Biological activity of the receptor for macrophage colony-stimulating factor in the human endometrial cancer cell line, Ishikawa

S Takeda*, WP Soutter, NJ Dibb and JO White

Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, UK.

Summary
Previously we found that the Ishikawa endometrial cancer cell line expresses macrophage colony-stimulating factor (M-CSF) and c-fms transcripts and that its proliferation is enhanced by the addition of recombinant M-CSF. This suggested that Ishikawa cells are constitutively stimulated by M-CSF. In support of this we now show that Ishikawa cells secrete M-CSF and that known stimulators of M-CSF production increase the amount detected in Ishikawa cell conditioned medium. Using retroviral infections to introduce and express exogenous c-fms genes in Ishikawa cells we also demonstrate proliferation to be partially inhibited by a dominant negative, mutant c-fms gene, yet enhanced approximately 3-fold by a normal c-fms gene, under conditions in which the only source of M-CSF was that produced by the cells. The data provide evidence for the existence of an active M-CSF/receptor loop in these endometrial cancer cells and suggests the possibility of such activity in tumours of the endometrium and ovary that aberrantly express M-CSF and fms genes.

Keywords: macrophage colony-stimulating factor; c-fms gene; Ishikawa endometrial cancer cell line

The sex steroid hormones oestradiol and progesterone regulate the synthesis of locally acting polypeptide growth factors and their receptors (Pollard, 1991; Giudice, 1994), and therefore potentially have indirect as well as direct effects upon uterine growth and differentiation. Macrophage colony-stimulating factor (M-CSF) was initially demonstrated to be under sex-steroid hormone control in the mouse uterus and to be elevated during pregnancy (Pollard et al., 1987; Arceci et al., 1989) whereas its receptor, and that encoded by the proto-oncogene c-fms (Sherr et al., 1985), was expressed in trophoblast cells (Arceci et al., 1989; Regenstrief and Rossant, 1989). Subsequently, M-CSF and its receptor have been suggested as local mediators at the feto-maternal interface on the basis of their expression in the pregnant endometrium and trophoblast respectively (Kauma et al., 1991; Daiter et al., 1992; Pampfer et al., 1992; Jokhi et al., 1993). The expression of M-CSF and c-fms is not however restricted to pregnancy as low-level expression of each transcript has been detected in normal endometrium (Kauma et al., 1991; Daiter et al., 1992; Pampfer et al., 1992).

The level of expression of M-CSF and c-fms in endometrial cancer is greater than in normal and benign tissue specimens; co-expression of M-CSF and c-fms is frequently observed in endometrial adenocarcinomas and is correlated with adverse prognostic indicators (Kacinski et al., 1988; Baiochchi et al., 1991; Leiserowitz et al., 1993). Elevated serum M-CSF is a feature of endometrial cancer patients and is suggested to be a circulating tumour marker of neoplastic disease activity (Kacinski et al., 1990). Such observations have led these authors to suggest that overexpression of M-CSF and c-fms contributes to the development and progression of endometrial cancer. We have previously reported on the expression of M-CSF and c-fms mRNA in the human endometrial adenocarcinoma cell line, Ishikawa, and demonstrated increased cellular proliferation in response to recombinant human (rh) M-CSF (Croxtall et al., 1992). We speculated, therefore, that locally produced M-CSF regulated proliferation of Ishikawa cells through activation of the M-CSF receptor (M-CSFR) (Croxtall et al., 1992). Several reports have shown that mutant receptors with defective tyrosine kinase activity, such as those for epidermal growth factor (EGF) and M-CSF (Kashles et al., 1991; Reith et al., 1993), can form inactive heterodimers with normal receptors expressed in the same cell or tissue. This approach, together with the demonstration of M-CSF production by Ishikawa cells, has been used in the present study to generate evidence of the functional importance of c-fms and M-CSF expression in endometrial cancer cells. We found that the expression of a mutant M-CSFR from a retroviral vector suppressed the proliferation of Ishikawa cells, indicating that the endogenous normal M-CSF actively stimulates growth. Furthermore, the proliferation of Ishikawa cells was enhanced by the increased expression of the normal M-CSFR, as a result of retroviral infection. The results strongly indicate the functional importance of the c-fms and M-CSF transcripts present in Ishikawa endometrial cancer cells and suggests that their overexpression in endometrial cancer compared with normal endometrium may contribute to the process of malignant transformation.

