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Macrophage Colony-stimulating Factor Induces the Growth and Differentiation of Normal Pregnancy Human Cytotrophoblast Cells and Hydatidiform Moles but Does Not Induce the Growth and Differentiation of Choriocarcinoma Cells

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In the present study, we examined whether or not macrophage colony-stimulating factor (M-CSF; CSF-1) is involved in the growth and differentiation of human chorionic, hydatidiform mole and choriocarcinoma cells. M-CSF promotes the growth of early gestation chorionic cells, hydatidiform mole cells, and a human term placenta cell line (tPA30-1). However, the growth of choriocarcinoma cells, BeWo, Jar, Jeg-3, and NUC-1, was not influenced at all by M-CSF. M-CSF promoted the secretion of human chorionic gonadotropin (hCG) and human placental lactogen (hPL), which are secreted from differentiated trophoblast, from early gestation chorionic cells and from hydatidiform mole cells. However, the secretion of hCG and hPL from choriocarcinoma cells was not affected by M-CSF. When M-CSF localization was examined by immunohistochemical staining, M-CSF was detected in chorionic and hydatidiform mole cells, but was absent in choriocarcinoma cells. These results suggest that the growth and differentiation of normal chorionic and hydatidiform mole cells are M-CSF-dependent, while the growth and differentiation of choriocarcinoma cells are not.

Key words: M-CSF — Trophoblast — Growth factor — Differentiation — Choriocarcinoma

Chorionic cells express numerous growth factor receptors which control growth and differentiation. c-*fms*, a receptor for macrophage colony-stimulating factor (M-CSF), induces the growth and differentiation of macrophages.[1] Although c-*fms* is expressed in chorionic and choriocarcinoma cells,[2,3] the physiological action of M-CSF (i.e., its role in the growth and differentiation of chorionic cells) has, until now, remained obscure. It has recently been shown, that uterine tissue M-CSF levels in pregnant mice are approximately 1000 times greater than in nonpregnant mice,[4,5] that c-*fms* expression in endometrial stromal cells is directly related to M-CSF mRNA expression[6,7] and that murine placenta-like cell DNA synthesis is elevated by M-CSF.[8] These investigations have led us to study the physiological actions of M-CSF on chorionic cells. It has also been recently revealed that M-CSF is present in human placenta and decidual tissue,[9–12] that it promotes the secretion of human chorionic gonadotropin (hCG)[13] and human placental lactogen (hPL)[14] from chorionic cells, and that maternal blood M-CSF levels increase gradually during the course of pregnancy.[15] With those observations in mind, we deal here with the effects of M-CSF on the growth and differentiation of human chorionic and choriocarcinoma cells.

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MATERIALS AND METHODS

Separation and incubation of cytotrophoblasts According to the method of Kliman *et al.*,[16] cytotrophoblasts were isolated from chorions collected from women who had undergone artificial abortion in early pregnancy (week 7–11 of pregnancy). When examined with a cell sorter, these cytotrophoblasts were found to contain about 5% monocytes/macrophages. The contaminant monocytes/macrophages, were eliminated from cytotrophoblasts through the following two steps: (1) incubation of cytotrophoblasts on ice for 30 min in the presence of 2 μg/ml of anti-CD14 antibody (LeuM3, Becton Dickinson, USA); and (2) subsequent incubation of the cytotrophoblasts at 37°C for 30 min in the presence of 1/4 their amount of rabbit complement. The cytotrophoblasts thus obtained were suspended in a 10% fetal calf serum (FCS)-supplemented RPMI1640 medium to a concentration of 2×10^5 cells/ml. The suspension (200 μl) was poured into each well of a 96-well flat culture plate coated with laminin (Iwaki Co., Ltd., Tokyo). Informed consent was obtained from each woman before her chorion was sampled.

