Differentiation-Dependent Changes in Human Trophoblast Expression of Decay-Accelerating Factor Are Modulated by 3′,5′ Cyclic Adenosine Monophosphate

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OBJECTIVE: *Decay-accelerating factor (DAF) is a complement regulatory protein that prevents complement-mediated cell lysis. Its expression in human trophoblasts depends on their anatomic location and gestational age. The mechanisms that govern the regulation of its expression in trophoblasts are not understood. The objective of the present study was to investigate the effects of trophoblast differentiation and cyclic adenosine monophosphate (cAMP) on DAF expression.*

METHODS: *Human trophoblasts were isolated from chorionic villi, cultured, and harvested at timed intervals for total RNA extraction and Northern analysis. Expression of DAF was also assessed by immunocytochemistry. In some cultures, the trophoblasts were exposed to the cAMP agonists 8-bromo-cAMP and Sp-cAMPs or the antagonist Rp-cAMPs.*

RESULTS: *Expression of DAF mRNA increased as the cells differentiated in culture, with the 2.2-kb transcript of membrane-bound DAF appearing first. As differentiation proceeded, the 1.5-kb transcript became the predominant mRNA form. Exposure of the cells to Rp-cAMPs delayed this process; 8-bromo-cAMP accelerated it. Sp-cAMPs selectively up-regulated the 2.2-kb mRNA transcript. Immunocytochemistry confirmed the effects of differentiation on DAF protein expression.*

CONCLUSION: *Expression of DAF in human trophoblasts is dependent on the state of cell differentiation, and cAMP is an intracellular modulator of this process. This effect may be mediated through alternative processing of DAF mRNA in its 3′UT region, which in turn affects mRNA stability.* (J Soc Gynecol Invest 1997;4:47–53. Copyright © 1997 by the Society for Gynecologic Investigation.)

KEY WORDS: Cell lysis, complement, cyclic AMP, differentiation, RNA splicing.

The human conceptus is in proximate contact with maternal tissue and/or maternal blood from the time of implantation until the end of pregnancy. The trophectoderm of the blastocyst, which initiates contact with the maternal endometrium, consists of trophoblasts, the cells that form the fetomaternal interface throughout pregnancy.[1] Because trophoblastic tissue, like the entire conceptus, is semiallogenic to maternal tissues, it is potentially susceptible to hostile maternal immune response mechanisms. However, such adverse reactions are extremely rare. This is all the more remarkable because maternal antibodies to trophoblast are abundant in maternal blood and in the placenta.[2,3] It thus appears that trophoblasts possess mechanisms for avoiding rejection mediated by maternal humoral or cell-mediated immunologic mechanisms. Cytotoxic effects associated with the immune response are generally complement mediated. One mechanism by which the fetal allograft is protected from maternal immunologic attack is expression of specific proteins that regulate complement activation.[4,5]

Decay-accelerating factor (DAF) is one of the complement regulatory proteins known to be expressed by trophoblasts.[3] Decay-accelerating factor (also known as CD55) is a glycoprotein that exists in both membrane-bound (hydrophobic) and secretory (hydrophilic) forms, which are translated from two alternatively spliced classes of DAF mRNA.[6] Through its interaction with complement factor 3 (C3) convertases, it prevents complement assembly and accelerates the decay of both

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the classic and alternative pathways of complement activation, thereby preventing complement-mediated cell lysis.[7] There is direct evidence that DAF protects human trophoblasts from complement-mediated attack,[8] and it probably acts in concert with membrane cofactor protein (MCP, CD46) and protectin (CD59) for this purpose.[9] The three transcripts of DAF mRNA that are translated into the membrane-bound and soluble forms of the protein have been shown to be expressed in human trophoblasts obtained from the chorion laeve at term.[3] Although evidence from immunocytochemical studies suggests that DAF is present in both first-trimester and term placenta and that its distribution in trophoblasts may depend on the anatomic location and state of differentiation,[4,10,11] the mechanisms by which its expression is regulated in these cells are not yet characterized.

It is now well established that when mononuclear cytotrophoblasts are isolated from human chorionic villi and placed in culture, they differentiate in a fashion parallel to the morphogenetic process of trophoblast differentiation in vivo.[12–14] We therefore sought to apply this model to investigate the effects of differentiation on DAF expression in trophoblasts. Because a 3′,5′cyclic adenosine monophosphate (cAMP) response element has been found in the 5′-flanking region of the human DAF gene[15] and it has been clearly established that aggregation and differentiation of trophoblasts in culture are associated with a marked increase in cAMP within the cells,[16] we explored the effects of cAMP agonists and antagonists on trophoblast DAF expression to ascertain whether cAMP is a mediator of the process.