Materials and methods

Cell culture
Ishikawa cells were maintained in Dulbecco’s Modified Eagle medium/F12 (DMEM/F12, Sigma) containing 10% fetal calf serum (FCS) (Gibco). Serum-free medium was used for the assay of cell growth and the production of M-CSF; it consisted of DMEM/F12 supplemented with insulin (6.25 µg ml⁻¹), transferrin (6.35 µg ml⁻¹), selenium (6.25 ng ml⁻¹), bovine serum albumin (1.25 µg ml⁻¹) and linoleic acid (5.35 µg ml⁻¹) (ITS), all from Sigma.

Kinase activity of fms protein
For in vitro kinase assays a rabbit polyclonal antibody was used to immunoprecipitate fms proteins from lysates of Ishikawa cells (5 x 10⁶ cells grown to near confluence in DMEM/F12 + 5% FCS). The fms proteins were labelled with γ-ATP in vitro and analysed by SDS–PAGE as previously described (Dibb et al., 1990).

Determination of M-CSF in conditioned medium
The immunological detection of M-CSF was accomplished by dot-blot assay of serum-free culture medium conditioned by Ishikawa cells following treatment with tumour necrosis

Correspondence: JO White

*Present address: Department of Obstetrics and Gynaecology, Saitama Medical Centre, Kawagoe, Saitama 350, Japan

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factor α (TNF-α) (1, 2.5 and 5 ng ml⁻¹), 8-bromo cAMP (5, 7.5 and 10 mM) or phorbol 12-myristate 13-acetate (TPA, 0.5 and 1 mM) for 24 h. Media were blotted onto zeta-probe membrane (Bio-Rad) by microfiltration, blocked in 5% milk powder solution, washed and incubated with rabbit antihuman M-CSF polyclonal antibody at a final dilution of 1:7500 (Genzyme Corporation). The immunoblots were visualised using alkaline phosphatase (Bio-Rad) according to the manufacturer’s specifications. In preliminary experiments this procedure detected rhM-CSF but not IL-1β, IL-2, granulocyte colony-stimulating factor (G-CSF) or granulocyte–macrophage colony-stimulating factor (GM-CSF).

Infection of cells with fms constructs

The retroviral construct vsn2c-fms contains the complete cDNA for the human M-CSFr, whereas vsn2v-fms carries an oncogenic derivative, and was made previously (Dibb et al., 1990). Retroviral construct vsn2c-fmsK612A was prepared here and is otherwise identical to vsn2c-fms except that the lysine residue 612 of the M-CSFr (Cousens et al., 1986) was changed to alanine by oligo-directed mutagenesis using the oligonucleotide TGCTGTCTTCGCCGTGGCTGTGA. Ishikawa cells were infected by the addition of supernatant from the retroviral packaging cell line PA317 that was separately transfected with each of the retroviral constructs vsn2; vsn2c-fms; vsn2v-fms and vsn2c-fmsK612A, as previously described (Baker et al., 1994). The recombinant retroviruses vsn2c-fms; vsn2v-fms and vsn2c-fmsK612A all had equivalent infection frequencies of Ishikawa cells in the order of 10⁻²–10⁻³ G418 resistant colonies per ml of retroviral supernatant. As expected, the control retrovirus vsn2 had a high infection frequency owing to its smaller size (Dibb et al., 1990). Cells infected with each of the constructs were selected in 1 mg ml⁻¹ G418 (Gibco), resistant colonies pooled and then expanded for experimentation. Growth experiments were performed under serum-free conditions in DMEM/F12/ITS and assessed either by measurement of cell number after trypsinisation or by assaying DNA after solubilisation of cells in 0.2% SDS and incubation with 1 mg ml⁻¹ Hoechst 33258 in SSC (Labarca and Paigen, 1980).

