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Characterization of the Endocannabinoid System in Early Human Pregnancy

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In recent years, it has been demonstrated that high circulating levels of the endogenous cannabinoid anandamide, resulting from low expression of its metabolizing enzyme fatty acid amide hydrolase (FAAH), may contribute to spontaneous miscarriage and poor outcome in women undergoing *in vitro* fertilization. The site of action of this compound, however, has not been determined. In this study, we examined the distribution of the cannabinoid receptors, CB1 and CB2, and the endocannabinoid-metabolizing enzyme FAAH in first trimester human placenta. Here, we show that FAAH is expressed throughout the human first trimester placenta, in extravillous trophoblast columns, villous cytotrophoblasts, syncytiotrophoblasts, and macrophages. Furthermore, FAAH mRNA levels appear to be regulated during gestation, with levels peaking at 11 wk before declining again. The immune system-associated cannabinoid CB2 receptors were localized only to placental macrophages. Interestingly, the cannabinoid receptor CB1 was not identified in first trimester placenta despite having previously been shown to be present in placental tissues at term. These findings suggest that the placenta may form a barrier preventing maternal-fetal transfer of anandamide and/or modulate local levels of anandamide by regulation of FAAH expression with gestation. (*J Clin Endocrinol Metab* 89: 5168–5174, 2004)

ANANDAMIDE IS AN endogenous cannabinoid that is believed to have a role in reproductive processes (1). It produces its effects by acting on the G protein-coupled CB1 and CB2 cannabinoid receptors. Termination of the signal is thought to be achieved by uptake of anandamide into the cell, followed by rapid degradation by the enzyme fatty acid amide hydrolyase (FAAH). There appears to be a link between FAAH activity and early pregnancy success; for example, a strong correlation between decreased circulating FAAH in maternal peripheral blood mononuclear cells and spontaneous miscarriage in women has been reported (2). Similarly, the circulating level and activity of FAAH are significantly lower in patients undergoing *in vitro* fertilization-embryo transfer who fail to achieve an ongoing pregnancy than in those who become pregnant (3). This decrease in circulating FAAH expression was paralleled by increased anandamide levels, suggesting that FAAH expression is important in the control of circulating anandamide concentrations (3).

These clinical studies are supported by experimental models suggesting that low FAAH activity and subsequently increased anandamide levels are detrimental in early pregnancy. Thus, it has been demonstrated that anandamide levels in the mouse uterus are inversely related to uterine receptivity for implantation, with elevated anandamide and low FAAH levels correlating with uterine refractoriness to blastocyst implantation (4, 5). Furthermore, FAAH levels are highest and anandamide levels are lowest at implantation sites (6).

In light of the above observations, we hypothesized that anandamide may have important local actions on the developing human placenta. We recently demonstrated the localization of cannabinoid CB1 receptors and FAAH immunohistochemically in human term placenta and gestational membranes (7). The CB1 receptor was found in all layers of the gestational membrane, with particularly strong expression in amniotic epithelium and reticular cells and in cells of the maternally derived decidua. Moderate CB1 expression was observed in chorionic cytotrophoblasts. The expression of FAAH was high in amniotic epithelial cells and trophoblasts of the chorion and maternal decidua. This finding agreed with previous studies that used RT-PCR to identify the presence of CB1 receptors and FAAH in human term placenta (8). CB1 receptors have also been identified in human myometrium at term (9). However, the expression of these proteins in the developing placenta has not yet been elucidated. In this study we have examined the expression of CB1 and CB2 receptors and FAAH in human first trimester placental tissues by RT-PCR and immunohistochemistry. We have also quantified the expression of FAAH in first trimester tissues by real-time PCR.

Materials and Methods

Tissue collection

This study was approved by the regional ethics committee. First trimester placentas were collected from elective termination of normal pregnancies with informed signed consent. Tissue was collected from 16 placentas between 9 wk, 3 d and 13 wk, 2 d gestation, as determined by

Abbreviations: 2AG, 2-Arachidonyl glycerol; FAAH, fatty acid amide hydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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ultrasound. Placental tissue was frozen in isopentane on dry-ice in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and stored at −80 C until required.

Because the CB1 receptor and FAAH localization have been characterized in brain and term placenta, sections from adult rat brain and human term placenta were used as positive controls for immunohistochemistry. Rat brains were donated by Dr. Jian Guan (Liggins Institute). Brains were snap-frozen as for placenta and stored at −80 C before sectioning. Term placenta sections were collected as previously described (7).

Seven-micrometer cryostat sections were cut and mounted onto polysine-coated microscope slides (VWR International, Dorset, UK) and air-dried. Sections were fixed for 10 min in cold acetone, air-dried, and stored at −20 C until required.

RT-PCR for FAAH and CB1 and CB2 receptors

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. After isolation, the RNA was dissolved in nuclease-free water (Zaxis Inc., Hudson, OH) and stored at −80 C.