MATERIALS AND METHODS

Tissue Preparation and Cell Culture

Utilization of human tissue, including placentas, for our ongoing studies has been approved by the Institutional Review Board in our university. All human tissue used in this study was "residual" or excess tissue that was not required for histopathologic diagnosis. Term placentas were used because DAF expression has been shown to be more intense in the third trimester than earlier in gestation.[3]

Cytotrophoblasts were prepared from term human placentas as described previously.[12] Briefly, chorionic villi were minced and then digested with trypsin and deoxyribonuclease in calcium- and magnesium-free Hank's balanced salt solution (GIBCO-BRL, Grand Island, NY), followed by centrifugation on a 5–70% Percoll gradient. Cells banding at a density of 1048–1065 g/mL represents a highly enriched (up to 95% pure) and viable preparation of cytotrophoblasts. These cells were collected and cultured in Dulbecco's modified Eagle's medium containing 25 mmol/L glucose and 25 mmol/L HEPES (GIBCO BRL), supplemented with 10% heat-inactivated fetal calf serum and 50 μg/mL gentamicin, at 37C in an atmosphere containing 5% carbon dioxide. In some experiments, we added 1.5 mmol/L 8-bromo-cAMP (Sigma, St. Louis, MO), 100 μmol/L Rp-cAMP triethylamine salt (specific membrane-permeable inhibitor of activation of protein kinase I and II by cAMP; Research Biochemicals International, Natick, MA), or 100 μmol/L Sp-cAMP triethylamine salt (membrane-permeable activator of cAMP-dependent protein kinase I and II; Research Biochemicals International) to the culture media from the time of initial planting of the cells.

Cells were seeded onto glass coverslips (2 × 2 cm) and placed in six-well plates (Nunclon, Delta, Copenhagen, Denmark) at a density of 1.25×10^5 cells/cm^2 for indirect immunofluorescence studies. Cytokeratin staining confirmed that macrophage contamination of the trophoblast preparation was less than 5%. For total RNA preparation, cells were cultured in 100-mm culture dishes (Nunclon Delta) at a density similar to that used for the coverslips. The experiments were all done in triplicate.

Immunohistochemistry

For indirect immunofluorescence, cells grown on coverslips were washed three times in phosphate-buffered saline (PBS) and fixed for 15 minutes at room temperature in Bouin's solution. Fixed cells were washed in PBS and incubated in 20% normal goat serum for 30 minutes at room temperature. The cells were incubated for 2 hours with mouse monoclonal anti-human DAF IgG at a concentration of 4 μg/mL at room temperature. This antibody has been characterized previously.[17] Cells were then washed thoroughly with PBS-0.4% bovine serum albumin and incubated with fluorescein-conjugated anti-mouse IgG (Jackson Immunoreserch Laboratories Inc., West Grove, PA) at a concentration of 12 μg/mL for 30 minutes. Coverslips were mounted on glass slides with fluoromount (Southern Biotechnology Associates, Birmingham, AL) containing DABCO (Polysciences Inc., Warrington, PA) to prevent rapid quenching of fluorescence. Cells were examined and photographed in a Nikon Microphot microscope (Tokyo, Japan).

RNA Isolation

Total RNA was extracted from mononuclear cytotrophoblast cells after Percoll gradient isolation and at appropriate time intervals during primary cell culture using the acid guanidine thiocyanate phenol chloroform method.[18] Concentrations of RNA were determined by spectrophotometry at 260 nm. The RNA samples were stored at −70C until use.

Preparation of cDNA Probes

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR). Total RNA was isolated from term human placentas as described above after pulverizing the tissue in a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). Using 1 μg of placental total RNA, reverse transcription was performed with murine reverse transcriptase (GIBCO BRL), and an aliquot of the RT product was amplified by PCR. Reactions were performed using recombinant *Taq* polymerase and two PCR primers that were designed according to the published human DAF cDNA sequence.[6,19] These 20-mer oligonucleotides were 5′-GCGCAGTCAATGGTCAGATA-3′ (sense) and 5′-CCACTCCACTCTCCTTCATC-3′ (antisense), comple-

mentary to positions 314–333 and 879–898, respectively. After completion of PCR, the product was subjected to electrophoresis in an agarose gel. Fragments of the expected length of 584 base pairs were purified, subcloned into the PCR-TMII vector (Invitrogen, San Diego, CA), and sequenced to confirm their identity.

