Endothelin-1 and macrophage colony-stimulating factor are co-localized in human amnion membrane cells and secreted into amniotic fluid

Gabriel Fried¹, Anna Sand, Eva Östlund, Eva Andersson, Birgitta Byström and Berit Ståbi

Department of Woman and Child Health, Division of Obstetrics and Gynecology, Karolinska Institute, S-171 76 Stockholm, Sweden

¹To whom correspondence should be addressed. E-mail: gabriel.fried@kbh.ki.se

We have examined the cellular localization and human amniotic fluid content of endothelin-1 (ET-1) and macrophage colony-stimulating factor (M-CSF). The study material consisted of amniotic fluid from 20 patients referred for amniocentesis, and placental samples from normal deliveries. ET-1 and M-CSF were analysed by radioimmunoassay and enzyme-linked immunosorbent assay respectively. The cellular localization of ET-1 and M-CSF in the amnion membranes was analysed by double-labelling immunocytochemistry using fluorescein isothiocyanate- and Cy3-labelled secondary antibodies. Release of ET-1 and M-CSF was studied in cultured amniocytes. We found that the mean ± SD concentrations of ET-1 and M-CSF in fetal amniotic fluid were 45.6 ± 17.3 pmol/l (range 16.8–85.5) and 7323 ± 3415 ng/l (range 2640–12 110) respectively. Double-labelling immunocytochemistry showed that both M-CSF and ET-1 were co-localized in the same cells to a high extent. Further analysis revealed that levels of M-CSF, but not ET-1, were significantly correlated with pregnancy length. Both M-CSF and ET-1 were released from cultured amniocytes in response to interleukin-1. These findings show that ET-1 and M-CSF are partly co-localized to specific cells in the human amniotic membrane. As both M-CSF and ET-1 were released from cultured amniocytes in vitro, this suggests that they both may be secreted into fetal amniotic fluid in vivo as well.

Key words: amnion/endothelin-1/macrophage-stimulated colony factor/placenta/pregnancy

Introduction

Macrophage colony-stimulating factor (M-CSF), also called colony-stimulating factor-1 (CSF-1), was discovered in serum and urine as a factor that could stimulate growth of macrophage colonies from haematopoietic progenitor cells obtained from bone marrow (Stanley et al., 1977). It was later found to be produced in numerous different cell types, and one of its primary functions appeared to be to regulate growth, differentiation and function of mononuclear phagocytes. It also regulates decidual cells of the endometrium and trophoblasts of the placenta (Arceci et al., 1989).

M-CSF is a disulphide-linked homodimeric glycoprotein that occurs in several isoforms (Fixe and Praloran, 1998). The longest human isoform, M-CSF522, is a 554 amino acid residue Type I transmembrane preprotein containing a 32 amino acid residue signal peptide, followed by an additional 522 amino acid residue. Two shorter forms also exist, M-CSF224 and M-CSF406. M-CSF exerts its biological effects by binding to a high affinity cell surface receptor, c-fms (Sherr et al., 1985).

M-CSF levels were discovered to be high in amniotic fluid and found to be derived both from neonatal urine and from epithelial cells of the fetal membrane (Saito et al., 1992). The levels in amniotic fluid were found to be higher than in fetal cord blood (Saito et al., 1992).

In the feto-placental unit, M-CSF (CSF-1) was detected in serum, endometrium, placenta, chorion, amnion, and amniotic fluid, with significant increases in serum and endometrial samples from the first trimester compared with levels in non-pregnant controls (Daiter et al., 1992). M-CSF mRNA was demonstrated in these tissues with a significant increase within the first trimester endometrial samples over non-pregnant control values. In addition to the major 4.0 kilobase mRNA, other species of M-CSF mRNA were detected, shown to be due to alternative splicing within exon 6 and the alternative use of exon 9 or 10. In the endometrium, M-CSF was localized to glandular epithelial and endothelial cells. In first trimester placenta, M-CSF was found in the cytotrophoblasts lining the villous core and in the villous mesenchymal cells. By the third trimester, M-CSF was only detected in cells lining the villous vessels (Daiter et al., 1992).