Extracted RNA was treated with ribonuclease-free deoxyribonuclease I (Invitrogen Life Technologies) to remove residual genomic DNA. Deoxyribonuclease-treated RNA (1.0 μg) was reverse transcribed with 0.5 μg oligo(deoxythymidine)$_{12–18}$ primer using a SuperScript II kit (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA samples with no reverse transcriptase enzyme were used as controls to confirm the absence of genomic DNA contamination in the samples. The RT products (cDNA) were amplified by PCR. Paired oligonucleotides (Invitrogen Life Technologies), for amplification of human FAAH, CB1, and CB2, were designed using Oligo 4 primer-design software (National Biosciences, Plymouth, MN). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a housekeeping marker in parallel tubes using specific GAPDH primers (MWG-Biotech AG, Ebersberg, Germany). Intron-spanning primer pairs were designed for both GAPDH and FAAH; CB1 and CB2 receptors are intronless. The primer sequences and annealing temperatures used are listed in Table 1.

PCR was carried out with the following final concentrations: 1× PCR Gold Buffer (contains no $MgCl_2$; Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 200 μM deoxy-NTP, 0.2 μM forward primer, 0.2 μM reverse primer, 2 U/reaction of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1–4 ng/μl cDNA template. Nuclease-free water (Zaxis Inc.) was used to make the volume up to 50 μl. Aerosol-resistant tips were used, and blank control reactions containing no template were run to check that all reagents were clean. PCR conditions were one cycle of an initial denaturation step of 96 C for 5 min, followed by 45 cycles of 94 C (45 sec), 55.8–62.2 C (45 sec), and 72 C (90 sec), ending with one cycle of 72 C for 7 min. All PCR experiments were performed in an Eppendorf Mastercycler ep Gradient (Brinkmann Instruments, Inc., Westbury, NY). The PCR products were visualized on a UV transilluminator (UVP Inc., Upland, CA) after electrophoresis on 2% agarose gel and ethidium bromide staining. A 1-kb plus ladder (0.4 μg; Invitrogen Life Technologies) was run beside the PCR product to check the size of the band.

Sequencing

The PCR products were purified using the Concert Rapid Gel Extraction System (Invitrogen Life Technologies), according to the manufacturer's instructions, before sequencing (School of Biological Sciences Sequencing Unit, University of Auckland). The resulting sequence file was then Blast-searched to verify the sequence.

Quantitative real-time PCR

TaqMan probes and primers for FAAH (Hs00155015_m1) were designed by and obtained from Applied Biosystems as Assay on Demand Kits. TaqMan PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative expression of FAAH mRNA was normalized to the amount of 18S rRNA in the same sample as described by the manufacturer. A term placental sample was used as the positive control in these studies.

Antibodies and Western blotting

Primary antibodies to CB1, CB2, and FAAH were custom-manufactured by R&R Research & Development (Stanwood, WA). The CB1 antibody has previously been characterized (10). For the FAAH and CB2 antibodies, a glutathione-*S*-transferase fusion protein was generated by inserting the coding sequence for the terminal 103 amino acids of rat FAAH or the N-terminal 33 amino acids of human CB2 receptor into the pGEX-3X vector (Amersham Biosciences, Piscataway, NJ) and was used to generate polyclonal antiserum in rabbits by standard methods. Antibodies were enriched by affinity purification of the antiserum over a Sepharose resin bound with $(His)_6$ fusion proteins. The purified antibodies recognized recombinant FAAH or CB2 receptor expressed in HEK293 cells, but exhibited no detectable immunoreactivity to wild-type cells (data not shown) and recognized a single band by Western immunoblot.

Immunohistochemistry

All sections were incubated in a 50% methanol solution with 1% H_2O_2 to quench endogenous peroxidase activity. The CB1 receptor and FAAH antibodies were used at a dilution of 1:1000. The CB2 antibody was used at a dilution of 1:500. Sections were incubated with primary antibody overnight at 4 C, washed, and incubated with secondary antibody (biotinylated goat antirabbit IgG, Vector Laboratories, Inc., Burlingame, CA) at a 1:500 dilution for 3 h at room temperature, then with ExtrAvidin peroxidase conjugate (Sigma-Aldrich Corp., St. Louis, MO) at 1:1000 for 1 h at room temperature. All antibodies/conjugates were diluted in PBS containing 0.2% Triton-X-100 and 1% normal goat serum, and between each incubation, the sections were washed (three times, 10 min each time) in PBS containing 0.2% Triton-X-100. The sections were then reacted for up to 20 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp.) and 0.01% H_2O_2 in 0.1 M phosphate buffer (pH 7.4) to visualize the brown reaction product. Sections were washed in PBS, lightly counterstained in Mayer's hematoxylin for 10 sec, rinsed in tap water, dehydrated in graded alcohol to xylene, and cov-

