Marked Increase in Macrophage Migration Inhibitory Factor Synthesis and Secretion in Human Endometrial Cells in Response to Human Chorionic Gonadotropin Hormone

Ali Akoum, Christine N. Metz, and Mathieu Morin

Unité d’Endocrinologie de la Reproduction (A.A., M.M.), Centre de Recherche, Hôpital Saint-François d’Assise, Centre Hospitalier Universitaire de Québec, Faculté de Médecine, Université Laval, Québec, Canada G1L 3L5; and North Shore Long Island Jewish Research Institute (C.N.M.), Manhasset, New York 11030

Human chorionic gonadotropin (hCG), a placental glycoprotein hormone mainly produced by the syncytiotrophoblasts in the chorionic villi, is one of the major and earliest embryonic signals. In a normal pregnancy, hCG is detectable in maternal serum as early as 1 d after the initiation of embryo implantation, then rapidly rises to a peak value around 60–90 d gestation, and gradually decreases within the second trimester (1). The main role of hCG is considered to stimulate the corpus luteum in the ovary to produce progesterone and maintain embryo implantation (2). Newest evidence, however, showed a wide spectrum of cell targets and biological properties, by which hCG plays a crucial role in the implantation and growth of human embryo. Interestingly, it has been shown that the human endometrium, the natural host site where the embryo implants and develops, contains functional membrane-bound hCG/LH in glandular and luminal epithelial cells as well as in stromal cells (3, 4). Additional studies demonstrated that hCG promotes the decidualization of human endometrial stromal cells (5). It is now well documented that hCG directly modulates numerous endometrial functions, thereby promoting endometrial receptivity as well as the implantation and growth of human embryo (6).

Implantation of human embryo involves a complex process of tissue remodeling. Deep morphological, structural, and functional changes occurring within the uterus, particularly in the endometrium, are orchestrated primarily by embryonic factors/signals that facilitate and enable embryonic invasion and growth (7). Angiogenesis represents a key feature of such a tissue remodelling. hCG has been shown to possess intrinsic angiogenic properties (8) and to promote the production of angiogenic factors, such as vascular endothelial growth factor, by the placenta (9) as well as by decidual macrophages (8). However, it is still unknown whether hCG can stimulate endometrial cells to produce angiogenic factors. Cumulative evidence also indicates a critical role for immune cells in tissue remodeling occurring within the endometrial tissue during embryo implantation and growth. During normal pregnancy, the decidua is populated by a variety of leukocytes and macrophages that constitute 20–30% of the decidual cells at the site of implantation (10). Macrophages are believed to protect the embryo against infection and to play an important role in maternal tolerance and maintenance of pregnancy (10).

Originally described as a product of activated T lymphocytes that inhibits the random migration of cultured macrophages, macrophage migration inhibitory factor (MIF) is now known for being involved in a variety of biological processes, including inflammatory and immune responses, and angiogenesis. Currently, MIF is considered to play a key role in the initiation and maintenance of pregnancy, marking a new mechanism by which hCG sustains human pregnancy and promotes embryonic growth. (J Clin Endocrinol Metab 90: 2904–2910, 2005)
cell proliferation, angiogenesis, and tumor progression (for reviews, see Refs. 11–13). Recent studies showed MIF expression in the human endometrium as well as in trophoblasts in the first trimester of pregnancy, which suggests a role for this factor during implantation and early embryonic development (14, 15).

The present study showed that hCG induces MIF synthesis and secretion by endometrial stromal cells. Our findings revealed that hCG had no significant effect on MIF mRNA stability and mainly acts by increasing MIF gene transcription. In view of the proangiogenic properties and immunomodulatory effects of MIF, particularly those of activation and inhibition of macrophage migration and immunosuppression of natural killer (NK) cell activity, it is quite plausible that MIF represents an important effector cell mediator of hCG-induced endometrial changes during embryo implantation, growth, and development.

