipoproteins

The cells were cultured for 3 days on 12-well plates in 1 ml HAM containing 10% NCS and 50 ng/ml M-CSF. After incubation for 36 h in medium containing 5 mg/ml LPDS and 50 ng/ml M-CSF, cells were incubated for 12 h in medium in the presence or absence of 10 μg/ml LDL. 10 μg/ml [^{125}I]lipoproteins were then added to the culture medium and incubated for a further 4 h. Degradation of [^{125}I]lipoproteins was measured according to the method of Goldstein et al. [7,16].

2.6. Quantitative analysis of LDL receptor mRNA

The competitive PCR method was adopted to measure mRNA levels [17]. After incubation for 36 h on 10 cm dishes in 10 ml HAM containing 5 mg/ml LPDS, cells were incubated for 48 h in medium containing LPDS. 1 μg of total RNA isolated from the cells was reverse-transcribed with random hexamer primers (Boehringer Mannheim Biochemica). The DNA fragment of the Chinese hamster LDL receptor gene (356 bp) was preparatively amplified from genomic DNA of CHO cells with a pair of primers (primer A, 5′-TCGCCTCACAGGCTCAGATG; primer B, 5′-TTGGCCAGTAGCATGCCATC) and used in the competitive PCR assay as a control template. Aliquots of the cDNA products and the control DNA were co-amplified in a series of ratios as indicated using the same set of primers (primer A and B). The final products were run on a 1.5% agarose gel, followed by ethidium bromide staining.

3. Results

3.1. Transfection and expression of human c-fms gene into CHO cells

The highest expression clone was designated CHO-*fms*. CHO cells transfected with the vector without c-*fms* cDNA were designated CHO(−) and used as control cells. In Northern blot analysis with a full-length c-*fms* cDNA probe, human c-*fms* mRNA of the same size (3.2 kbp) as in human macrophages was detected in CHO-*fms* (Fig. 1). Human c-*fms* protein was demonstrated in these cells as a 160 kDa protein by immunoblot analysis (Fig. 2), confirming the expression of the introduced gene.

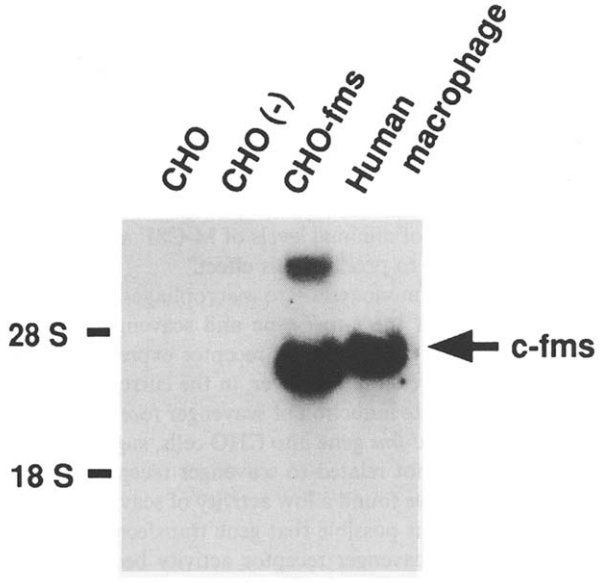

Fig. 1. Northern blot analysis of human c-*fms* gene-transfected CHO cells. Cellular RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform method from CHO cells (wild-type CHO-K), CHO cells transfected with the c-*fms* gene (CHO-*fms*), CHO cells transfected with the empty vector (CHO(−)) as a negative control, and human monocyte-derived macrophages as a positive control. Total RNA (20 μg) was subjected to 1% formaldehyde gel-electrophoresis and transferred to nitrocellulose filters. The filter was hybridized with the human c-*fms* cDNA probe.

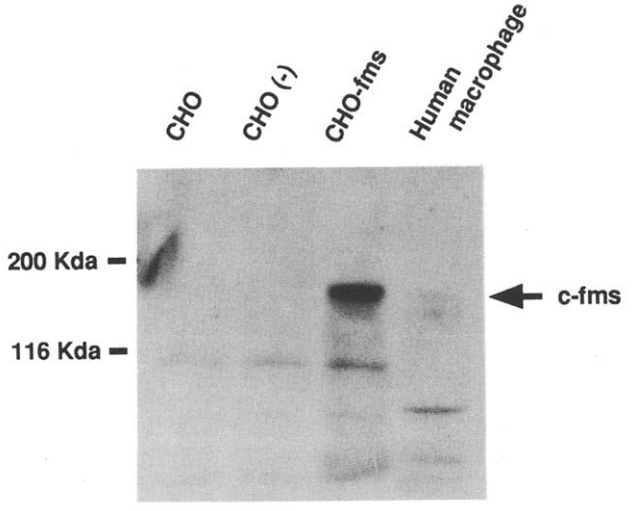

Fig. 2. Immunoblot analysis of human c-*fms* gene transfected CHO cells. Total cell protein (100 μg) was extracted from the cells as described in Fig. 1 in lysis buffer and analyzed by 7% SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with anti-c-*fms* polyclonal antibody and then with [^{125}I]protein A.