TABLE 1. Primer sequences

Product (size)	Annealing temperature (C)		Primer sequence (5′–3′)
GAPDH (473 bp)	56	Forward	TGGAAGTCCTCCAAAAGCCCAG
		Reverse	TGTCCATAGACACAGCCCTTCAG
CB1 (1.5 kb)	60	Forward	ATGAAGTCGATCCTAGATGGCCTTGCAGA
		Reverse	TCACAGAGCCTCGGCAGACGTG
CB2 (676 bp)	55.8	Forward	GACCTTCACAGCCTCTGTGGGTA
		Reverse	GATTTTCCCATCAGCCTCTGTCT
FAAH (303 bp)	62.2	Forward	TGGAAGTCCTCCAAAAGCCCAG
		Reverse	TGTCCATAGACACAGCCCTTCAG

erslipped using Hystomount (Hughes and Hughes, Somerset, UK) mounting medium. Control sections for nonspecific labeling were also processed as described above, except that the primary antibody was either omitted or preincubated for 1 h at room temperature with an excess of its blocking peptide (3 μg/ml). Digital images were obtained using a DMR microscope (Leica, Deerfield, IL) and a Jenoptik ProgRes 3008 digital camera (Jenoptik, Jena, Germany).

Double-immunofluorescent labeling followed by confocal microscopy were used to determine whether FAAH and CB2 were expressed on placental macrophages. Macrophages were identified using a mouse monoclonal anti-CD14 antibody (gift from Dr. G. Vince, University of Liverpool, Liverpool, UK) at a dilution of 1:100 together with the appropriate antibody against FAAH or CB2 as described above. For the colocalization of CD14 and FAAH, specific labeling was identified using directly labeled goat antimouse Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR) and goat antirabbit Alexa Fluor 594 (Molecular Probes) antibodies. For the colocalization of CD14 and CB2, an amplification step was included. Specific labeling was identified using goat antimouse Alexa Fluor 488 (1:200; Molecular Probes) and goat antirabbit biotin (1:500; Vector Laboratories, Inc.), followed by streptavidin-Alexa Fluor 594 (1:200; Molecular Probes) antibodies. Sections were counterstained with Hoechst 33258 (8 μg/ml; Sigma-Aldrich Corp.) to label the cell nuclei and then were mounted in Citifluor (Agar Scientific, Essex, UK). The fluorescent-labeled sections were imaged using a confocal laser scanning microscope (TCS SP2, Leica) equipped with UV, argon, argon/krypton, and helium/neon lasers (Biomedical Imaging Research Unit, University of Auckland). Each fluorescent label was imaged serially to eliminate detection of bleed-through.

Results

RT-PCR

cDNA from 16 first trimester human placental samples were subjected to PCR amplification using oligonucleotide primer sets corresponding to either FAAH or cannabinoid receptors, as shown in Table 1. The integrity of the cDNA samples was established using RT-PCR with primers for GAPDH, which yielded a band of the expected size (473 bp) in 14 of the 16 samples, which were then used in future experiments. For all primer sets studied, no bands were amplified in the absence of reverse transcriptase- or cDNA-negative control samples, and a single band was observed for each reaction demonstrating the absence of genomic DNA or other contamination (data not shown). PCR products from at least two placentas for each primer set were sequenced and verified to be part of the gene of interest.

As demonstrated in the *upper panel* of Fig. 1, the FAAH primer set yielded a PCR product of the expected size (303 bp) in 13 of the 14 first trimester placental samples and in the term amnion positive control sample. In contrast to FAAH, no CB1 receptor mRNA was detected in samples extracted from first trimester tissues (n = 14) despite a band of the correct size (1.5 kb) and sequence being detected in the term amnion positive control under identical conditions (Fig. 1). The CB2 receptor primer set yielded a PCR product of the expected size (676 bp) in 10 of the 14 first trimester placentas tested (Fig. 1).

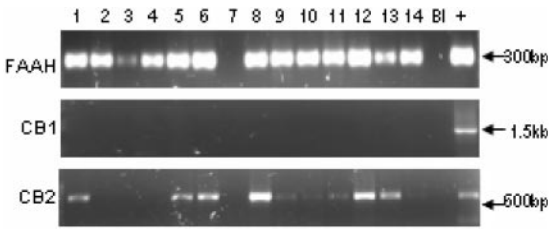

FIG. 1. RT-PCR products using primers specific for FAAH, CB1, and CB2 transcripts from first trimester placenta samples (lanes 1–14). Lane 15, The no cDNA negative control (Bl, blank); lane 16, the positive control (+), which was an amnion sample from a term human placenta for FAAH and CB1, and bone marrow for CB2.