ET-1, a 21 amino acid polypeptide, is produced and released primarily from endothelial cells (Yanagisawa et al., 1988), but is also produced by glandular epithelium in various tissues including the amnion cells (Hemsen 1991). It is one of the most potent vasoconstrictors known, and it contracts vessels from most vascular beds including the placenta (Fried et al., 1991, 1994; Haegerstrand et al., 1989). The physiological actions of ET-1 in the placental vessels are likely to be related to regulation of blood flow. We have previously found an increased contractile response to ET-1 in placental vessels from patients with intrauterine growth restriction (IUGR) of the fetus as compared to vessels from normal pregnant women (Liu et al., 1995) and therefore hypothesized that ET-1 may be involved in the pathophysiology of IUGR.

ET-1 is also found in the endometrium, amniotic membranes and amniotic fluid of both humans and experimental animals (Mitchell et al., 1991; Cameron et al., 1992; Economos et al., 1992; Salamonsen et al., 1992; Riley et al., 1995). In the endometrium, the strongest staining for ET-1 was found in the luminal epithelium throughout the
secretory phase and in the glandular epithelium in the late secretory phase. In the umbilical cord, the most intense immunoreactivity was present on the amnion cells on the outer cord, with some staining in intertissent cells in the Wharton’s jelly and in umbilical vein cells (Salamonsen et al., 1992).

IUGR is a condition with a heterogeneous pathophysiology with an incidence in Sweden of 2-3%. In severe IUGR, analysis of fetal blood after sampling by amniocentesis offers the clinician information regarding possible different aetiologies of IUGR, such as isoimmunization. Blood after sampling by amniocentesis offers the clinician information on the growth factor M-CSF in the regulation of placental development. Studies in experimental animals such as the mouse have strongly implicated the growth factor M-CSF in the regulation of placental development (Daiter et al., 1992).

In order to evaluate the possible relevance of M-CSF and ET-1 in the context of intrauterine fetal growth, we have in the present study analysed the amniotic fluid levels of M-CSF and ET-1, their cellular localization in the amniotic membrane, and whether they could be released from cultured amniocytes. In the release studies, we used interleukin (IL)-1α as agonist. Previous data have shown that inflammatory agents, including IL-1α and IL-1β, regulate M-CSF as well as endothelin in the placenta (Mitchell et al., 1991; Garcia-Lloret et al., 1994; Sagawa et al., 1994).

Materials and methods

Subjects and samples

Twenty pregnant women with suspected IUGR because of an estimated fetal weight below 22% (corresponding to –2 SD, and approximately corresponding to the 3rd centile) were included in the study. The definitions of IUGR have been used in detail in Marsel et al. (1996) (definitions used in Sweden and parts of Europe) and Alexander et al. (1996) (definitions used in the USA and UK). Clinical data for the 20 patients are shown in Table I. Gestational length was calculated from an ultrasound in the first trimester. An estimation of fetal weight was performed in the third trimester. The weight estimation was performed using ultrasound measurements of fetal biparietal diameter, femur length and abdominal circumference. The measurement gave information on the estimated fetal weight at the time of admittance to the hospital. The estimated fetal weight in relation to the gestational length gave information on whether the fetus followed the normal growth curve or not. The difference between the estimated fetal weight and the normal fetal weight for the gestational length was called weight deviation. The weight deviations of the 20 patients based on ultrasound at the time of sampling ranged from –22 to –50%, and the pregnancy length from 34 to 40 weeks (238 to 280 days). Weight deviations at birth ranged from 0 to –50%. Thirteen out of the 20 women were nullisara. Eight out of the 20 women were smokers.

Table I. Clinical data for the patients

| Age (years) | 31.0 ± 6.9 (18–44) |
| Gestational age (days) | 260 ± 12 (203–280) |
| Primipara (n) | 13 |
| Weight deviation at amniocentesis (%) | –29 ± 6 (–22 to –50) |
| Weight deviation at delivery (%) | –27 ± 12 (0 to –50) |

In order to karyotype the fetus, and to perform viral tests (toxoplasma, rubella, cytomegalovirus, herpes), amniocentesis was performed. Amniotic fluid was obtained from the mothers between gestational weeks 28 and 38 using ultrasound monitoring and local anaesthesia (10 ml Xylocain s.c.). All karyotypes were normal, and viral tests were negative. Between 20 and 30 ml amniotic fluid was withdrawn and stored at –70°C until analysis. All participants in the study gave informed consent and all procedures were approved by the local ethics committee.