3.2. Degradation of lipoproteins in CHO-fms cells

LDL degradation activity was analyzed in CHO(−) and CHO-*fms* (Fig. 3). The cells were incubated for 48 h in medium containing LPDS to increase the LDL receptors. Degradation of LDL was significantly less (30%) in CHO-*fms* than in CHO(−). To analyze the effect of c-*fms* expression on LDL receptor activity more precisely, we calculated the sterol-sensitive portion of LDL receptor activity as the difference between LDL degradation in the assay in the presence or absence of LDL. LDL degradation in the presence of 10 μg/ml LDL was designated as the sterol-insensitive portion of LDL receptor activity (Fig. 3). Sterol-insensitive activity was not significantly different between the two cell types, whereas the sterol-sensitive activity was significantly lower in CHO-*fms* than in CHO(−). This indicates that introduction of the c-*fms* gene suppressed

Table 1
Degradation (ng/mg cell protein) of LDL and acetyl-LDL in CHO-*fms* and CHO(−)

	LDL	acetyl-LDL
CHO(−)	2649 ± 748*	38
CHO-*fms*	1846 ± 668	19
Mouse macrophages	Not done	10,570

Cells were incubated with 10 μg/ml LDL or acetyl-LDL for 4 h. Data represent mean ± S.D. of 4 experiments or mean of two experiments. Each experiment was performed in triplicate. *$P < 0.001$, as compared to CHO-*fms*.

LDL receptor activity by reducing the sterol-sensitive portion of LDL receptor activity. These results were similar both in the presence and absence of M-CSF in the medium.

Furthermore, acetyl-LDL degradation activity was examined to study the effect of c-*fms* expression on scavenger receptor activity in CHO cells (Table 1). We found no increase in acetyl-LDL degradation activity in CHO-*fms* as compared to CHO(−). The data indicate that the introduction of the c-*fms* gene did not induce acetyl-LDL receptor activity in CHO cells, although CHO(−) had low scavenger receptor activity. LDL and acetyl-LDL degradations were further evaluated in another CHO cell line which was transfected with c-*fms*, and the results were reproducible.

3.3. *Quantitative analysis of LDL mRNA on* c-fms *overexpressing CHO cells*

To determine whether suppression of LDL receptor activity by c-*fms* gene introduction was at the transcriptional level, we estimated the mRNA level of LDL receptor in CHO(−) and CHO-*fms* using a competitive PCR method (Fig. 4). RT-PCR with the two specific primers produced the 222 bp targeted sequence of the LDL receptor derived from mRNA and the 356 bp fragment derived from genomic DNA. During the PCR reaction containing both LDL receptor cDNA and genomic DNA, the two fragments were competitively amplified in a concentration-dependent manner. In this method, the relative concentrations of mRNA from both cell lines were calculated from the relative concentration of the cDNA to authentic genomic DNA. It was estimated that the amount of LDL receptor mRNA in CHO-*fms* was approximately 25% of that of CHO(−). The data indicate that expression of LDL receptor is decreased in CHO-*fms* at the transcriptional level.

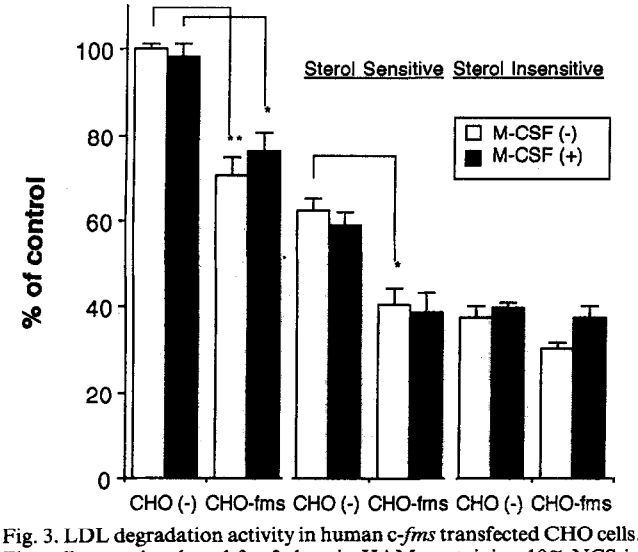

Fig. 3. LDL degradation activity in human c-*fms* transfected CHO cells. The cells were incubated for 3 days in HAM containing 10% NCS in the presence (black columns) or absence (open columns) of 50 ng/ml M-CSF. After incubation for 36 h in HAM containing 5 mg/ml LPDS in the presence or absence of 50 ng/ml M-CSF, cells were incubated for 12 h in the medium in the presence (Sterol Insensitive) or absence of 10 μg/ml LDL. 10 μg/ml [^{125}I]LDL was added to the cells and the cells were incubated for 4 h, and cell-degraded [^{125}I]LDL was measured. The sterol-sensitive portion was determined as the difference between the degradation activities in the presence and absence of 10 μg/ml LDL. 100% indicates [^{125}I]LDL degradation of control CHO cells in the absence of both M-CSF and LDL, i.e. 2.65 μg/mg cell protein. Data represent mean±S.E.M. of four experiments. $*P < 0.05$, $**P < 0.001$ compared with degradation values of control cells, CHO(−).