Incubation of tPA30-1 cells tPA30-1 cells were purchased from Dainippon Pharmaceutical Co., Ltd., Osaka. These cells were established by transformation of the human term placenta using a temperature-sensitive simian virus 40 mutant of the A class.[17] They lose the

Table I. Primer Sequences Used for PCR

	5′ sense primer	3′ antisense primer
c-*fms* Leu301	5′-TCCCACTAATGCCAGATGCTTG-3′	5′-TAGGCCTCCACCATGACTTTGA-3′
c-*fms* Tyr969	5′-GAGAGCTCTAGTGAGCACCTG-3′	5′-TGGTACTCCCTGTCGTCAAC-3′
M-CSF	5′-CTCTCTTTGAGGCTGAAGAGC-3′	5′-GGGGTTGAAGGCCCCGACAG-3′

transformed phenotype at 40°C and begin to function like placenta. In the present study, therefore, tPA30-1 cells were incubated at 40°C in a 10% FCS-supplemented RPMI1640 medium.

Quantification of M-CSF, hCG and hPL M-CSF was quantified by the method of Hanamura *et al.*[18] The lower limit of M-CSF detection with this method was 0.1 ng/ml. hCG and hPL were quantified by using EIA kits (Mochida Pharmaceutical Co., Ltd., Tokyo). The detection limit was 5 mIU for hCG, and 2.5 ng/ml for hPL. M-CSF, hCG and hPL were each quantified in duplicate.

M-CSF, anti-M-CSF antibody and anti-*fms* antibody Urinary-derived purified M-CSF was supplied by Morinaga Milk Industry Co., Ltd., Tokyo. Its specific activity was $1–2\times10^8$ U/mg protein, when assessed by the soft agar colony formation method using mouse bone marrow cells. Anti-human M-CSF rabbit IgG was also obtained from Morinaga Milk Industry Co., Ltd. It was prepared by immunizing rabbits with recombinant human M-CSF. Its neutralizing activity was 5.6×10^5 U/mg. It was added to the culture media for cytotrophoblasts, hydatidiform mole cells and choriocarcinoma cells at a concentration of 2 μg/ml. Rabbit anti-*fms* serum (Cambridge Biochemicals, USA) was used at a concentration of 1 μg/ml.

Immunohistochemical examination Six-μm paraffin-embedded sections were collected on slides coated with chrom alum gelatin adhesive. After deparaffinization with xylene, these sections were exposed to 0.3% H_2O_2 in methanol for 30 min. The sections were then subjected to ABC immunoperoxidase staining (Vecstatin Elite ABC kit, Vector Lab., USA), using rabbit anti-M-CSF antibody as a primary antibody. These sections were incubated in 0.1 *M* Tris HCl buffer (pH 7.2) containing 0.02% H_2O_2 and 0.1% diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Of the antibodies used for this immunohistochemical examination rabbit, anti-M-CSF specific IgG (purified through a recombinant human M-CSF coupled CL4B column) was supplied by Morinaga Milk Industry, Co., Ltd., and anti-hCGα monoclonal antibody was supplied by Mochida Pharmaceutical Co., Ltd.

Reverse transcriptase polymerase chain reaction (RT-PCR) Total RNA (4 μg), extracted from tPA30-1 cells, BeWo cells, Jar cells, Jeg-3 cells or NUC-1 cells was incubated at 42°C for 1 h in 20 μl of 50 m*M* Tris HCl buffer (pH 8.3) containing 20 U of reverse transcriptase (Takara Shuzo Co., Ltd., Kyoto), 50 U of ribonuclease inhibitor (Takara Shuzo Co., Ltd.), 100 m*M* KCl, 3 m*M* $MgCl_2$ and 10 m*M* DTT. The solution was then heated at 95°C for 5 min. The cDNA solution thus prepared (2 μl) was combined with 48 μl of 1×PCR buffer (Stratagene, USA) containing 200 μM dNTP, 0.5 μM primer (Table I), and 2.5 U of *Taq* polymerase (Stratagene). This solution was subjected to 30 cycles of amplification (each cycle being composed of 50 s at 94°C, 1 min at 55°C and 1 min at 72°C). The solution was then electrophoresed on 6% polyacrylamide gel and stained with ethidium bromide.

Examination for c-*fms* point mutation DNA (1 μg), extracted from normal chorions, hydatidiform mole tissues, tPA30-1 cells, BeWo cells, Jar cells, Jeg-3 cells or NUC-1 cells was mixed with 1×PCR buffer containing 200 μM dNTP, 0.5 μM primer (Table I), and 2.5 U of *Taq* polymerase to make a final volume of 50 μl. This solution was subjected to 30 cycles of amplification (each cycle being composed of 50 s at 94°C, 1 min at 55°C and 1 min at 72°C). The PCR products were inserted into PCR™ vector, using a TA cloning kit (Invitrogen, USA), followed by DNA sequence determination with a DNA sequence kit (Pharmacia Co., USA).