Biochemical determinations

M-CSF in amniotic fluid and in the supernatants from the amniocyte cultures was determined by immunoassay, using a commercially available kit (M-CSF Quantikine kit; R&D Systems Inc., USA). The assay recognizes both natural human M-CSF and recombinant human M-CSF. Sensitivity was 20 pg/ml, intra-assay coefficient of variation (CV) was 5% and inter-assay CV was 6%. ET-1 in amniotic fluid was assayed by radioimmunoassay (Herness, 1991); intra-assay CV was 6% and interassay CV was 14%. ET-1 in the supernatants of the cell cultures was determined by enzyme-linked immunosorbent assay, using a commercially available kit (Chemiluminescent Immunoassay, Quantigen; R&D Systems Inc.). Blood acid-base balance, Kleihauer-Betke test and karyotyping were performed according to routinely used conventional methods of the Department of Clinical Chemistry and the Department of Clinical Genetics, Karolinska Hospital.

Immunohistochemistry

For immunohistochemical analysis, amniotic membrane tissue was cut into small transverse pieces comprising both fetal- and maternal-facing surfaces, rinsed from blood in phosphate-buffered saline (PBS) and fixed for 4 h in 4% paraformaldehyde with 0.4% picric acid in 0.1 mol/l sodium phosphate buffer, pH 7.4. Tissue was then processed for indirect immunofluorescence. Briefly, tissue was washed three times in phosphate buffer, snap-frozen in liquid nitrogen, and then sectioned at 14 μm in a cryostat. Tissue sections were then double-labelled by incubation in two primary antisera diluted in PBS 1:300 with 0.3% Triton for 24 h at 4°C. Mouse monoclonal anti-endothelin-1 IgG was from Affinity Bioreagents Inc. (USA). Goat polyclonal M-CSF (N-16) IgG was from Santa Cruz Biotech (USA). After rinsing in PBS, the slides were incubated with two secondary antibodies 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (H+L) (Vector Laboratories, USA) and 1:100 dilution of Cy3-conjugated affinity-purified donkey anti-goat IgG (H+L) (Jackson Immunoresearch Laboratories, USA). After incubation at 30°C for 1 h, slides were washed in PBS and mounted in a mixture of glycerol and PBS (3:1) containing p-phenylenediamine.

Negative staining controls were performed by incubation only with secondary antibody, in order to demonstrate that the observed fluorescence was not due to non-specific binding of the secondary antibody. The sections were viewed through a Zeiss confocal laser scanning microscope, LSM 510, using the appropriate wavelengths (543 and 488 nm) and filter combinations. The images were further processed on a Silicon Graphics Indy computer.

Ammniocyte cell culture

Primary cultures of human amnioncytes (Chang et al., 1992; Mathews and Verma, 1991) were obtained from the Department of Molecular Medicine, Clinical Genetics Unit at Karolinska Hospital. The cells were washed with PBS and then digested with Trypsin–EDTA ( Gibco/Invitrogen, USA) for 3 min. Chang-D-medium (Irvine Scientific, USA) was added, cell suspensions were pooled, the cell concentration was determined and the cells were plated in 17 mm culture wells (24 Well Cell Culture Cluster; Costar) and incubated for 24 h in humidified 5% CO2 and 95% air at 37°C. Samples of the culture media were then collected and IL-1 (1, 10 or 50 ng/ml) was added to the wells. After a further 72 h of incubation the culture media were collected and prepared for analysis of the ET-1 and M-CSF contents.