Subjects and Methods

Subjects and tissue handling

Tissue specimens were obtained from normal fertile women with regular menstrual cycle who underwent laparoscopy for tubal ligation and had not received hormonal or antiinflammatory therapy for at least 3 months before surgery (mean age ± sd, 33.60 ± 6.20 yr; n = 19). Seven women were in the proliferative phase of the menstrual cycle, and 12 women were in the secretory phase. The cycle phase was determined according to the histological criteria of Noyes et al. (16). A written informed consent was obtained from these women under a study protocol approved by the Ethical Committee on Human Research at Laval University, Quebec, Canada. Endometrial biopsies were obtained by aspiration using a probe with a lumen (Unimar Inc., Prodimed, Neufly-sur-Tarchelle, France). They were immediately placed in sterile Hanks’ balanced salt solution containing 1% antibiotics-antimycotics at 4°C (Brewin et al., En-Tchelle, France). They were immediately placed in sterile Hanks’ balanced salt solution containing 1% antibiotics-antimycotics at 4°C (Invitrogen Life Technologies, Burlington, Ontario, Canada) and transported to the laboratory.

Cell culture and treatment

Endometrial stromal cells were obtained and characterized according to our previously described procedure (17). Stromal cells were plated and subcultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen) to eliminate contamination by macrophages or other leukocytes. All experiments were initiated after 24-h incubation in FBS-free medium. Extensive characterization of cell cultures prepared using this protocol previously confirmed more than 95% purity, with cells retaining cytoskeletal markers of their endometrial stromal origin (17). At confluence, the complete medium was discarded and replaced overnight with FBS-free medium, and cells were cultured for additional periods of time (0–24 h) with fresh FBS-free medium containing different concentrations of hCG (0–1 μg/ml) (recombinant expressed in mouse cell line, 10,000 IU/mg; Sigma Chemical Co., St. Louis, MO). In some experiments, cycloheximide and actinomycin D (Sigma) were added to the cell culture at the same time as hCG, at a final concentration 100 and 10 μg/ml (355 and 8 μm), respectively. hCG-induced MIF protein expression/secretion was evaluated by immuno- cytofluorescence and by ELISA in the culture medium, whereas MIF induced MIF protein expression/secretion was evaluated by immunocytofluorescence and by ELISA in the culture medium, whereas MIF mRNA was evaluated by RT-PCR and nuclear transcription (run-on).

Immunocytofluorescence

Stromal cells were plated onto Lab-Tek 8-chamber slides (Nalge Nunc International, Naperville, IL), treated for 24 h in the absence or presence of 0.1 μg/ml (2.6 nm) hCG, and fixed for 15 min at room temperature in 95% cold ethanol. Cultures were then rinsed three times in PBS/0.1% Tween 20 (PBS-Tween); incubated for 90 min at room temperature with polyclonal anti-MIF goat antibody (0.66 μg/ml of PBS-Tween; primary antibody) (R&D Systems, Minneapolis, MN); rinsed three times with PBS-Tween; incubated for 1 h at room temperature with 1:50 dilution in PBS-Tween of fluorescein isothiocyanate-conjugated donkey anti-goat IgG (second antibody) (ICN Biomedicals, St. Laurent, Quebec, Canada); rinsed three times with PBS-Tween; covered with mounting medium (Mowiol containing 10% paraformaldehyde, an antifading agent; Sigma), and sealed. The specificity of the immunostaining was confirmed by immunosorption of the primary anti-MIF antibody with 2 μg/ml (0.16 μl) recombinant human (rh) MIF (R&D Systems) before incubation with cells. Cells incubated without the primary antibody or with goat IgG (Sigma) at the same concentration as the primary antibody were included as negative controls in all experiments. Slides were observed under a microscope equipped for fluorescence (Leica Mikroskopie und Systeme GmbH, Model DMRB; Postfach, Wetzlar, Germany) and photographed.