LABELING OF PROBE FOR NORTHERN ANALYSIS. The purified cDNA served as the template for synthesis of labeled DNA using random priming[18] with [α^{32}P]-dCTP (NEN, Boston, MA), a mixture of the other deoxynucleoside triphosphates, and Klenow enzyme (Random Primed DNA Labeling Kit; Boehringer Mannheim Biochemicals, Indianapolis, IN) to a specific activity of 1×10^6 to 1×10^7 cpm per ng probe. We also used a 1.2-kb cDNA encoding the 28S rRNA subunit or a 300-bp cDNA encoding the 18S rRNA to prepare probes for normalization of the blots.

Northern Analysis

Denatured total RNA (20 μg) was separated by size electrophoretically in a formaldehyde agarose denaturing gel, transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) by capillary action using standard saline citrate (SSC) solution, and cross-linked to the membrane by ultraviolet radiation (Stratalinker 1800, Stratagene, La Jolla, CA) using 12×10^4 μjoules radiation. The membrane was prehybridized with 50% (v/v) formamide, standard saline phosphate in ethylenediamine tetra-acetate buffer, Denhardt's solution, and 0.1% sodium dodecyl sulfate (SDS) at 42C for 2 hours. Hybridization was with 2×10^6 cpm of denatured probe per mL hybridization solution for 18 hours at 42C. The blots were washed by four 15-minute incubations at 55C with SSC (containing 0.1% SDS). Autoradiography was done using Kodak XAR film for 1–72 hours at −70C until the desired exposure was obtained. The intensity of the autoradiographic images of DAF mRNA relative to those of 28S rRNA was determined by scanning densitometry using a desktop scanner and the ImageQuant computer program (Molecular Dynamics, Kemsing, UK).

RESULTS

DAF mRNA Expression Is Dependent on Trophoblast Differentiation

Freshly isolated cytotrophoblasts expressed very little DAF mRNA, and only the variant that codes for the membrane-bound DAF seems to be expressed in these undifferentiated cells. This variant of DAF mRNA displays heterogeneity at its 3′ end, resulting in two alternatively spliced 3′ noncoding regions that differ in size by 788 bp, and that migrate on Northern analysis as two separate bands of 1.5 and 2.2 kb.[6] Both of these bands were present in Northern blots from the trophoblasts (Figure 1A). As the cells aggregated in culture, there was a dramatic increase in the intensity of expression of this membrane-bound DAF mRNA, with the 2.2-kb variant, the initially prevalent form, undergoing a fourfold increase in expression as assessed by densitometry (Figure 1B). As the cells continued to aggregate, the abundance of the 1.5-kb transcript soon matched that of the 2.2-kb form and eventually became the predominant form expressed. As its peak, there was an approximately eightfold increase in the expression of the 1.5-kb mRNA transcript. The unspliced 1.6-kb mRNA, which codes for the soluble secretory form of DAF, was undetectable in freshly isolated trophoblasts. It was expressed at a much lower level of intensity than the mRNA for the membrane-bound protein even in the differentiated cells, constituting about 12% of all DAF mRNA (as assessed by densitometry) at the peak of its expression. It is often difficult to distinguish the 1.6-kb band from the 1.5-kb band because of their closeness on the Northern blot; but, with densitometric scanning, its expression could be seen to increase as the cells differentiated in culture. Its expression later declined slightly as the process of differentiation continued (Figure 1B).