RESULTS

Effect of M-CSF on DNA synthesis in primary cultured human cytotrophoblast from normal pregnancy and hydatidiform mole cells ^{3}H-thymidine uptake by early gestation human cytotrophoblast primary cultures was elevated in a dose-dependent manner in response to M-CSF. It rose to 114±11%, 155±49% or 161±54% of the control, when 0.2, 2 or 20 ng/ml M-CSF was added to the culture medium (mean±SD, n=5). Cytotrophoblast DNA synthesis was reduced to 66±24% of control in the presence of anti-M-CSF antibody, and to 60±19% in the presence of anti-*fms* antibody (Fig. 1). Hydatidiform mole cell DNA synthesis was also elevated by M-CSF in a dose-dependent manner (119% of the control with 0.2 ng/ml, 146% with 2 ng/ml, and 144% with 20 ng/ml M-CSF, respectively)(n=1). DNA synthesis by the same cells was reduced to 83% of the control after treatment with anti-M-CSF antibody and to 67% after anti-*fms* antibody treatment (Fig. 1).

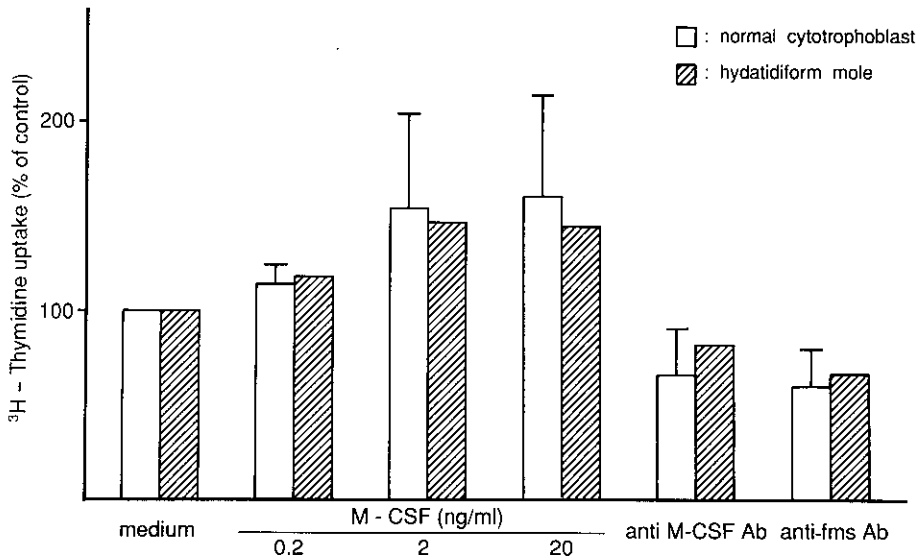
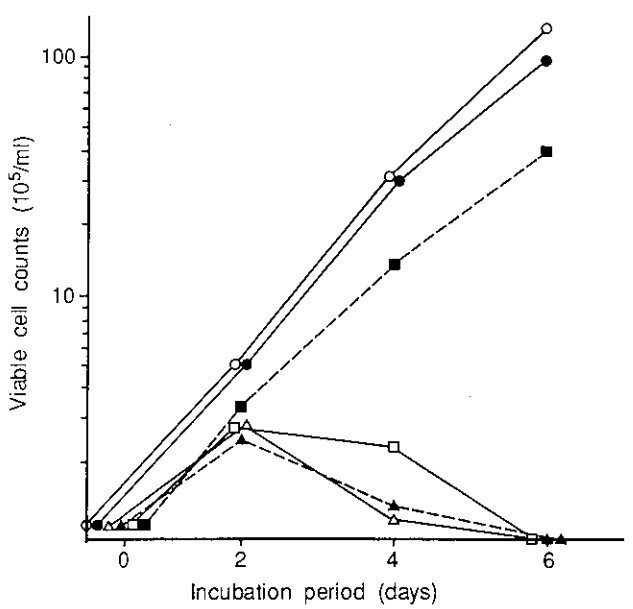


Fig. 1. Effect of M-CSF, anti-M-CSF antibody, and anti-*fms* antibody on ^{3}H-thymidine uptake by primary cultured human cytotrophoblast and hydatidiform mole cells. Normal pregnancy cytotrophoblast, vertical bar shows the SD (n=5); hydatidiform mole, the average of three assays from one hydatidiform mole is shown.