Quantitative real-time PCR

Quantitative differences in FAAH mRNA levels were examined using real-time PCR with results normalized to 18S rRNA expression. As demonstrated in Fig. 2, a range of FAAH expression levels was observed in the tissues from different cases. Although small numbers of samples prevent any statistical analysis of gestational age-related trend, it appears that expression levels increase up to 11 wk of gestational age before decreasing again beyond this time point. As shown in Fig. 2, a particularly high level of FAAH mRNA expression was observed in one case (11 wk, 1 d gestation) relative to all other cases. A review of the clinical data available for this case did not show anything unusual, nor did subsequent histological analysis of the tissue, so the reason for this difference is not clear at present.

Immunohistochemistry

Specific FAAH immunoreactivity was investigated in three of the first trimester cases. Despite the variable level of expression, the cellular localization of specific immunolabeling was very similar in all cases. Strong FAAH immunoreactivity was localized to the villous cytotrophoblasts (Fig. 3, A and C). Intense labeling was also observed in extravillous trophoblast columns (Fig. 3A). Weaker FAAH immunoreactivity was localized to the syncytiotrophoblast layer (Fig. 3, A and C). In all cases, specific labeling was completely abolished by preincubation of the primary antibody with its blocking peptide (Fig. 3B). A subset of cells within the placental villous stroma was strongly immunoreactive,

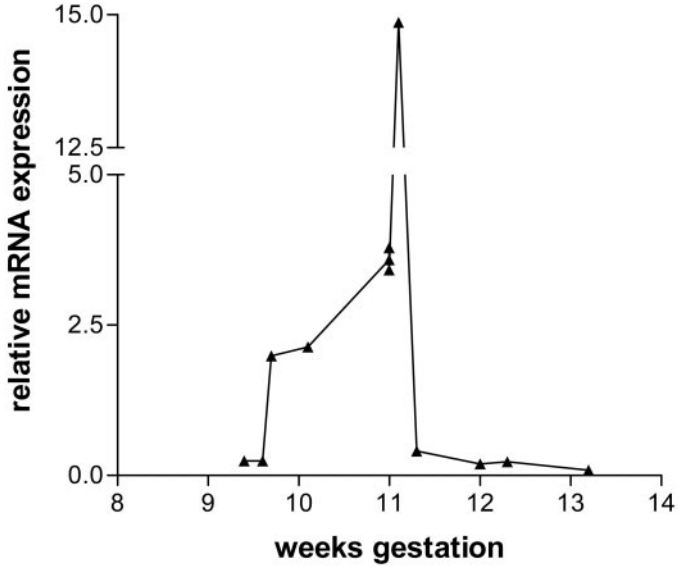

FIG. 2. FAAH expression levels (relative to 18S rRNA measured in the same samples) in first trimester tissues of differing gestational age, as determined by real-time PCR (see *Materials and Methods* for details).