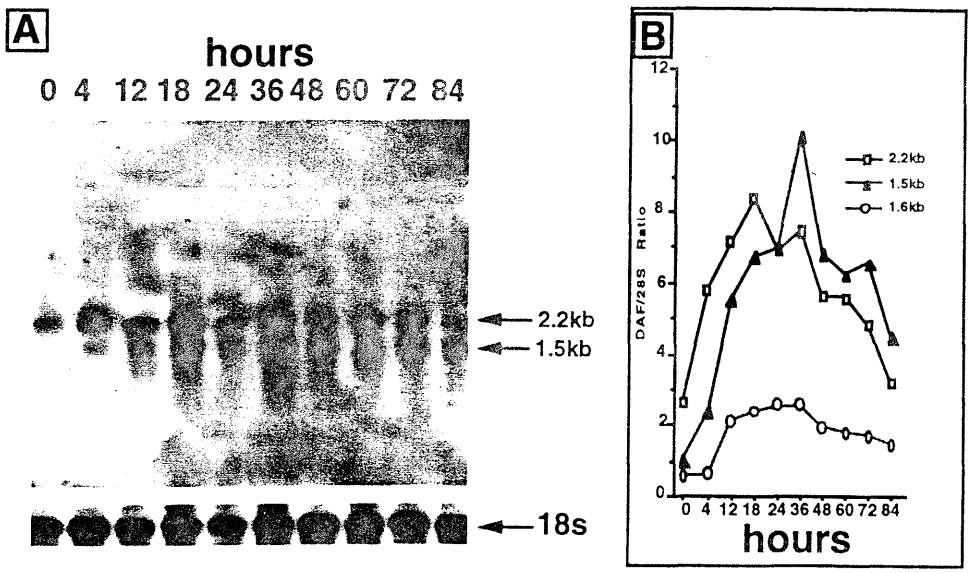

Figure 1. Northern blot analysis of DAF during the differentiation of human trophoblasts in vitro. Total RNA extracted at the time of trophoblast isolation (0 hours) and at time points up to 84 hours in culture was processed as described in Materials and Methods. An 18S ribosomal RNA probe was used for normalization of total RNA loading. B) Mean values from the quantitative analysis of densitometric ratios of the three transcripts of DAF mRNA to 18S from three experiments. Both the 1.5-kb and 2.2-kb transcripts code for the membrane-bound DAF protein. The 1.6-kb transcript is difficult to distinguish visually from the 1.5-kb transcript because of their proximity.

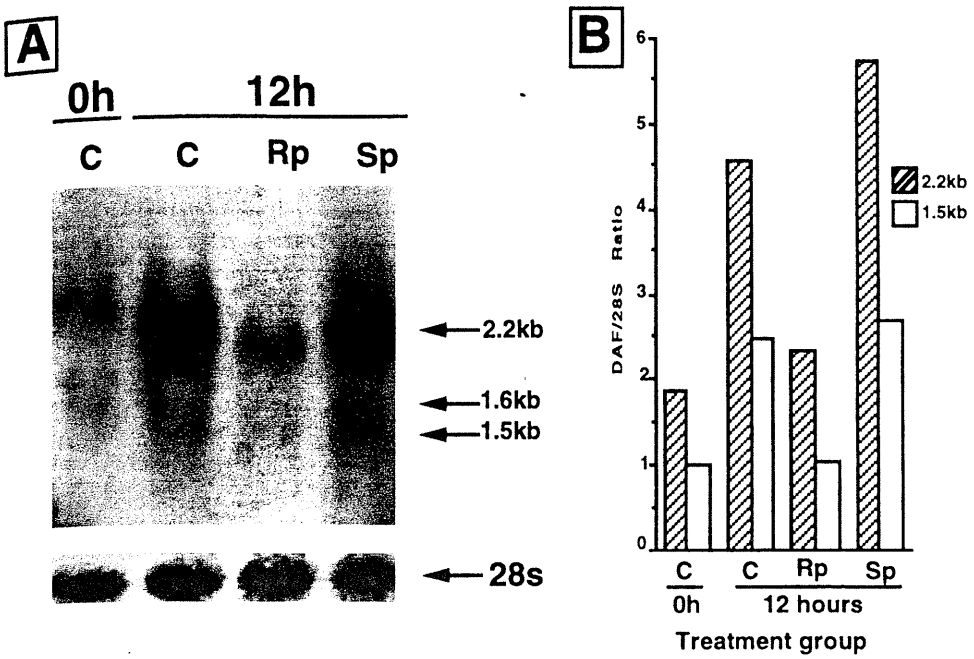

Figure 2. Effects of the cAMP antagonist (Rp) and agonist (Sp) on DAF mRNA expression in trophoblasts. Total RNA was extracted at the time of isolation and at 12 hours of culture in the absence (C) or presence of cAMP antagonist (Rp) or agonist (Sp) for Northern analysis. Normalization of RNA for densitometry was achieved by probing the blot for 28S ribosomal RNA. Note that up to 12 hours of culture, the antagonist Rp-cAMPs blocked the increase in DAF mRNA expression for both the 1.5-kb and 2.2-kb transcripts.