Effect of M-CSF, anti-M-CSF antibody and anti-*fms* antibody on the growth of tPA30-1 cells The growth of tPA30-1 cells in medium supplemented with M-CSF (20 ng/ml) was not significantly different from that in medium alone (Fig. 2). When anti-M-CSF antibody was added to the medium, tPA30-1 cell growth was markedly suppressed on the fourth day of incubation, and most tPA30-1 cells were nonviable by the sixth day of incubation (Fig. 2). When M-CSF was added to the medium in an amount sufficient to overcome the neutralizing ability of anti-M-CSF antibody, tPA30-1 cell growth resumed, and a considerable number of viable tPA30-1 cells were observed on the sixth day of incubation (Fig. 2). Almost all tPA30-1 cells were unable to grow and were nonviable during the first four days of incubation in the medium containing anti-*fms* antibody (Fig. 2). This effect of the anti-*fms* antibody was not neutralized by the simultaneous addition of M-CSF (Fig. 2).



Effect of anti-M-CSF and anti-*fms* antibodies on the growth of BeWo, Jar, Jeg-3 and NUC-1 choriocarcinoma cell lines Growth of the choriocarcinoma cell lines such as BeWo, Jar, Jeg-3, and NUC-1 was not affected by the addition of either anti-M-CSF antibody or anti-*fms* antibody.

Effect of M-CSF on hCG secretion from normal placenta cytotrophoblast, hydatidiform mole cells and choriocarcinoma cells The hCG level in cytotrophoblast culture supernatant increased 1.19±0.17, 2.36±0.46 and

Fig. 2. Effect of M-CSF, anti-M-CSF antibody, and anti-*fms* antibody on the growth of tPA30-1 cells. Control viable cell counts (●); viable cell counts with M-CSF (○); viable cell counts with anti-M-CSF antibody (□); viable cell counts with anti-M-CSF antibody and M-CSF (■); viable cell counts with anti-*fms* antibody (△); viable cell counts with anti-*fms* antibody and M-CSF (▲).

Table II. Effect of M-CSF, Anti-M-CSF Antibody and Anti-*fms* Antibody on hCG Secretion by Primary Culture Human Trophoblast, Hydatidiform Mole, and Choriocarcinoma Cells[a]

	M-CSF (ng/ml)				anti-M-CSF Ab (2 μg/ml)	anti-*fms* Ab (1 μg/ml)
	0	0.2	2	20		
Normal pregnancy						
7 w	290[b]	370	745	910	205	180
8 w	415	445	895	1180	243	204
9 w	485	725	1485	1590	270	245
9 w	840	915	1960	2020	720	710
10 w	1120	1155	1885	2000	680	690
Hydatidiform mole						
9 w	2590	3765	5610	3880	2015	2160
Choriocarcinoma cell lines						
BeWo	3300	NT[c]	NT	3115	3200	3150
Jar	330	NT	NT	352	310	315
Jeg-3	285	NT	NT	260	290	270
NUC-1	178	NT	NT	160	170	185

a) Human trophoblast and hydatidiform mole cells were cultured in serum-free medium. Choriocarcinoma cell lines, BeWo, Jar, Jeg-3, and NUC-1, were cultured in RPMI-1640 medium supplemented with 10% FCS.
b) Numbers represent mIU/ml of hCG/2×10^4 cells/48 h.
c) Not tested.