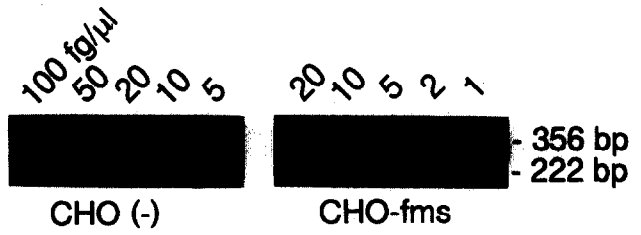

Fig. 4. Quantitative analysis of LDL receptor mRNA in human c-*fms*-transfected CHO cells. After incubation for 48 h in HAM containing 5 mg/ml LPDS, total RNA of the cells was isolated. A portion of total RNA (2.8 μg) was subjected to reverse-transcription with random hexamer primers. The control DNA template of the Chinese hamster LDL receptor gene (356 bp) was prepared from PCR with genomic DNA. cDNA products and the control DNA were mixed in a series of ratios as indicated and co-amplified by PCR, yielding a 222 bp fragment derived from LDL receptor mRNA as well as the control fragment (356 bp). The relative amount of the two products relfects the original content of LDL receptor mRNA and control template DNA.

4. Discussion

In the current study, we found that introduction of the c-*fms* gene into CHO cells decreased LDL receptor activity. It has been reported that differentiation of macrophage progenitors such as the HL-60 cells, a human promyelocytic leukemic cell line, and THP-1, a human acute monocytic leukemia cell line, into macrophages by phorbol esters is associated with induction of c-*fms* gene and decreased LDL receptors [9,11,18]. Our results suggest that induction of c-*fms* gene expression in macrophages primarily causes suppression of LDL receptor expression and that the two events are not coincidental during differentiation. Suppression occurred at the transcriptional level and affected the sterol-sensitive regulation of the LDL receptor. Introduction of the c-*fms* gene might affect the activity of the sterol-regulatory element in the promotor region of the LDL receptor gene. Interestingly, this effect was observed irrespective of M-CSF treatment. This suggests that expression of c-*fms* itself might be important for suppression of the LDL receptor, or that the presence of minimal levels of M-CSF activity in the medium is sufficient to produce this effect.

Differentiation of monocytes into macrophages is accompanied by induction of the c-*fms* gene and scavenger activities [9,11,18], suggesting that scavenger receptor expression is also related to c-*fms* expression. However, in the current study, we could not demonstrate induction of scavenger receptor activity after transfection of c-*fms* gene into CHO cells, suggesting that c-*fms* expression is not related to scavenger receptor activity. On the other hand, we found a low activity of scavenger receptor in CHO cells. It is possible that gene transfection of c-*fms* may not influence scavenger receptor activity because of the substantial absence of receptors in CHO cells. CHO cells may lack some intracellular mediators in the cascade of M-CSF signal transduction to the expression of scavenger receptors, or other signals may be required for induction of scavenger activity.

In the present study, we found that introduction of the c-*fms* gene affects the expression of LDL receptors, one of the most important lipoprotein receptors regulating plasma and intracel-

lular cholesterol metabolism. This may explain why macrophages have relatively low LDL receptor activity as compared to fibroblasts and hepatocytes which lack c-*fms* expression. The latter cells have strict regulatory systems for homeostasis of intracellular cholesterol content, including sterol-responsive regulation of LDL receptors, HMG-CoA reductase and synthetase, and acyl-CoA acyltransferase [19]. In contrast, macrophages take up modified LDL without this type of regulation, forming foam cells. We previously reported the formation of foamy cells from CHO cells over-expressing LDL receptors after incubation with LDL. This indicates that when LDL receptors are expressed without sterol regulation, native LDL could also produce foamy cells and might be involved in the development of early phases of atherosclerosis as well as modified LDL [20]. However, decreased expression of LDL receptors with c-*fms* expression in macrophages suggests that native LDL contributes much less to early phases of atherogenesis than modified LDL or apoE-rich lipoproteins such as remnants. Since CHO cells are widely used in investigations, CHO-*fms* will be a valuable tool to study the relationship between M-CSF receptors and lipoprotein metabolism or atherosclerosis.

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