cAMP Regulation of DAF mRNA Expression

A cAMP response element has been found in the 5′-flanking region of the DAF gene.[15] Many of the cell differentiation-dependent endocrine functions of human trophoblasts have been shown to be regulated by cAMP.[20] Moreover, in vitro studies have suggested that intracellular cAMP increases as cytotrophoblasts undergo differentiation.[16] Hence, we investigated the possibility that cAMP was a mediator of the differentiation-related changes in DAF expression. Exposure of the cells to the cAMP antagonist Rp-cAMP in the early stages of trophoblast aggregation inhibited the up-regulation observed in DAF mRNA expression as the cells differentiated in culture (Figure 2A), suggesting that endogenous cAMP regulated the observed increase in DAF mRNA in untreated cells. As the cells continued the process of differentiation, this effect of Rp-cAMPs became less noticeable, presumably because an increasing endogenous cAMP concentration overrode the effect of Rp-cAMPs as they competed for the same sites on protein kinase A. Thus, at steady state, Rp-cAMPs maintained DAF expression at approximately the same level as that of untreated cells (Figure 3A). Remarkably, exposure of the cells to two different cAMP agonists led to widely divergent effects

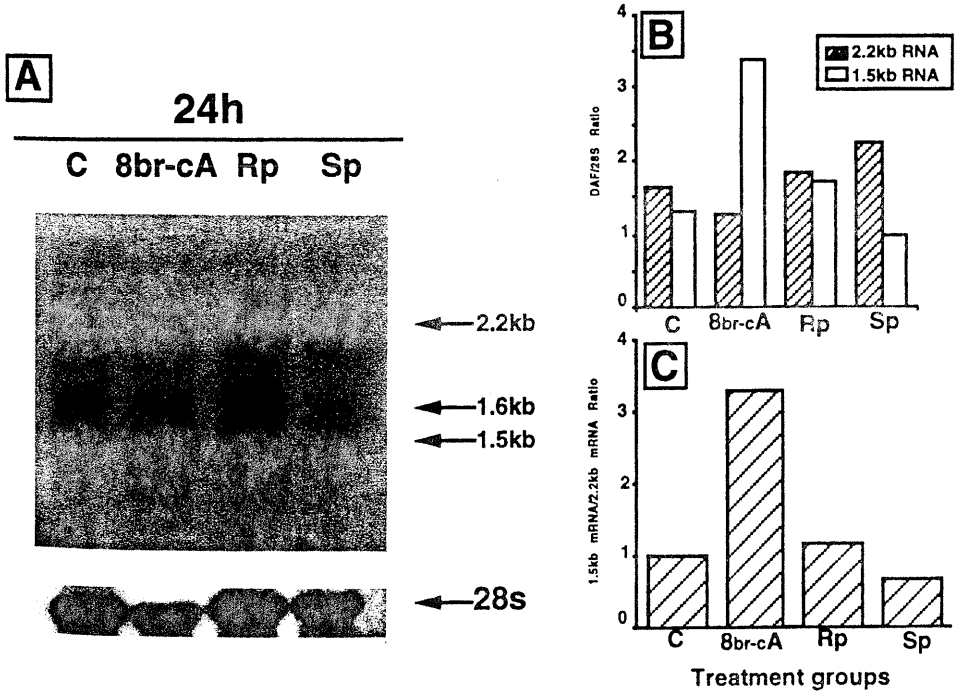

Figure 3. Differential effects of different cAMP analogues on DAF mRNA expression in human trophoblasts. Cells were cultured for 24 hours in the absence (C) or presence of the cAMP agonists (8br-cA and Sp) and the antagonist (Rp), and were processed for total RNA extraction as described in Materials and Methods. Note that 8-bromo-cAMP selectively up-regulated the 1.5-kb transcript, whereas Sp-cAMPs selectively inhibited the same. This is quantitatively demonstrated by the ratios of the 1.5/2.2-kb transcripts (C).

on membrane-bound DAF mRNA expression (Figure 3A). Addition of 8-bromo-cAMP led to selective up-regulation of the 1.5-kb mRNA transcript, while the 2.2-kb transcript was relatively unaffected. Conversely, exposure of the cells to another cAMP agonist, Sp-cAMPs, produced selective inhibition of the 1.5-kb species of mRNA, with the 2.2-kb species being slightly up-regulated. Thus, the ratio of 1.5-kb mRNA to 2.2-kb mRNA was much higher in the cells exposed to 8-bromo-cAMP (Figure 3C).