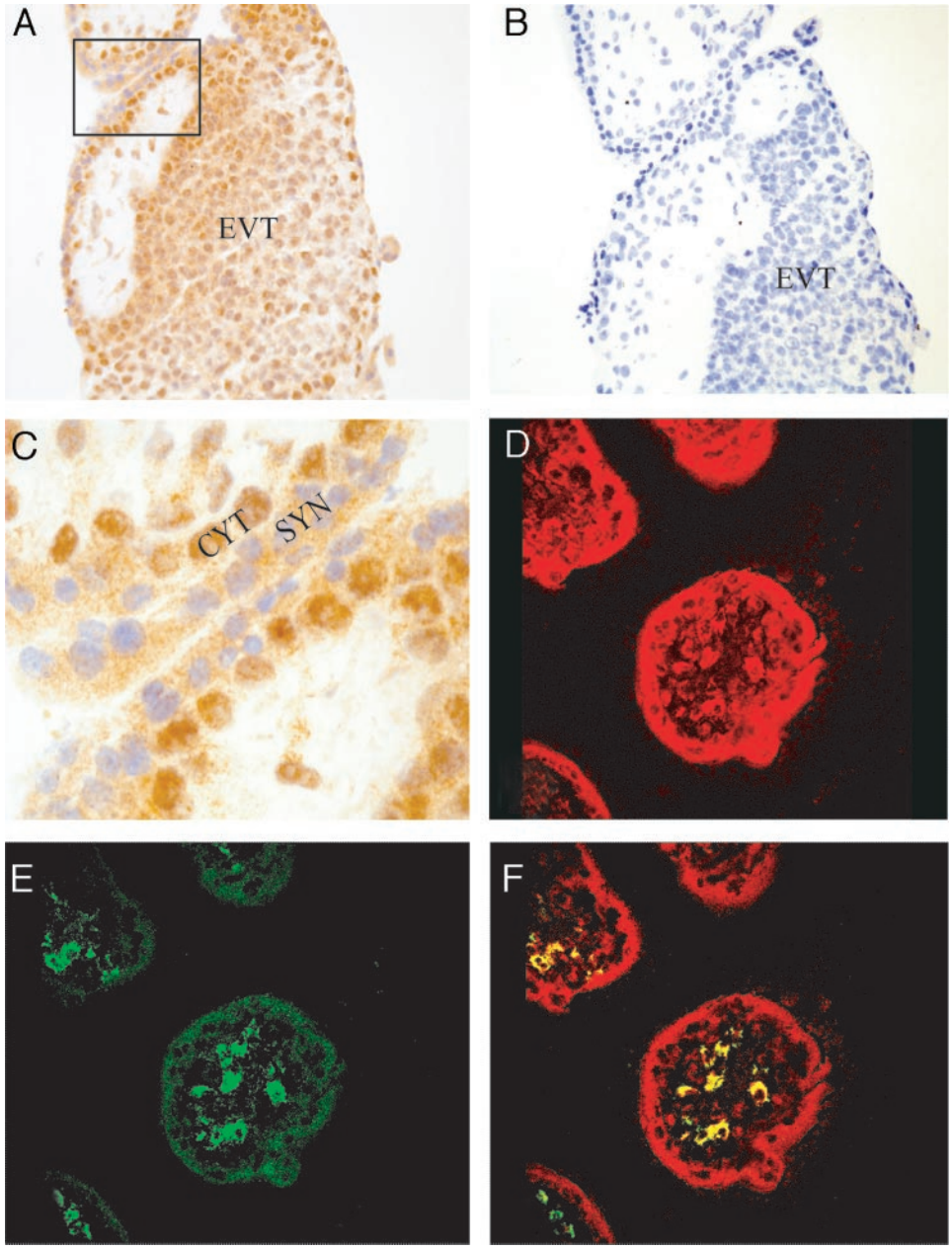

FIG. 3. FAAH immunoreactivity in first trimester placenta. A, Intense labeling was observed in extravillous trophoblast (EVT) columns (×25 objective) of anchoring villous. B, In all cases, specific labeling was completely abolished by preincubation of the primary antibody with its blocking peptide. C, Syncytiotrophoblast of the anchoring villous in A is shown at higher magnification (×100 objective). Strong FAAH immunoreactivity was localized to the villous cytotrophoblasts (CYT). Weaker FAAH immunoreactivity was localized to the syncytiotrophoblast (SYN). D, FAAH-positive macrophages were identified by double-label immunofluorescence for FAAH (*red*) and E, CD14 (*green*); F, merged images, demonstrating double labeling (*yellow*).

and these were subsequently identified as macrophages by double-label immunofluorescence for FAAH and CD14 (Fig. 3, D–F).

Consistent with the lack of CB1 expression by RT-PCR, no CB1 immunoreactivity was observed in first trimester tissues (Fig. 4B) under conditions that produced strong immunoreactive labeling in human term placenta (data not shown) and rat brain (Fig. 4A).

CB2 immunoreactivity was markedly different from that of FAAH in the first trimester placental samples. CB2 positive labeling was only observed in a subset of cells within the villous stroma (Fig. 4, C and D, *arrows*). These cells were identified as villous macrophages by double immunofluorescence labeling with antibodies to CB2 and CD14 (Fig. 4, E–H).

Discussion

This study reveals for the first time evidence for a local action of endocannabinoids in the human placenta during early pregnancy. Previous studies suggested that circulating FAAH and anandamide levels might be critical to the outcome of early pregnancy (2, 3). Indeed, it has been shown that decreased expression and activity of FAAH in peripheral blood lymphocytes is an early marker of human spontaneous abortion (2, 3). From those studies it was assumed that lymphocyte FAAH plays a key role in modulating the T helper cell type 1/2 cytokine balance at the maternal-fetal interface (11). Here we reveal for the first time, that the endocannabinoid-metabolizing enzyme, FAAH, and CB2 but not CB1, cannabinoid receptors are expressed in human first trimester