Results

To establish that the M-CSF transcript is translated in Ishikawa cells conditioned medium was assayed for immunoreactive protein with a rabbit polyclonal anti-human M-CSF antibody, as shown in Figure 1. This assay demonstrated the presence of immunoreactive M-CSF in Ishikawa cell conditioned medium when the cells had been grown under basal serum-free conditions. No immunoreactive material was detected in serum-free medium that had not been exposed to Ishikawa cells (data not shown). The amount of M-CSF detected under basal conditions was inversely proportional to the density of cells (Figure 2), suggesting that M-CSF production decreased as the cells reached confluence. To establish whether the control of M-CSF production in Ishikawa cells was similar to that reported in other systems cells were treated with TNF-α and phorbol ester (TPA), which increase the titre of M-CSF (Ralph et al., 1986; Yamada et al., 1991). This resulted in an increase in immunoreactive M-CSF present in conditioned medium (Figure 1). Elevation of intracellular cAMP by incubation of Ishikawa cells with 8-bromo cyclic AMP also resulted in an increase in immunoreactive M-CSF (Figure 1).

The role of the M-CSF in the growth of Ishikawa cells was investigated by the expression of retroviral wild-type c-fms, which potentially could enhance the response to M-CSF, and by the introduction of a kinase inactive, mutant receptor, K612A, which would be expected to inhibit the activity of the endogenous M-CSFr. The construct vsn2c-fms encodes a functional M-CSFr as evidenced by its activity in FDC-P1 cells and Rat-2 fibroblasts (Dibb et al., 1990; Baker et al., 1994). Ishikawa cells infected with vsn2c-fms grew signifi-

cantly faster than control cells (Figure 3a). The only source of M-CSF for receptor activation under these serum-free conditions was that produced endogenously by the cells. Under identical culture conditions, cells infected with the loss-of-function construct c-fmsK612A demonstrated a decrease in cell growth compared with control cells (Figure 3b).

Figure 1 Ishikawa cell conditioned medium contains immunoreactive M-CSF. Serum-free media collected from Ishikawa cell cultures treated for 24 h with TNF-α (a), 8-bromocyclic AMP (8-Br) (b) or phorbol 12-myristate 13-acetate (TPA) (c), at the indicated concentrations, were analysed for immunoreactive M-CSF. Densitometry indicated that induction by each treatment was in the range: TNF, 700–900%; 8-Br, 200–350%; TPA 350–400%.

Figure 2 M-CSF immunoreactivity is inversely proportional to cell density. Ishikawa cells were plated at increasing concentrations in serum-containing medium and then changed to serum-free conditions for 4 days to achieve the final cell density indicated. Medium was analysed for M-CSF immunoreactivity at the end of the culture period. Regression analysis of arbitrary densitometric units vs cell density indicated a correlation coefficient (r²) of 0.93 (P<0.001).
Discussion

A relatively large number of chromosomal loci are likely to play a role in the genesis of endometrial cancer (Fujino et al., 1994). Mutational activation of c-Ki-ras (Sasaki et al., 1993, and references therein) and mutations in p53 (Inoue et al., 1994, and references therein) have been reported in this disease, but only in approximately one-third of cases. In contrast, the majority of endometrial cancers overexpress c-fms (Kacinski et al., 1988, 1990; Baiocchi et al., 1991; Leiserowitz et al., 1993), and this expression is associated with clinicopathological features of aggressive disease (Kacinski et al., 1988; Leiserowitz et al., 1993).