Immunocytochemical Localization of DAF in Human Trophoblasts

Freshly isolated cytotrophoblasts showed no immunoreactivity to DAF (Figure 4A). With differentiation over time, the cells became immunoreactive, showing particularly strong positive staining for the protein in the cell membranes of the trophoblasts (Figure 4B, 4C). The temporal pattern of immunoreactivity in the cells closely paralleled that of DAF mRNA expression. Treatment with 8-bromo-cAMP was not associated with any specific changes in the pattern of immunolocalization of DAF (Figure 4D). Where the membranes had fused in the process of syncytialization, there was no DAF immunoreactivity.

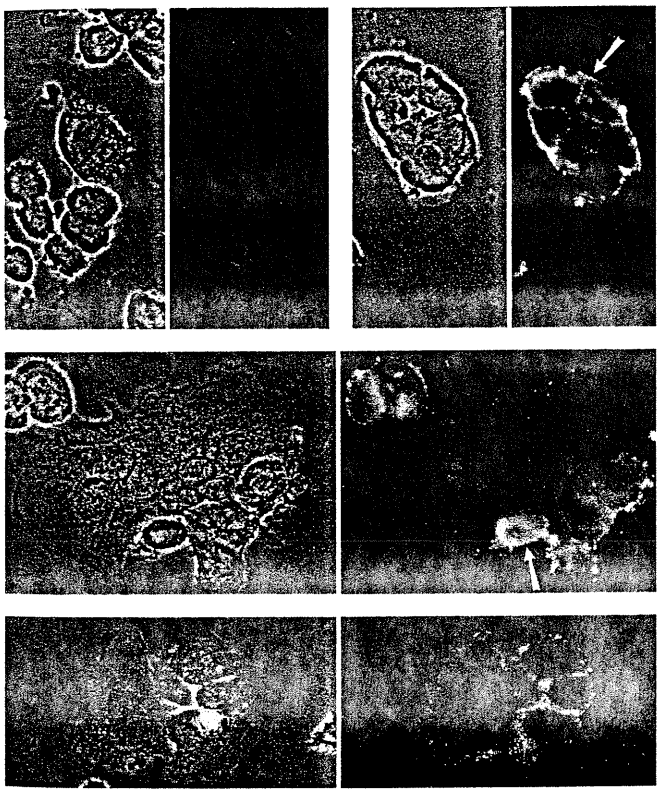

Figure 4. Immunocytochemical localization of DAF in human trophoblasts. Cytotrophoblasts isolated from term placentas were cultured and fixed shortly after isolation (A), at 12 hours (B), and at 24 hours (C). Some trophoblasts were treated with 8-bromo-cAMP and fixed at 24 hours (D). All were stained with a specific DAF antibody, as described in the text. Note that as the cells differentiated, immunoreactive DAF is seen on the cell membrane (B). With syncytialization, immunoreactivity was lost as the membranes fused, but was still present in the mononuclear cells at the edges of the syncytium (*arrow* in C).

DISCUSSION

The results of this study clearly demonstrate that the expression of DAF in trophoblasts is regulated by the state of differentiation of the cells. It has been demonstrated earlier, by immunohistochemistry, immunoblotting, and radioimmunoassay, that DAF protein expression in human chorionic tissue increases multiple-fold between the first trimester and term and that DAF is expressed primarily in trophoblasts that are in contact with maternal tissue or blood.[3] It has been shown that C3 is expressed in the human endometrial epithelium only during the luteal phase of the menstrual cycle.[21] Taken together, these findings suggest that DAF may play a role in the process of placentation, and its regulation may be critical for the successful establishment of pregnancy.

Our data demonstrate that cell differentiation affects the relative abundance of the two transcripts (1.5 kb and 2.2 kb) of membrane-bound DAF mRNA in trophoblasts. Both transcripts code for the same protein and differ only in the lengths of their 3′ untranslated (UT) regions, with the longer transcript having four polyadenylation signals in this segment, compared with two in the shorter one.[6] Regulation of such alternative processing of mRNA is poorly understood, but these alternative sequences may arise from differential utilization of promoters[22] or from the activity of splicing factors.[23] The process of trophoblast differentiation may be associated with changes in the activity of such factors, leading to the observed differences in the pattern of expression of the two species of membrane-bond DAF mRNA. In this study, investigation of secretory DAF, encoded by the 1.6-kb mRNA transcript, did not show substantial regulation.