Table III. Effect of M-CSF, Anti-M-CSF Antibody, and Anti-*fms* Antibody on hPL Secretion by Primary Cultured Human Trophoblastic Cells, Hydatidiform Mole Cells and Choriocarcinoma Cells

	M-CSF (ng/ml)				anti-M-CSF Ab (2 μg/ml)	anti-*fms* Ab (1 μg/ml)
	0	0.2	2	20		
Normal pregnancy						
7 w	72[a]	102	134	188	60	55
8 w	77	87	123	141	52	40
9 w	68	82	108	144	45	41
9 w	147	151	267	282	98	102
10 w	185	171	263	255	125	118
Hydatidiform mole						
9 w	106	98	124	172	82	70
Choriocarcinoma cell lines						
BeWo	13.9	NT[b]	NT	14.2	14.3	15.0
Jar	<2.5	NT	NT	<2.5	<2.5	<2.5
Jeg-3	<2.5	NT	NT	<2.5	<2.5	<2.5
NUC-1	<2.5	NT	NT	<2.5	<2.5	<2.5

a) Numbers represent μg/ml of hPL/2×10^4 cells/48 h.
b) Not tested.

2.69 ± 0.54 times in the presence of 0.2, 2, and 20 ng/ml of M-CSF, as compared with that in the absence of M-CSF. It was reduced to 0.66 ± 0.11 times by the addition of anti-M-CSF antibody, and to 0.62 ± 0.13 times by the addition of anti-*fms* antibody, as compared with the control level (Table II). The hCG secretion from hydatidiform mole cells was promoted by the addition of M-CSF in a dose-dependent manner, but was suppressed by the addition of either anti-M-CSF antibody or anti-*fms* antibody. The hCG secretion from four choriocarcinoma cell lines was not affected by the treatment with M-CSF, anti-M-CSF antibody, or anti-*fms* antibody (Table II).

Table IV. M-CSF Secretion by tPA30-1, BeWo, Jar, Jeg-3, and NUC-1 Cells and the Concentration of M-CSF in Hydatidiform Mole Vesicle Fluid

	M-CSF (ng/ml/2×10^5 cells/24 h)
tPA 30-1	1.6
BeWo	<0.1
Jar	<0.1
Jeg-3	<0.1
NUC-1	<0.1
Hydatidiform mole vesicle fluid	
case 1	60
case 2	134

Effect of M-CSF on hPL secretion from normal placental cytotrophoblast, hydatidiform mole, and choriocarcinoma cells The hPL levels in cytotrophoblast culture supernatants increased 1.14 ± 0.17, 1.66 ± 0.16, and 1.92 ± 0.40 times in the presence of 0.2, 2, and 20 ng/ml M-CSF, as compared with that in the absence of M-CSF. On the other hand, treatment with anti-M-CSF antibody or anti-*fms* antibody reduced hPL secretion to 0.70 ± 0.07 or 0.64 ± 0.08 times, respectively, as compared with the control level (Table III). The hPL secretion from hydatidiform mole cells was elevated by the addition of M-CSF in a dose-dependent manner, but was suppressed by the addition of anti-M-CSF antibody or anti-*fms* antibody (Table III). hPL secretion from the four choriocarcinoma cell lines was not affected by the addition of either M-CSF, anti-M-CSF antibody, or anti-*fms* antibody (Table III).

M-CSF production by tPA30-1 and choriocarcinoma cells tPA30-1 cell supernatants contained a detectable amount of M-CSF, while the supernatants of the four choriocarcinoma cell lines did not (Table IV). M-CSF could not be assayed in hydatidiform mole cell culture supernatants, although the fluid pooled within the hydatidiform mole vesicles contained large amounts of M-CSF. (Note: since hydatidiform moles have no fetal blood vessels, molar fluid pools within vesicles.) Amplification using RT-PCR did not detect M-CSF mRNA in any of the four choriocarcinoma cell lines (Fig. 3).

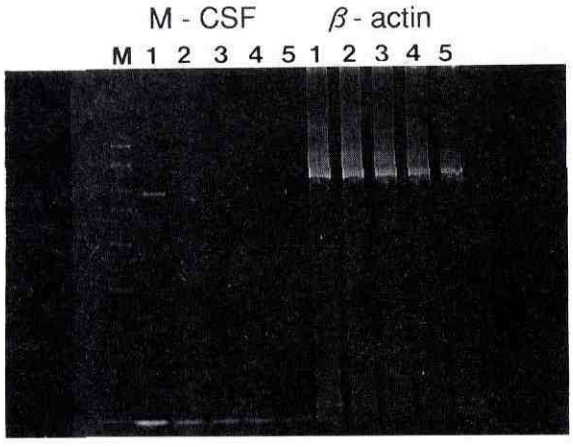


Fig. 3. M-CSF and β-actin mRNA expression in tPA30-1 cells (lane 1), BeWo cells (lane 2), Jar cells (lane 3), Jeg-3 cells (lane 4), and NUC-1 cells (lane 5). M represents BioMarker™ low double-stranded DNA bands of 1000, 700, 500, 400, 300, 200, 100, and 50 bp, respectively.