ELISA

MIF ELISA was performed using a mouse monoclonal antihuman-MIF antibody (R&D Systems) as a capture antibody, a rabbit polyclonal antihuman-MIF antibody for detection, an alkaline phosphatase-conjugated goat antirabbit antibody, and paranitrophenyl phosphate (Sigma) as substrate (18). The OD was measured at 405 nm, and MIF concentrations were extrapolated from a standard curve using rhMIF. The sensitivity limit of the assay was 300 pg/ml (24 pm), with intra- and interassay coefficients of variation less than 4%.

RT-PCR

Total RNA was extracted from stromal cell cultures using the TRIzol reagent kit (Invitrogen). The conditions for preparing the internal standards, the RT-PCR, the primers used for semiquantitative RT-PCR, and Southern blotting have been described previously (19). Briefly, specific oligonucleotide primers were designed to amplify sequences from human MIF mRNA (255 bp) and human glyceraldehyde phosphate dehydrogenase (GAPDH) (240 bp) as internal control, and reaction products were separated on 1.8% agarose gels. Southern blot hybridization was performed using 32P-labeled MIF and GAPDH cDNAs [American Type Culture Collection (ATCC), Manassas, VA]. The intensity of the hybridization signals was determined by computer-assisted densitometry, using Quantity One Quantitation Software (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The quantity of the PCR products was determined by densitometric analysis of the intensity of the hybridization signal, the relative level of MIF mRNA normalized to GAPDH mRNA was calculated, and the results were expressed as percentage of control (MIF mRNA level in cells cultured without any stimulus).

mRNA stability and half-life experiments

Cells were incubated with the culture medium alone (control) or containing 0.1 μg/ml (2.6 nm) hCG for 24 h, as described earlier. Transcription was then stopped with 10 μg/ml (8 μM) actinomycin D, and cells were harvested after different times (0–8 h) of incubation with actinomycin D for RNA extraction and RT-PCR analysis. The relative level of MIF mRNA normalized to GAPDH mRNA was calculated at each time point, and the time required for 50% diminution of MIF mRNA level was determined by linear regression using GraphPad Software, Prism 3.0 (GraphPad Software, San Diego, CA).

Nuclear run-on assay

Cell culture and treatment with hCG were performed as mentioned above. Isolation of nuclei, nuclear transcription, and extraction of radiolabeled RNA were performed as described previously (20). The radioactive RNA was used to probe 5 μg alkali denatured plasmid MIF and 28S DNAs (ATCC) immobilized on nylon membranes, using a slot blot apparatus (Hoefer, San Francisco, CA). Empty plasmid vector was used as a negative control. Hybridization was carried out for 3 d at 42°C using 5–10 million cpm/ml of hybridization buffer. Membranes were washed four times with 2× sodium saline citrate, 0.1% sodium dodecyl sulfate at 65°C for 30 min; incubated with 10 μg/ml RNase A and 100 μg/ml protease K for 30 min at 37°C, respectively; washed twice again with 2× sodium saline citrate, 0.1% sodium dodecyl sulfate at 65°C for 1 h.
Statistical analysis

Data followed a parametric distribution and were therefore expressed as mean ± SEM. An unpaired t test was used for comparing the means of two groups, and one-way ANOVA followed by the Dunnett test was used for multiple comparisons. Differences were considered as statistically significant whenever a P value less than 0.05 occurred. All analyses were performed using GraphPad Software.

Results

Endometrial stromal cells were first examined for MIF protein expression by immunocytofluorescence. Confluent monolayers of endometrial stromal cells incubated with anti-MIF antibody showed faint staining under conditions in which the culture medium contained no stimulus (Fig. 1A). After culturing the cells in the presence of 0.1 μg/ml (2.6 nM) hCG for 24 h (Fig. 1C), an increase in staining intensity was noted to be mainly localized in perinuclear cytoplasm. Staining was virtually absent in cells incubated with goat IgGs instead of anti-MIF antibody or with anti-MIF antibody preabsorbed with an excess 2 μg/ml (0.16 μμ) rhMIF (Fig. 1, B and D). These data are representative of three stromal cell cultures; one from proliferative phase endometrial tissue and two from secretory phase tissues.