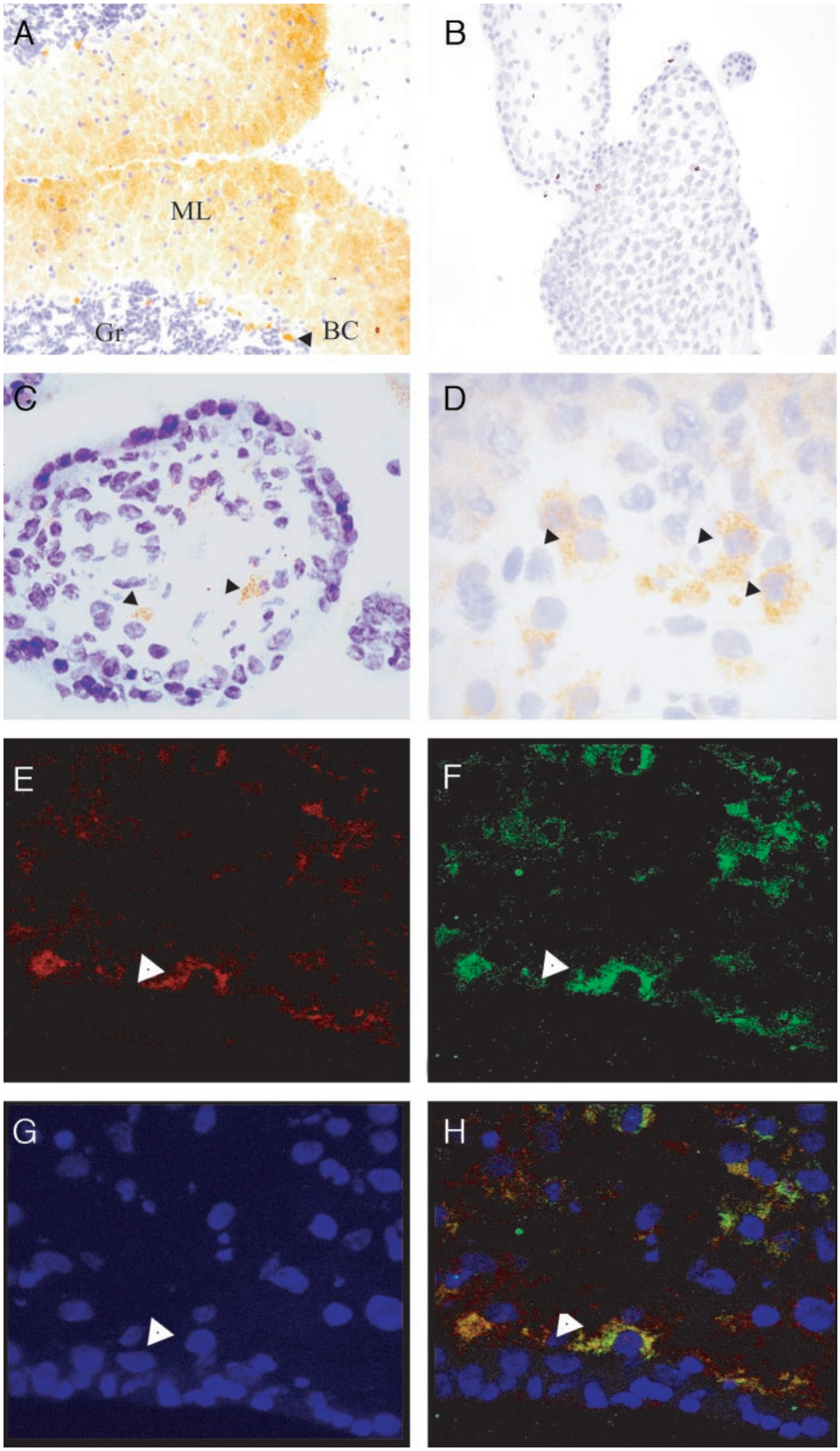

FIG. 4. Cannabinoid receptor immunoreactivity in first trimester placenta. A, Rat cerebellum was used as a positive control for CB1 immunoreactivity. Consistent with previous reports (27), CB1 immunoreactivity was strong in the molecular layer (ML) and in basket cells (BC), but was absent from the granule cell layer (Gr; ×25 objective). B, No CB1-positive immunoreactivity was identified in first trimester placental samples (×25 objective). C and D, CB2-positive labeling was observed in only a subset of cells within the villous stroma (*arrows*; ×63 and ×100 objectives, respectively). These cells were identified as villous macrophages by double immunofluorescence labeling with antibodies to CB2 (*red*; E) and CD14 (*green*; F). G, Hoechst-stained nuclei (*blue*). H, Double labeling demonstrating colocalization of these signals (*yellow*; ×100 objective).

placental tissues. Our results provide evidence of endocannabinoid regulation in fetal cells of the placenta that is independent of the maternal immune system.

Animal models suggested that low anandamide levels, maintained by high FAAH expression, are important for successful implantation (6). Indeed, a recent study has shown

a differential regulation of implantation in the mouse via cannabinoid receptor signaling (12). Specifically it was shown that a low (7 nM) anandamide level makes the blastocyst competent for implantation, whereas a higher concentration (28 nM) inhibits blastocyst competency. These effects were mediated by alterations in MAPK signaling and calcium channel activity through the CB1 receptor (12). Corresponding alterations in CB1 receptor expression have been described, with dramatic up-regulation of CB1 protein in the trophectoderm of dormant blastocysts and its rapid down-regulation with blastocyst activation by estrogen (12). Given these findings, the absence of detectable CB1 receptor expression by either RT-PCR or immunohistochemistry in first trimester human placenta may reflect down-regulation of placental receptors, whereas FAAH is up-regulated, to minimize placental cannabinoid activity at this early stage in placental development (9–13 wk gestation).