Immunohistochemical staining of M-CSF in normal placenta, hydatidiform mole, and choriocarcinoma tissue Immunohistochemical staining, performed using rabbit IgG specific anti-M-CSF, revealed that normal chorion and hydatidiform mole cytotrophoblast tissue were intensely stained, while normal chorion and hydatidiform mole syncytiotrophoblast tissue showed only weak staining (Fig. 4B and D). When choriocarcinoma tissue was examined for M-CSF staining, only fibroblasts surrounding the choriocarcinoma tissue exhibited weak staining, while the choriocarcinoma cells themselves exhibited no staining (Fig. 4F). On the other hand, choriocarcinoma cells exhibited intense staining with an anti-hCGα monoclonal antibody (Fig. 4G).

Point mutation of c-*fms* at Leu301 and Tyr969 Point mutations of c-*fms* at Leu301 and Tyr969 result in ligand-independent c-*fms* kinase activity, similar to the activity possessed by v-*fms*.[19] When hydatidiform mole and Jeg-3 cells were examined, there were no c-*fms* mutations at Leu301 or Tyr969 (Fig. 5 and 6). Other choriocarcinoma cell lines, BeWo, Jar, and NUC-1, also did not exhibit point mutations in c-*fms* at Leu301 or Tyr969 (data not shown).

DISCUSSION

A growth factor, M-CSF, is known to regulate the growth, differentiation, and survival of monocytes/macrophages.[20] c-*fms*, a receptor for M-CSF, has been reported to be expressed on not only monocytes, but also placental and choriocarcinoma cells.[2, 3] Prior to the pres-