In most primary sites of endometrial cancer and in all metastatic lesions (Kacinski et al., 1990; Baiocchi et al., 1991), c-fms and M-CSF were co-expressed. In comparison with the low-level expression of c-fms and M-CSF in normal endometrium observed in each of the above studies, this suggests that aberrant activation of the M-CSF/receptor signalling pathway may contribute to endometrial carcinogenesis. Data obtained in NIH-3T3 cells in which the enforced expression of c-fms and M-CSF resulted in transformation (Rettenmeier et al., 1987) supports this hypothesis. We have previously demonstrated that Ishikawa cells express M-CSF and c-fms mRNA and are responsive to recombinant human M-CSF when grown under serum-free conditions (Croxall et al., 1992). The increase in growth of Ishikawa cells following retroviral infection with a c-fms construct (Figure 3a) suggests that activation of the M-CSFr and of its signalling intermediates is associated with proliferation in these cells. Activation of the M-CSFr is
associated with its internalisation and the disappearance of the mature form of the receptor (Downing et al., 1989; Sariban et al., 1989). The relatively low abundance of the mature M-CSF in Ishikawa cells (Figure 4) is therefore consistent with its turnover as a result of M-CSF stimulation. However, we have not as yet ruled out the alternative possibility that the mature form of the M-CSF is regulated in these cells by the activity of protein kinase C, which is also known to influence M-CSF activity, but by a mechanism independent of that stimulated by M-CSF (Downing et al., 1989).

The ability of the loss-of-function mutant M-CSF, encoded by vsn2K612A, to retard the growth of Ishikawa cells (Figure 3b) further strengthens the proposal that the growth of Ishikawa cells is responsive to, but not dependent upon, endogenously produced M-CSF. The mutant M-CSF presumably inhibits M-CSF-induced growth by forming inactive heterodimers with the normal cellular M-CSF. However, the alternative possibility that homodimeric, mutant, loss-of-function M-CSF receptors may act as a sink for endogenous M-CSF also needs to be considered. Regardless of the mechanism involved the data provide evidence of the importance of activation of M-CSF signalling pathways in the proliferation of endometrial cancer cells.

Detection of M-CSF in the conditioned medium of Ishikawa cells, under conditions in which there is no other source, indicates that the M-CSF transcript previously detected in these cells (Croxtall et al., 1991) is translated into protein. Regulation of M-CSF expression by TNF and the phorbol ester, TPA, is consistent with such regulation in monocytes (Ralph et al., 1986; Sherman et al., 1990; Yamada et al., 1991). TNF regulation of M-CSF in HL-60 cells is the result of transcriptional and post-transcriptional mechanisms, in which cAMP is capable of antagonising the effects of TNF (Sherman et al., 1990). Although cAMP was not used in combination with TNF in the present study, its ability to induce M-CSF suggests that it would be unlikely to antagonise the effects of TNF in Ishikawa cells. Such stimulation of M-CSF by cAMP is more typical of the response observed in endothelial cells (Parhami et al., 1993). The extrapolation of this data to the in vivo situation suggests that there is a potential functional consequence to the co-expression of M-CSF and c-fms in the majority of endometrial carcinomas. Taken together with evidence of the role of this loop in stimulating cellular invasion (Fidlerman et al., 1992) it seems likely that deregulation of M-CSF/c-fms expression and/or function has a pivotal role in growth and metastasis of endometrial cancer. The ability of interactive signalling pathways to regulate the expression of M-CSF, as shown here (Figure 1), and the capacity of such signalling pathways also to be involved in the regulation of c-fms expression (Yue et al., 1993), provide avenues to explore the molecular mechanism underlying such overexpression in endometrial and ovarian cancer.

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References

ARCECI RJ, SHANAHAN F, STANLEY ER AND POLLARD JW. (1989). The temporal expression and location of colony-stimulating factor-1 (CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1 regulated placental development. Proc. Natl Acad. Sci. USA, 86, 8818 – 8822.

BACIOCCHI G, KAVANAGH JJ, TALPAZ M, WHARTON JT, GUTTERMAN JU AND KURZROCK R. (1991). Expression of the macrophage colony-stimulating factor and its receptor in gynecologic malignancies. Cancer, 67, 990 – 996.

BAKER DA, GLOVER HR AND DIBB NJ. (1994). Synergy between SCF or M-CSF with IL-3 or GM-CSF in FDC-P1 cells: a sensitive assay of transforming mutations of c-fms. Leukemia, 8, 141 – 150.

COUSSENS L, VAN BEVEREN C, SMITH D, CHEN E, MITCHELL RL, ISACKE CM, VERMA IM AND ULLRICH A. (1986). Structural alteration of the viral homologue of receptor protein tyrosine kinase fms at the carboxy-terminus. Nature, 320, 277 – 280.