These findings prompted us to quantitatively assess MIF secretion by endometrial cells in response to hCG treatment. Thus, endometrial cell cultures were exposed to varying concentrations of hCG for different time periods. Our data showed a spontaneous secretion of MIF in the culture medium devoid of any stimulus (control) and that hCG significantly stimulates MIF secretion over control and in a dose- and time-dependent manner. Cell stimulation with 0.1 and 1 μg/ml (2.6 and 26 nm) hCG for 24 h resulted in 190.30 ± 20.53% and 240.20 ± 34.45% increase over control (P < 0.01 and P < 0.001, respectively) (data from nine different patients; four in the proliferative phase and five in the secretory phase) (Fig. 2A). The hCG-induced MIF secretion gradually increased throughout the incubation period, reaching statistical significance after 24 h of exposure to 0.1 μg/ml (2.6 nm) hCG (193.40 ± 17.29% increase over control; P < 0.01) (data from four different patients; three in the proliferative phase and one in the secretory phase) (Fig. 2B). Analysis of cell responsiveness according to the menstrual cycle phase showed that endometrial cells from the secretory phase of the menstrual cycle were significantly more responsive to 1 μg/ml (26 nm) hCG compared with cells from the proliferative phase (297.60 ± 54.75% vs. 168.60 ± 18.61% increase in MIF secretion over control, respectively; P < 0.05).

In some experiments, endometrial cells were incubated with 0.1 μg/ml (2.6 nm) hCG together with 100 μg/ml (355 μμ) cycloheximide, an inhibitor of protein synthesis. Data illustrated in Fig. 2A showed that cycloheximide completely blocked hCG-induced MIF secretion (Fig. 2A), suggesting that MIF secretion in response to hCG did not result from preformed and stored MIF but rather from de novo MIF synthesis.

We then assessed MIF mRNA synthesis by endometrial cells after treatment with hCG and again found a dose- and time-dependent increase in MIF mRNA as shown in RT-PCR autoradiograms (data from one patient in the secretory phase) (Figs. 3A and 4A). Data from four patients (three in the proliferative phase and one in the secretory phase) showed 218.80 ± 26.33%, 284.60 ± 44.10%, and 306.70 ± 83.11% increase in MIF mRNA levels over control after cell exposure to 0.01, 0.1, and 1 μg/ml (0.26, 2.6, and 26 nm) hCG (P < 0.05, P < 0.01, and P < 0.05, respectively) for 24-h period (Fig. 3B). Cell stimulation with 0.1 μg/ml (2.6 nm) hCG for 12 and 24 h of culture resulted in 183.50 ± 20.76% and 284.60 ± 44.10% increase over control, respectively (P < 0.05 and P < 0.01) (Fig. 4B). Moreover, adding 100 μg/ml (355 μμ) cycloheximide together with 0.1 μg/ml (2.6 nm) hCG to endometrial cell cultures had no noticeable effect on MIF mRNA levels compared with cultures incubated with 0.1

Fig. 1. Immunochemical analysis of hCG-induced MIF expression in endometrial cells. Cells cultured in chamber slides were incubated for 24 h with the culture medium alone or supplemented with 0.1 μg/ml (2.6 nm) hCG, and MIF was detected by immunocytofluorescence. Using a MIF-specific polyclonal goat antibody. Note the faint staining in cells incubated in the culture medium without stimulus (A) and the marked increase in the intensity of staining in the presence of hCG (C). Staining was virtually absent in the presence of goat Igs used instead of the primary antibody (B) or anti-MIF antibody preabsorbed with an excess of rhMIF (D). Scale bar, 30 μm.
hCG alone (Fig. 3), suggesting that hCG-induced MIF mRNA synthesis does not require de novo protein synthesis.