Statistical analysis

Means are given ± SD. Means between two groups were compared using Student’s t-test. Differences between more than two groups were analysed by analysis of variance using appropriate post-hoc tests. Regression analysis was performed using standard methods.
Results

The mean ± SD concentrations of ET-1 and M-CSF in fetal amniotic fluid in the 20 patients analysed were 45.6 ± 17.3 pmol/l (range 16.8–85.5) and 7323 ± 3415 ng/l (range 2640–12110) respectively. At term, birthweight for these fetuses was 2167 ± 524 g (range 1770–3550 g, n = 20), and placenta weight was 392 ± 99 g (range 200–530 g, n = 20). Weight deviation at delivery was −27 ± 12%, range 0 to −50%. Weight deviation at the time of amniocentesis was −29 ± 6%, range −22 to −30%. Clinical data for the patients are shown in Table I.

Further analysis showed that M-CSF in amniotic fluid correlated positively with pregnancy length (P < 0.05, R² = 0.1990, n = 20). Amniotic fluid ET-1 did not correlate with pregnancy length (P = 0.56, R² = 0.019, n = 20) (Figure 1a, b).

In order to display graphically the relationship between M-CSF/ET-1 and intrauterine fetal growth, we sorted the patients according to the weight deviation at delivery in three subgroups, designated group A: > −22%; group B: −22 to −33%; group C: < −33%. The distribution of individual M-CSF and ET-1 levels in these three subgroups is shown in Figure 2. For M-CSF, it is clearly seen that there is a wide range of M-CSF levels in the three groups, but that the average levels in group C, containing the most growth-retarded fetuses, appear lower than in group A (Figure 2), although there was no statistically significant difference.

We then analysed the cellular localization of M-CSF and ET-1 by double-labelling immunocytochemistry. We found that both substances were localized in amnion membrane cells; both in elongated, spindle-shaped cells present below the amniotic epithelial lining (Figure 3a), as well as in the rounded-cuboidal cells in the amnion epithelial lining (Figure 3b). Some cells contained both M-CSF and ET-1, some cells were only M-CSF positive, and some cells were only ET-1 positive. Both M-CSF and ET-1 had maximal immunostaining in the cytoplasm, with no or only weak staining in the nuclei and plasma membrane (Figure 3, lower panel). Also in underlying trophoblast layers, there were some cells immunostained with M-CSF and ET-1 (not shown).
In order to test whether M-CSF and ET-1 in amniocytes could be synthesized and released together in response to inflammatory agents, we performed experiments using cultured amniocytes, using IL-1α as an agonist. We found that incubation of cultured amniocytes with IL-1α (1 ng/ml-50 ng/ml) resulted in a dose-dependent release of both M-CSF and ET-1 (Figure 4).

**Discussion**

These results clearly demonstrate that ET-1 and M-CSF are localized to specific cells in human amniotic membrane, and that both may be secreted into the fetal amniotic fluid. The immunohistochemical results show that there is a partial overlap of M-CSF and ET-1 staining in some amniocytes, indicating co-localization of both substances in some amniocytes. Previous results with immunohistochemical staining indicated that production of M-CSF as well as another growth factor, granulocyte-colony stimulating factor (G-CSF) was localized in the epithelial cells of fetal membrane (Saito et al., 1992). Similarly, previous results with ET-1 have shown immunostaining of endothelial cells in all placental villous vessels and in the fetal membranes. Also, intensive immunopositive staining was observed in the chorionic trophoblast following vaginal deliveries in term and preterm tissues. This suggested that amnion and trophoblast represents a source of ET-1 production or, alternatively, a site for ET-1 binding (Salamonsen et al., 1992; Marinoni et al., 1995). The present results show for the first time that M-CSF and ET-1 are partially co-localized in human amniotic membrane cells.

The average level of M-CSF in fetal amniotic fluid was 7323 ± 3415 ng/l (range 2640–12110). This is lower than the previously published levels of 17 300 ± 8500 ng/l (Saito et al., 1992), but in agreement with earlier data (Ringler et al., 1989) which reported 9000 ng/l in amniotic fluid obtained from pregnancies at 33–40 weeks gestation. This study also noted a 2-fold rise from levels in early pregnancy, weeks 16–18. Our levels also compare well with recent data of Murakawa (1998), who found levels of 7400 ± 1600 ng/l in a normal pregnancy group, and 5000 ± 1400 ng/l in a IUGR-complicated pregnancy group. In agreement with our results, the latter study showed lower levels of M-CSF in amniotic fluid from pregnancies complicated by IUGR and suggested that amniotic fluid M-CSF was one of the regulators of human placental development and was related to fetal growth (Murakawa, 1998). A very recent study has analysed maternal serum levels of M-CSF in IUGR pregnancies, and found that serum levels of M-CSF in week 36 were elevated in IUGR pregnancies compared with normal pregnancies (Hayashi, 2002). Since production and clearance of M-CSF is different in blood compared with amniotic fluid, the observed differences may not be inconsistent (see Hayashi, 2002).