CROXTALL JD, POLLARD JW, CAREY F, FORDER RA AND WHITE JO. (1992). Colony-stimulating factor-1 stimulates Ishikawa cell proliferation and lipoprotein II synthesis. J. Steroid Biochem. Mol. Biol., 42, 850 – 858.

DAITRE E, PAMPER R, YEUNG YG, BARAD D, STANLEY ER AND POLLARD JW. (1992). Colony-stimulating factor-1 stimulates colony-stimulating factor-1 in the human uterus and placenta. J. Clin. Endocrinol. Metabol., 74, 850 – 858.

DSTEKER TM, GARLAND J, SCOTT D, SCOLNICK E AND METCALF D. (1980). Growth factor-dependent hemopoietic precursor cell lines. J. Exp. Med., 152, 1036 – 1047.

DIBB NJ AND RALPH P. (1992b). Expression of v-fms and c-fms in the hemopoietic cell line FDC-P1. Growth Factors, 2, 301 – 311.

DOWNING JR, ROUSSELL MF AND SHERR CJ. (1989). Ligand and protein kinase C downmodulate colony-stimulating factor 1 receptor by independent mechanisms. Mol. Cell. Biol., 9, 2890 – 2896.

FIDLERMAN AE, BRUCKNER A, KACINSKI BM, DENG N AND REMOLD HG. (1992). Macrophage colony-stimulating factor (CSF-1) enhances invasiveness in CSF-1 receptor-positive carcinoma cell lines. Cancer Res., 52, 3661 – 3666.

FUJINO T, RISINGER JH, COLLINS NK, FU-SHING L, NISHI H, TAKAHASHI H, WESTPHAL EY, BARRETT JC, SASAKI H, KOHLER MF, BARCHUCK A AND BOYD J. (1994). Allelotypes of endometrial cancer. Cancer Res., 54, 4294 – 4298.

GIUDICE LC. (1994). Growth factors and growth modulators in human uterine endometrium: potential relevance to reproductive medicine. Fertil. Steril., 61, 1 – 17.

INOUE M, OKAYAMA A, FUJITA M, ENOMOTO T, SAKATA M, TANIZAWA O AND UESHIMA H. (1994). Clinicopathological characteristics of p53 overexpression in endometrial cancer. Int. J. Cancer, 58, 14 – 19.

JOKHI PP, CHUMBLEY G, KING A, GARDNER L AND LOLE YW. (1993). Expression of the colony stimulating factor-1 receptor (c-fms product) by cells at the human uteroplacental interface. Lab. Invest., 68, 308 – 320.

KACINSKI BM, CARTER D, DMITTAL K, KOHORN EI, BLOODGOOD RS, DONAHUE J, DONOFORIO L, EDWARDS R, SCHWARTZ PE, CHAMBERS JT AND CHAMBERS SK. (1990). High level expression of fms protooncogene mRNA is observed in clinically aggressive human endometrial adenocarcinomas. Int. J. Radiat. Oncol. Biol. Phys., 15, 823 – 829.

KACINSKI BM, CHAMBERS SK, STANLEY ER, CARTER D, TSENG P, SCATA KA, CHANG DHY, PiRRO MH, NGUYEN JT, ARIZA A, ROHRSCHNEIDER LR AND ROTHVELL VM. (1990). The cytokine CSF-1 (M-CSF) expressed by endometrial carcinomas in vivo and endometrial disease activity in endometrial carcinoma patients. Int. J. Radiat. Oncol. Biol. Phys., 19, 619 – 626.

KASHLES O, YARDEN Y, FISCHER R, ULLRICH A AND SCHLESINGER J. (1991). A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. Mol. Cell. Biol., 11, 1454 – 1463.

KAUMA SW, AUERKAMER SL, EIERMAN D AND TURNER T. (1991). Colony-stimulating factor-1 and c-fms expression in human endometrial tissues and placenta during the menstrual cycle and early pregnancy. J. Clin. Endocrinol. Metab., 73, 746 – 751.