To determine whether the effect of hCG was exerted at the posttranscriptional level and/or the transcriptional level, we evaluated MIF mRNA stability and nuclear transcription. Cells were treated or not with 0.1 μg/ml hCG (2.6 nM) for 24 h, as previously described. Figure 5 shows that pretreatment with hCG had no significant effect on MIF mRNA stability (P > 0.05). Without pretreatment with hCG, the half-life of MIF mRNA was approximately 6.56 ± 0.20 h, whereas in hCG-treated cells, it was 7.53 ± 0.38 h (data from three separate cultures; one from proliferative phase endometrial tissue and two from secretory phase tissues). Furthermore, it would appear that there are two phases in the degradation of mRNA, and after 4 h, there appears to be little if any decrease in mRNA. Nuclear run-on analysis showed that hCG treatment markedly increased MIF nuclear transcription (Fig. 6). The mean increase ± SEM in MIF gene transcription over control as determined by densitometric analysis was 220.70 ± 27.72% (data from three separate cultures from secretory phase endometrial tissues).

**Discussion**

Molecular interactions at the embryo-maternal interface during the time of adhesion and subsequent invasion are crucial to the process of embryonic implantation. Available data indicate that the embryo participates intensively in this early embryo-maternal signaling. hCG represents one of the major and earliest embryonic signals and seems to influence...
mediated induction of MIF mRNA synthesis does not require de novo protein synthesis by endometrial cells.

To further investigate the mechanisms underlying hCG-mediated increase in MIF expression by endometrial cells, we assessed the effects of hCG on MIF mRNA stability. Cytokines are generally known for having a short half-life, and one of the mechanisms that underlie their sustained and prolonged action is that related to their prolonged mRNA stability under specific physiological and pathological conditions (23, 24). Our study did not detect a statistically significant change in MIF mRNA stability in response to hCG. However, nuclear transcription run-on assays revealed a marked increase in MIF mRNA nuclear transcripts in endometrial cells in response to hCG, which clearly indicates a transcriptional regulation of MIF expression by hCG in endometrial stromal cells. Therefore, our data indicate that hCG up-regulation of MIF mRNA synthesis by endometrial stromal cells is exerted more predominantly at the transcriptional than at the posttranscriptional level. At the present time, it is still unknown how hCG can up-regulate MIF gene transcription. Recent data from our laboratory indicate that the transcription factor nuclear factor-κB (NF-κB) is a major factor involved in MIF gene transcription in endometrial cells (our unpublished data). Furthermore, NF-κB appeared to be activated and translocated in some cell types as a result of hCG stimulation (25), but it remains unknown whether hCG had any effect on NF-κB in endometrial cells.

In the present study, we further showed that endometrial cells from the secretory phase of the menstrual cycle were significantly more responsive than cells from the proliferative phase to high but physiological hCG concentration. This is in keeping with the finding of higher levels of hCG receptors in the endometrium in the second phase of the cycle and the fact that progesterone, the secretion of which is maintained by hCG during the first trimester of pregnancy (2), up-regulates hCG receptors in the endometrium (3), therefore making this tissue more responsive to elevated hCG concentrations. Taken together, these findings further support a role for hCG-mediated MIF secretion in uterine adaptation during implantation and placentation.

These findings may have a considerable physiological significance, considering the biological properties of MIF and the critical role of hCG in human pregnancy.

First, they clearly demonstrate that MIF can be produced locally in the endometrial tissue in response to the major embryonic signal hCG. There is little information on the role of MIF in early pregnancy, and the present results suggest that the human embryo has the capacity for local modulation of the production of cytokines that, in turn, may sustain development and maintain pregnancy. Data available to date point toward a role for MIF in reproduction. This factor has been found in human ovary (26), human endometrium, and chorionic villi from first-trimester human placenta (14, 15) and has been shown to be up-regulated in bovine epithelial cells in response to interferon-γ, a bovine embryonic signal (27). In human pregnancy, the major binding protein of the interferon antagonist sarcolectin, the properties of which correspond to those of MIF, has been described in term placenta (28). However, there is no previous report on the mechanisms underlying MIF expression and involvement in
embryonic implantation and growth. The fact that hCG directly up-regulates MIF by human endometrial stromal cells provides evidence that MIF expression may, at least in part, be under embryonic control.