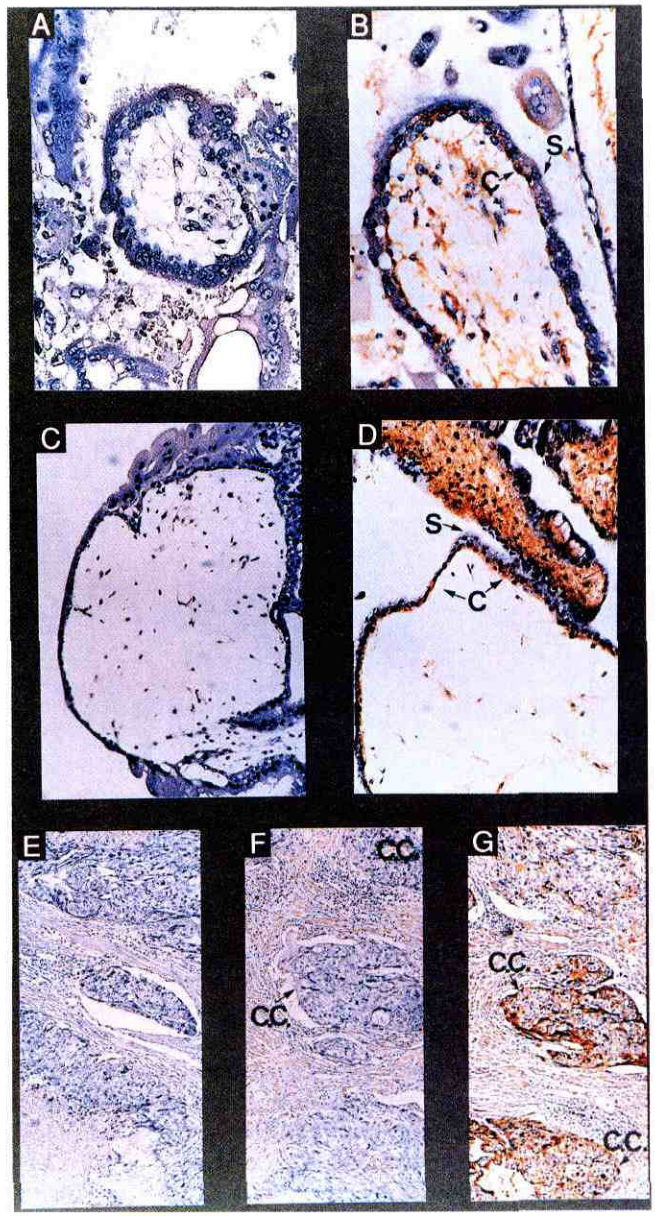


Fig. 4. Localization of M-CSF in the early pregnancy human placenta (A and B), hydatidiform mole (C and D) and choriocarcinoma cells (E, F and G) by immunohistochemical staining with normal rabbit serum (A, C and E), rabbit anti-human M-CSF antibody (B, D and F) and mouse anti-hCGα monoclonal antibody (G). Cytotrophoblasts (C) showed intense staining while syncytiotrophoblasts (S) were only weakly stained with anti-human M-CSF antibody. Choriocarcinoma cells (CC) were not stained with anti-M-CSF antibody, but were strongly stained with anti-hCGα antibody. Magnification, ×400 (A and B), ×200 (C and D) and ×100 (E, F and G).

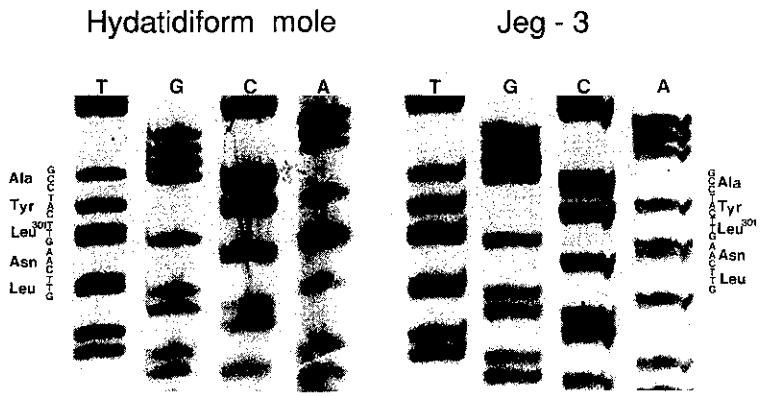


Fig. 5. c-*fms* DNA sequence at Leu301 in hydatidiform mole cells and the choriocarcinoma cell line, Jeg-3.

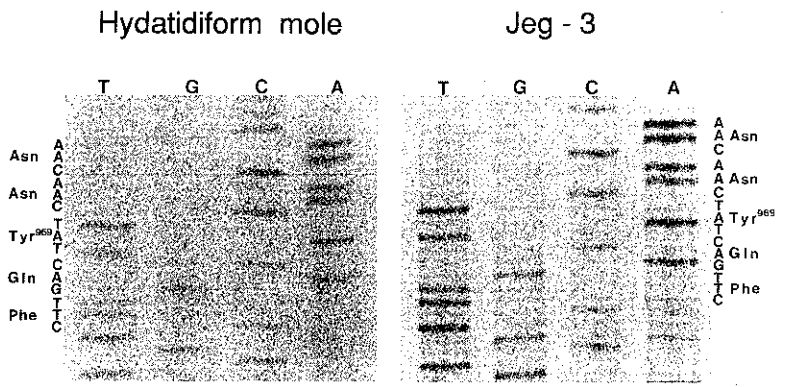


Fig. 6. c-*fms* DNA sequence at Tyr969 in hydatidiform mole cells and the choriocarcinoma cell line, Jeg-3.

ent study, the effect of M-CSF on placental cells was not well known, although experiments in mice suggested the involvement of M-CSF in placenta-like cell growth.[8] The M-CSF-deficient *op/op* mouse is consistently infertile.[21] These data suggest that M-CSF is required for pregnancy. The present study demonstrated that the growth of human placental cells in early gestation was induced by the addition of exogenous M-CSF. On the other hand, the growth of placental cells in later gestation (tPA30-1 cell) was not affected by exogenous M-CSF treatment, but was suppressed by treatment with anti-M-CSF or anti-*fms* antibody. These results indicate that the growth of tPA30-1 cells is not further promoted by the addition of exogenous M-CSF because these cells secrete M-CSF in a sufficient amount to promote their own proliferation (Table II).