The placenta forms a barrier between fetal and maternal blood flow and separates the fetus from uterine tissue; therefore, it was our hypothesis that if high levels of maternal anandamide were detrimental to early placental and fetal development, FAAH expression would be high in the placenta during normal early pregnancy. Real-time PCR enables precise quantification of mRNA expression levels. Although larger subject numbers would be required to confirm our findings, this study suggests that FAAH levels in the human placenta may increase from 9 wk of age, peaking between 10 and 11 wk gestation before declining again by 12 wk. This is similar to a previous study that examined maternal plasma FAAH levels in 50 women who had normal pregnancies between gestational ages 7–12 wk (2). That study found that the levels of FAAH activity peaked at 9–10 wk gestation. There is growing evidence that a major transition in placental physiology occurs at approximately 10 wk gestation with the dissipation of trophoblast plugs from the spiral arteries consequently allowing maternal blood to perfuse the placenta for the first time (13, 14). Thus, at about 10 wk gestation, the human fetus must begin to protect itself against circulating maternal anandamide.

In the human, the syncytiotrophoblast is the placental cell layer that is in direct contact with the maternal blood, and villous cytotrophoblast lie directly underneath the syncytiotrophoblast layer. Thus, substances in the maternal blood must first pass through the syncytiotrophoblast, then the villous cytotrophoblast, before they can cross the remainder of the placenta and enter the fetal circulation via the placental capillaries. The high levels of FAAH that we observed in the villous cytotrophoblast and expression in the syncytiotrophoblast suggest that FAAH in these cells will prevent the transfer of anandamide from maternal blood to the fetus. Given the previous inverse relationship between anandamide and FAAH levels in the mouse uterus (5), it is also possible that FAAH in the first trimester placenta regulates local (placental) anandamide levels, but whether anandamide is produced in the human placenta is as yet unknown and will require additional study. In contrast, cytotrophoblast in the trophoblast columns invades uterine tissue to physically anchor the placenta to the uterus and therefore comes into direct contact with the uterine tissue, which has been shown in the mouse to express high levels of anandamide (15). It has also been shown in the mouse that reducing the level of uterine anandamide is important for successful implantation (15). Thus, the strong expression of FAAH in these invasive trophoblasts may reflect the need to reduce the high levels of anandamide present in the uterus to allow pregnancy to succeed.

Double-label immunofluorescent staining revealed the colocalization of CB2 receptors and FAAH on fetal macrophages resident in the placental villi. This is the first demonstration of these proteins in human fetal macrophages. In the adult human, the CB2 receptor is known to have a restricted tissue distribution, being almost exclusively localized to cells of the immune system, including B cells, T cells, and macrophages (16). Recently, it has been shown that the endocannabinoid 2-arachidonyl glycerol (2AG) induces migration of macrophage-like cells and human blood peripheral monocytes via a CB2, G_i/G_o-dependent pathway (17). Anandamide failed to induce migration in these cells (17), probably due to it acting only as a weak partial agonist on CB2 receptors (18, 19). Thus, it is possible that macrophages, which are believed to migrate into the placental villi from the embryonic mesenchyme, may be responding to secretion of 2AG from the trophoblasts, and that, unlike anandamide, 2AG is not detrimental to early pregnancy. Supporting this hypothesis, women undergoing *in vitro* fertilization-embryo transfer in whom implantation failed demonstrated raised anandamide, but not 2AG (3). Furthermore, in rodent models, the detrimental influence of anandamide on implantation appears to be CB1 receptor mediated (20). Thus, it is likely that CB2 receptors are involved in normal immune responses during early human pregnancy. Interestingly, LPS-stimulated macrophages secrete anandamide (21), suggesting that increased local anandamide may be one mechanism contributing to poor pregnancy outcome after bacterial infection (22, 23).

In the present study we could find no evidence of CB1 receptor mRNA or protein in placental samples from the first trimester of pregnancy. Neither villous trophoblast, villous macrophage, nor fetal vascular endothelium showed any evidence of CB1 immunolabeling by immunohistochemistry. Similarly, RT-PCR showed no evidence of CB1 receptor mRNA even at an extended cycle number. We have previously demonstrated the localization of CB1 receptors immunohistochemically in term gestational membranes and placental villi (7). At term, the CB1 immunoreactivity in gestational membranes was localized in amnion epithelial cells, chorionic trophoblast, and maternal decidua. In the placenta, CB1 immunoreactivity was identified throughout the villi. The absence of CB1 receptor in first trimester placental samples found in this study therefore suggests that CB1 receptor expression is induced at a later stage in placental development (*i.e.* after 13 wk). Thus, CB1 receptors appear to show differential regulation throughout the development of the human placenta.