The levels of ET-1 in amniotic fluid were 45.6 ± 17.3 pmol/l (range 16.8–85.5). These values also are comparable to previously published levels of ET-1 levels in amniotic fluid. For example, Bracero et al. (1995) found high levels in the amniotic fluid (33.3 ± 6 pmol/l) and compared this to the low levels in the maternal plasma (0.36 ± 0.08 pmol/l). These authors speculated that if elevated plasma ET-1 levels reflect increased bioactivity, the higher mean ET-1 levels in the cord vessels and in the amniotic fluid compared with maternal levels would suggest a role for ET-1 in the regulation of the feto-placental circulation and in the constriction of blood vessels in the uterus after parturition (Bracero et al., 1995). A detailed analysis of ET-1 levels in amniotic fluid found that the levels of ET-1 in amniotic fluid during the midtrimester of human pregnancy was higher at term (93.3 ± 7.4 pmol/l) than before the onset of labour (39.8 ± 4.1 pmol/l) (Casey et al., 1993). Furthermore, during labour, the levels in amniotic fluid...
were higher in the compartment between the fetal head and the cervix (the forebag) than in the upper compartment, indicating stimulation of ET-1 formation in the forebag possibly by inflammatory response mediators (Casey et al., 1993).

M-CSF in amniotic fluid has previously been suggested to regulate fetal growth (Tuo et al., 1995). In view of the partial co-localization of M-CSF and ET-1 in amniotic membrane cells, we hypothesized that both might be correlated to fetal growth and to birthweight. We found a correlation between M-CSF and pregnancy length, such that M-CSF levels increased with increasing pregnancy length. ET-1 levels, on the other hand, appeared to decrease with increasing pregnancy length, although not significantly in our patient material. These results present the possibility that fetal growth may be related to amniotic fluid levels of M-CSF, or vice versa. The mechanisms determining how substances present in amniotic fluid might affect the fetus are most likely to operate through effects on placental function. M-CSF has previously been shown to affect placental function (Arceci et al., 1989).

Since both substances were co-localized to some amniotic membrane cells, but not all, we hypothesized that some parameter other than fetal growth could contribute to the variable co-distribution of the two substances in amnion. We therefore looked for humoral factors that might regulate both M-CSF and ET-1 synthesis and release from amnioncytes. Inflammatory agents such as interleukins have previously been shown to up-regulate M-CSF as well as ET-1 in various systems (Mitchell et al., 1991; Garcia-Lloret et al., 1994; Sagawa, 1994). Therefore we analysed the effect of IL-1α on the levels of M-CSF and ET-1 in cultured amnioncytes. We found that both substances were released, with similar release patterns, in response to addition of IL-1α (1–50 ng/ml). There was an initial increase of both ET-1 and M-CSF, followed by a plateau phase and a slow decrease. The reason for this profile is unclear. We speculate that the pattern may be a result of several factors modulating the initial release, i.e. feedback inhibition of production, degradation of products and/or changes in turnover machinery. The finding of release of both ET-1 and M-CSF from amnioncytes in vitro indicates a strong likelihood of a release of both substances from amnioncytes also in vivo, where they would be secreted into the amniotic fluid.

In conclusion, the present experiments have shown that the presence of the M-CSF and endothelin in amniotic fluid may be due to the release of the two substances from amnioncytes, where they are partially co-localized. Pregnancy length appears to be significant for the levels of M-CSF, but not ET-1 in amniotic fluid. IL-1α appears to regulate release of both M-CSF and ET-1 from amnioncytes. The results suggest that M-CSF in amniotic fluid may be an interesting candidate for further evaluation as an adjunct indicator for the assessment of fetal growth from the perspective of placental development and IUGR.

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References


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