The data also indicate that tPA30-1 cell growth is suppressed if M-CSF produced by these cells is neutralized by anti-M-CSF antibody, or if M-CSF receptor is blocked by anti-c-*fms* antibody. Thus, early gestational human chorionic cells and tPA30-1 cells form an autocrine loop including M-CSF and c-*fms* product. It has been reported that the autocrine loop is completed by M-CSF production in c-*fms*-transfected fibroblasts.[22, 23] In addition, patients with acute myelocytic leukemia sometimes show simultaneous expression of M-CSF and c-*fms*,[24] suggesting the presence of an M-CSF-related autocrine loop. It is also known that the decidua contains a large amount of M-CSF.[9] Therefore, it seems likely that M-CSF regulates placental growth through an autocrine or paracrine mechanism. In the present study, the choriocarcinoma cell growth was completely independent of M-CSF. Interestingly, M-CSF production by choriocarcinoma cells, was seen at neither the protein level nor the mRNA level. Horiguchi *et al.* also reported that choriocarcinoma cell lines, BeWo and Jeg-3, did not express M-CSF mRNA, even after stimulation with TPA.[25] Similarly, in the present study using an immunohistochemical staining technique, choriocarcinoma tissue collected from clinical cases did not express cytoplasmic M-CSF. The term "hydatidiform mole" is used to indicate abnormal placental growth which can be considered as a gestational trophoblastic neoplasm, including abnormal chorionic epithelial growth as well as

edema of the villous stroma and avascular villi. In the present study, hydatidiform mole cytotrophoblast was immunohistochemically found to contain M-CSF. Hydatidiform mole vesicles were also observed to contain a large amount of M-CSF. Furthermore, hydatidiform mole cells with an increased proliferative ability showed M-CSF-dependent growth. We therefore note that normal chorionic and hydatidiform mole cells show M-CSF-dependent growth, while the growth of transformed chorionic cells in M-CSF-independent. The lack of M-CSF production of choriocarcinoma cells may, therefore, be a consequence of the fact that choriocarcinoma cell growth is M-CSF-independent.

Similar observations have been previously reported. Hepatocyte growth factor (HGF) is a potent mitogen of mature primary cultured hepatocytes, while it inhibits the growth of hepatocellular carcinoma derived cell line, Fao HCC.[26] In addition, HTLV-I-transformed T cells usually show IL-2-dependent growth, but IL-2-independent growth has also been observed in some cases.[27] In that report, growth of IL-2-independent T cells was suppressed by a two-week incubation with IL-2. In our study, a two-week incubation of choriocarcinoma cells with M-CSF revealed no influence of M-CSF on cell growth (data not shown).

hCG and hPL are only secreted from fused and differentiated syncytiotrophoblasts.[28,29] M-CSF promotes not only the growth of chorionic cells but also their differentiation to syncytiotrophoblast secreting hCG and hPL. In the present study, M-CSF induced hCG and hPL secretion from normal trophoblast and hydatidiform mole cells. On the other hand, M-CSF induced neither the secretion of hCG or hPL nor the growth and differentiation of choriocarcinoma cells.

c-*fms*, which possesses a tyrosine kinase domain, is important for cellular growth and differentiation. Point mutation at Leu301 or Tyr969 of c-*fms* protein is known to result in ligand-independent kinase activity similar to that of v-*fms*.[19,30] We surmised that M-CSF-independent growth of choriocarcinoma cells was a result of point mutation in the c-*fms* product. In the present study, however, none of the four choriocarcinoma cell lines possessed a point mutation at Leu301 or Tyr969 in c-*fms* product. It has been suggested that phosphatidylinositol-3 kinase,[31] phosphorylation of the *raf*-1 protein,[32] G-protein[33] and cyclin D[34] are involved in signal transduction. Although the signal transduction pathway in choriocarcinoma cells may be regulated by factors other than M-CSF, it would be of interest to study the choriocarcinoma cell signal transduction pathway in more detail.

In summary, we have shown that M-CSF induces the growth and differentiation of normal chorionic cells and hydatidiform mole cells, but not choriocarcinoma cells.

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