LABARCA C AND PAIGEN K. (1980). A simple, rapid and sensitive DNA assay procedure. Anal. Biochem., 102, 345 – 352.
LEISEROWITZ GS, HARRIS SA, SUBRAMANIAM M, KEENEY GL, PODRATZ KC AND SPELBERG TC. (1993). The protooncogene c-fms is overexpressed in endometrial cancer. Gynecol. Oncol., 49, 190 – 196.

PAMPFER S, DAUER E, BARAD D AND POLLARD JW. (1992). Expression of the colony-stimulating factor-1 receptor (c-fms protooncogene product) in the human uterus and placenta. Biol. Reprod., 46, 48 – 57.

PARHAM F, FANG ZT, FOGELMAN AM, ANDALIBI A, TERRITO MC AND BERLINER JA. (1993). Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. J. Clin. Invest., 92, 471 – 478.

POLLARD JW. (1991). Lymphohematopoietic cytokines in the female reproductive tract. Curr. Opin. Immunol., 3, 772 – 777.

POLLARD JW, BARTOCCIA A, ARCECI A, ORLOFSKY A, LADNER MB AND STANLEY ER. (1987). Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature, 330, 484 – 486.

RALPH P, WARREN MK, LEE M, CSEJTEY J, WEAVER J, BROXMEYER H, WILLIAMS D, STANLEY ER AND KAWASAKI E. (1986). Inducible production of human macrophage growth factor, CSF-1. Blood, 68, 633 – 639.

REGENSTIEF H AND ROSSANT J. (1989). Expression of the c-fms protooncogene and of the cytokine CSF-1 during mouse embryogenesis. Dev. Biol., 133, 284 – 294.

REITH AD, ELLIS C, MAROC N, PAWSON T, BERNSTEIN A AND DUBREUIL P. (1993). “W” mutant forms of the fms receptor tyrosine kinase act in a dominant manner to suppress CSF-1 dependent cellular transformation. Oncogene, 8, 45 – 53.

REUTENMEIER CW, ROUSSEL MF, ASHIMUN RA, RALPH P, PRICE K AND SHERR CJ. (1987). Synthesis of membrane-bound colony-stimulating factor-1 (CSF-1) and downmodulation of CSF-1 receptors in NIH 3T3 cells transformed by cotransfection of the human CSF-1 and c-fms (CSF-1 receptor) genes. Mol. Cell. Biol., 7, 2378 – 2387.

SARIBAN E, IMAMURA K, SHERMAN M, ROTHWELL V, PANTAZIS P AND KUFE D. (1989). Downregulation of c-fms gene expression in human monocytes treated with phorbol esters and colony-stimulating factor 1. Blood, 74, 123 – 129.

SASAKI H, NISHI H, TAKASHASHI H, TADA A, FURUSATO M, TERASHIMA Y, SIEGAL GP, PARKER SL, KOHLER MF, BERCHUCK A AND BOYD J. (1993). Mutation of the Ki-ras protooncogene in human endometrial hyperplasia and carcinoma. Cancer Res., 53, 1906 – 1910.

SHERMAN ML, WEBER BL, DATTA R AND KUFE DW. (1990). Transcriptional and posttranscriptional regulation of macrophage-specific colony stimulating factor gene expression by tumour necrosis factor. J. Clin. Invest., 85, 442 – 447.

SHERR CJ, RETENMEIER CW, SACCA R, ROUSSEL MF, LOOK AT AND STANLEY ER. (1985). The c-fms protooncogene product is related to the receptor for the mononuclear phagocytic growth factor, CSF-1. Cell, 41, 665 – 676.

YAMADA H, IWASI S, MOHRIM M AND KUFE D. (1991). Involvement of a nuclear factor – xB-like protein in induction of the macrophage colony-stimulating factor gene by tumour necrosis factor. Blood, 78, 1988 – 1995.

YUE X, FAYOT P, DUNN TL, CASSADY AI AND HUME DA. (1993). Expression of mRNA encoding the macrophage colony-stimulating factor receptor (c-fms) is controlled by a constitutive promoter and tissue-specific transcription elongation. Mol. Cell. Biol., 13, 3191 – 3201.