It has been demonstrated that the concentration of endogenous cAMP rises in trophoblasts as they aggregate and fuse in culture and that cAMP is a second messenger in many of the key processes during trophoblast differentiation.[16] The effects of cAMP analogues on DAF expression in this study also indicate that endogenous cAMP is an important mediator of DAF mRNA transcription. The presence of Rp-cAMPs, a competitive inhibitor of cAMP binding to protein kinase A (PKA), delayed the onset of DAF mRNA expression in the cells as they differentiated in culture. The effects of the agonist 8-bromo-cAMP on DAF mRNA expression resemble what is observed in the later stages of differentiation in untreated trophoblasts. When considered in conjunction with the effects of Rp-cAMPs on DAF expression , this provides strong evidence that endogenous cAMP modulates trophoblast DAF expression through its action on PKA. It is important to stress that the differentiation-dependent regulation and the cAMP effect described in this report involve the differential expression of the two mRNA transcripts encoding the membrane-bound form of DAF, and not the 1.6-kb transcript for secretory DAF.

One intriguing observation was the divergent effects of the two cAMP agonists used in these experiments on the expression of membrane-bound DAF mRNA. Exposure of the cells

to 8-bromo-cAMP appeared to channel the cells to favor expressing the mRNA with the shorter 3′UT region, whereas Sp-cAMPs preferentially up-regulated the mRNA with multiple polyadenylation signals in its 3′UT region, although they are both activators of PKA. One possibility is that these effects are mediated through different isoforms of PKA, which in turn exert their effects via different transcription factors on different response elements in the 5′-flanking region of the DAF gene. Such a postulated mechanism would regulate DAF mRNA transcription selectively either toward the 1.5-kb or the 2.2-kb species. In this regard, it has been established that the 5′-flanking region has several promoter elements that are susceptible to the action of PKA, including consensus sequences for a cAMP responsive element, and activator protein (AP)-1 and AP-2 sites.[15] Another plausible explanation for the differences in the responses to the two cAMP agonists may be the differences in their susceptibility to cyclic nucleotide-dependent phosphodiesterae (PDE) degradation. The Sp-cAMPs agonist is not as susceptible to PDE degradation as is 8-bromo-cAMP.[24] Additional studies are needed to broaden our understanding about the differences between the effects of these two cAMP agonists.

The effect of the heterogeneity within the 3′UT region of the membrane-bound DAF transcripts on trophoblast DAF production or function is unclear. Similar alternative mRNA processing occurs with many genes, often, but not exclusively, in association with differences in the coding regions.[22] These 3′UT sequences are known to be involved in the regulation of translational efficiency[25] and mRNA stability.[26] Hence, differences in these UT regions can be used in feedback suppression of translation or selective spatial targeting of a protein.[27] One example that is similar to DAF (in that it produces several transcripts, including two that are identical in the coding region but different in the 3′UT region) and that has been well characterized is the human macrophage-colony stimulating factor (M-CSF) gene. In one of these two M-CSF transcripts, the 3′UT region has a repetitive element that is associated with selective mRNA degradation, suggesting that M-CSF expression can be regulated at the level of mRNA turnover by selection of different 3′UT sequences.[28] Some of the putative polyadenylation sites in the 3′UT region of DAF mRNA are flanked upstream by AU-rich regions that regulate mRNA stability,[29] and it is possible that the differences observed in this region of the 1.5-kb and 2.2-kb mRNAs exist for fine-tuning the expression of the mRNA and DAF protein. It is interesting to note that in the later stages of trophoblast differentiation when the 1.5-kb mRNA becomes the predominant form, total DAF mRNA expression starts to decline. It is conceivable that this occurs because this shorter transcript is less stable than the longer one.

Immunocytochemical observations demonstrated that the pattern of DAF protein expression in human trophoblasts closely parallels that of DAF mRNA, showing that DAF gene expression is primarily under transcriptional control. This finding is consistent with what has been reported in many cell types.[15] Our findings show that the state of trophoblast differentiation influences DAF expression, and cAMP is an important mediator of this process. Our observations suggest that cAMP exerts this effect by selectively channeling DAF mRNA processing toward expression of the transcript with the shorter 3′UT region, which may be a less stable form of mRNA. Such an intricate scheme of regulation is additional evidence that DAF expression provides an important mechanism for protection of trophoblasts from maternal complement during pregnancy.

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