MIF is a multifunctional cytokine with a variety of biological properties that can be relevant for embryo implantation and development. In fact, one of the first identified properties of MIF is that of activating and inhibiting macrophage migration (11, 12, 22). Macrophages represent one of the predominant immune cell types in tissues around the embryo (10). The mechanisms and relevance of increased macrophage number at the embryo implantation site are still not clearly elucidated. It is believed, however, that these cells may provide protection for the embryo against infections and contribute to its expansion and growth via the secretion of growth, angiogenic, and tissue-remodeling factors (10, 29, 30). ICG-mediated MIF secretion may therefore represent a possible mechanism for macrophage accumulation at the embryo implantation site and suggests a role for the embryo in its own protection and a direct control of the local environment to create conditions that favor embryo survival and growth.

Both in vitro and in vivo data support an important role for MIF in angiogenesis and tumor growth development (11, 12, 31–33). Angiogenesis, or the development of new vessels, is critical for embryo growth. Due to a high demand for increased blood supply during pregnancy, the vasculature of the maternal tissue surrounding the embryo undergoes deep changes that involve vasodilation and increased permeability but also the development of new vessels (8) and a huge tissue remodeling comparable to some extent to tumor invasion (34, 35). Furthermore, this appears to be related to specific factors of trophoblast origin (36). Interestingly, recent data indicate a role for hCG in human endothelial cell migration and capillary formation via a direct effect (21), and the capability to stimulate the secretion of matrix metalloproteinases (MMP), such as MMP-9, by placental and endometrial cells (7, 37). This is of particular interest in view of our present data because it suggests that hCG may promote angiogenesis and embryo implantation through direct and indirect pathways. MIF is known for inducing MMP secretion (38) and for having potent angiogenic properties, which may, at least, be due to a direct mitogenic effect on endothelial cells (31–33) and to its capability of retaining macrophages, which are known for releasing numerous growth and angiogenic factors (10–12, 29, 30).

One of the intriguing and fascinating aspects of pregnancy is the capability of such a semiallograft, the embryo, to develop and to extract itself from rejection, the influence of the maternal immune system. Several lines of evidence show the ability of the embryo to modulate the maternal immune system so as to prevent rejection (10, 39, 40). During early pregnancy, the number of uterine leukocytes increases, and NK cells represent one of the main leukocyte types that are increased in the decidua (41), where they achieve peak numbers during the first trimester (42). NK cells play a key role in immune rejection, and, interestingly, it has been shown that NK cells from the embryo implantation site or the maternal serum are immunosuppressed and have lower cytotoxicity for fetal cells (39, 41, 43, 44). Our data showing an

hCG-induced MIF secretion by endometrial cells provide another example of the capability of the embryo to modulate the maternal immune system and to contribute to the development of maternal immunotolerance, because MIF is known for suppressing NK cell activity (45).

In conclusion, we have demonstrated that hCG directly up-regulates the synthesis and secretion of a multifunctional cytokine with potent immunomodulatory and angiogenic properties. hCG did not significantly prolong MIF mRNA stability but appeared to act predominantly at the transcriptional level. These findings may have an interesting significance in view of the biological properties of MIF, its expression by decidua of first-trimester placenta, and the paramount role of hCG in the initiation and maintenance of pregnancy, and point toward a role for MIF in the early events of embryo implantation and growth. Furthermore, they provide a new insight in the mechanisms of hCG action and reveal another feature of the endocrine/immune control of pregnancy and embryonic development.

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Address all correspondence and requests for reprints to: Ali Akoum, Ph.D., Laboratoire d’Endocrinologie de la Reproduction, Centre de Recherche, Hôpital Saint-François d’Assise, 10 rue de l’Espinay, Local D0-711, Québec, Québec, Canada G1L 3L5. E-mail: ali.akoum@crsf.ulaval.ca.

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