An alternative target site for the action of maternal or placental cannabinoids is the fetus itself. The ontogeny of the endocannabinoid system in the fetus has not been extensively studied. However, in the human fetus, cannabinoid receptors have been identified by quantitative autoradiography in the brain at 33 wk gestation (24), whereas in the rat

embryo, receptors have been detected at embryonic d 11 (25). Recently, anandamide was detected in human amniotic fluid at 16 wk gestation (26). If maternal/placental endocannabinoids can produce adverse effects on the developing fetus, then the need to regulate the maternal-fetal transfer of endocannabinoids would be clear. The localization of FAAH and CB2 receptors in the first trimester, as demonstrated in this paper, suggests that these proteins may have a role in human placentation, which warrants additional study.

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References

1. **Park B, McPartland JM, Glass M** 2004 Cannabis, cannabinoids and reproduction. Prostaglandins Leukotrienes Essent Fatty Acids 70:189–197
2. **Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agro A** 2000 Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet 355:1326–1329
3. **Maccarrone M, Bisogno T, Valensise H, Lazzarin N, Fezza F, Manna C, Di Marzo V, Finazzi-Agro A** 2002 Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Mol Hum Reprod 8:188–195
4. **Schmid PC, Paria BC, Krebsbach RJ, Schmid HH, Dey SK** 1997 Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. Proc Natl Acad Sci USA 94:4188–4192
5. **Paria BC, Deutsch DD, Dey SK** 1996 The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. Mol Reprod Dev 45:183–192
6. **Paria BC, Dey SK** 2000 Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. Chem Phys Lipids 108:211–220
7. **Park B, Gibbons HM, Mitchell MD, Glass M** 2003 Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta 24:990–995
8. **Kenney SP, Kekuda R, Prasad PD, Leibach FH, Devoe LD, Ganapathy V** 1999 Cannabinoid receptors and their role in the regulation of the serotonin transporter in human placenta. Am J Obstet Gynecol 181:491–497
9. **Dennedy M, Friel A, Houlihan D, Smith T, Morrison J** 2002 Cannabinoids and the human uterus during pregnancy. J Soc Gynecol Invest 9(Suppl):736
10. **Twitchell W, Brown S, Mackie K** 1997 Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J Neurophysiol 78: 43–50
11. **Maccarrone M, Falciglia K, Di Rienzo M, Finazzi-Agro A** 2002 Endocannabinoids, hormone-cytokine networks and human fertility. Prostaglandins Leukotrienes Essent Fatty Acids 66:309–317
12. **Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL, Dey SK** 2003 Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. Proc Natl Acad Sci USA 100: 14914–14919
13. **Jauniaux E, Watson A, Burton G** 2001 Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. Am J Obstet Gynecol 184:998–1003
14. **Burton GJ, Jauniaux E, Watson AL** 1999 Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. Am J Obstet Gynecol 181:718–724
15. **Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, Bonner TI, Zimmer A, Dey SK** 2001 Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 276:20523–20528
16. **Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P** 1995 Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem 232:54–61
17. **Kishimoto S, Gokoh M, Oka S, Muramatsu M, Kajiwara T, Waku K, Sugiura T** 2003 2-Arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism. J Biol Chem 278:24469–24475
18. **Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW** 2000 Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. Mol Pharmacol 57:1045–1050
19. **Glass M, Northup JK** 1999 Agonist selective regulation of G proteins by cannabinoid CB_1 and CB_2 receptors. Mol Pharmacol 56:1362–1369
20. **Wang L, Liu J, Harvey-White J, Zimmer A, Kunos G** 2003 Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice. Proc Natl Acad Sci USA 100:1393–1398
21. **Liu J, Batkai S, Pacher P, Harvey-White J, Wagner JA, Cravatt BF, Gao B, Kunos G** 2003 Lipopolysaccharide induces anandamide synthesis in macrophages via CD14/MAPK/phosphoinositide 3-kinase/NF-κB independently of platelet-activating factor. J Biol Chem 278:45034–45039
22. **Romero R, Espinoza J, Chaiworapongsa T, Kalache K** 2002 Infection and prematurity and the role of preventive strategies. Semin Neonatol 7:259–274
23. **Silver RM, Edwin SS, Trautman MS, Simmons DL, Branch DW, Dudley DJ, Mitchell MD** 1995 Bacterial lipopolysaccharide-mediated fetal death. Production of a newly recognized form of inducible cyclooxygenase (COX-2) in murine decidua in response to lipopolysaccharide. J Clin Invest 95:725–731
24. **Glass M, Dragunow M, Faull RL** 1997 Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. Neuroscience 77:299–318
25. **Buckley NE, Hansson S, Harta G, Mezey E** 1998 Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. Neuroscience 82:1131–1149
26. **Schuel H, Burkman LJ, Lippes JCrickard K, Forester E, Piomelli D, Giuffrida A** 2002 *N*-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121:211–227
27. **Egertova M, Giang DK, Cravatt BF, Elphick MR** 1998 A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. Proc R Soc Lond B Biol Sci 265:2081–2085