Increase in placental apolipoprotein D as an adaptation to human gestational diabetes

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ABSTRACT

The expression of apolipoprotein D (apo D), a lipocalin involved in defense mechanisms against oxidative stress, in placental tissue samples of pregnancies with gestational diabetes mellitus (GDM) was compared to non-diabetic controls. We have investigated the relationship of apo D with 4-HNE, a major propagation product of lipid peroxidation, in stressed tissues. We included 20 pregnant women with GDM and 30 women with normal ongoing pregnancies as the control group. Placentas were collected and frozen for Western blot or included in paraffin for immunohistochemistry. The intensity of immunostaining was higher for apo D and 4-HNE in GDM samples; however, the differences in expression between the groups was more intense for apo D. Positive signals for both antibodies was detected in the villous trophoblast and adventitia tunica around the large blood vessels for all groups. Specific immunostaining for apo D was noted in some mesenchymal and macrophagic-like cells and this signal increased in diabetic placentas. Densitometry analysis of Western blots showed no significant difference for 4-HNE, but was significantly more intense for apo D in diabetic women. The contradictory results for 4-HNE could be due to changes which are too small and are masked in tissue homogenates. The results for apo D showed a strong relationship with GDM in the placenta that may reflect its suggested function in defense mechanisms against oxidative stress.

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1. Introduction

The apolipoprotein D (apo D), a member of the lipocalin superfamily, is a protein with a wide distribution in mammal tissues and an apparently multifunctional role. Apo D is a component of high-density plasma lipoprotein (HDL) in blood plasma that is present in some corporal fluids, like breast cyst fluid, lachrymal secretions, apocrine axillary secretions, cerebrospinal fluid (CSF), and urine [1]. In humans, apo D is poorly expressed in the liver and intestines, and is highly expressed in placenta, ovaries, testes, brain, pancreas, and adrenal glands [2]. Apo D may have a local function in peripheral organs, in addition to or in conjunction with a potential role in the circulation [3]. It has been shown that apo D binds several hydrophobic molecules with high affinity for progesterone, pregnenolone, and arachidonic acid (AA), but low for cholesterol [4]. In this way, it has been reported that steroids, such as androgens, estrogens, and glucocorticoids, are very important in the regulation of apo D expression, suggesting that its expression could be directly or indirectly modulated by changes in cellular proliferation [5–10]. Furthermore, apo D expression is linked to cellular aging in nervous tissue; apo D is abundant in glial cells of aged individuals and in patients with neuronal cell aging disorders [11,12]. In normal human fibroblast cell lines, apo D is secreted when cells have reached a senescent stage [10]. Lack of mouse apo D function increases the sensitivity to oxidative stress and the levels of brain lipid peroxidation, and human apo D overexpression in the mouse brain produces opposite effects, increasing survival and preventing the increase in brain lipid peroxides after oxidant treatment [13,14]. Finally, direct addition of exogenous human apo D to rat hippocampal slice cultures after excitotoxic injury suggests apo D neuroprotective effects by binding to AA and cholesterol, thus preventing oxidation to neurotoxic products, such as 4-hydroxynonenal (4-HNE) and 7-ketocholesterol [15]. Taken together, these observations suggest that apo D plays a conserved role in response to stress, possibly managing or preventing lipid peroxidation.

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Gestational diabetes mellitus (GDM) is a common metabolic disease of pregnancy that is characterized by glucose intolerance and insulin resistance. This maternal diabetic environment alters embryonic and feto-placental development, increasing the risk for macrosomia. Hyperglycemia during pregnancy is associated with obesity and diabetes mellitus in adolescence and later life. However, the molecular mechanisms that link GDM and altered fetal growth are not clearly understood. Oxidative stress has recently been implicated in GDM; oxidative stress is defined as a preponderance of pro-oxidative forces over antioxidant defense [16]. The increased production of reactive oxygen species (ROS) has been attributed to protein glycation and glucose auto-oxidation in a hyperglycemic environment [17]. To avoid ROS-induced damage of cellular components, several biochemical safety mechanisms are present in the placenta and serum. It has been reported that anti-oxidant defense is impaired and oxidation reactions are accelerated in placentas of GDM patients; thus, there is an increase in xanthine oxidase (XO), the main free-radical producing enzyme [16]. The principal line of defense against ROS, superoxido-dismutase (SOD), glutathione peroxidase, and catalase (CAT), are decreased in diabetic animals [18], although the level of SOD in placental tissues of GDM patients was significantly higher [19] or did not significantly change [16]. In addition, other non-enzymatic scavengers, like vitamins E and C, have been shown to be decreased in diabetic pregnancies [18,20].

The main objective of this study was to determine the expression of apo D in pregnant women with GDM and to compare apo D expression with a marker of oxidative stress in the placenta (4-hydroxy-2-nonenal [4-HNE]). We have shows that in women with GDM, apo D is up-regulated and this increase could be correlated with levels of lipid peroxidation products present in the placentas of GDM pregnancies.

2. Materials and methods

2.1. Placental material

The human placentas used in the present study were collected at time = 0 immediately after caesarean section, and were provided by the Pathology Department of Virgen de la Concha Hospital (Zamora, Spain). The placentas had been examined previously in the fresh state after delivery in the Pathology Department and were immediately processed. Biopsy specimens from 30 women with uncomplicated pregnancies (N) and 20 women who developed GDM were obtained from the maternal (lobulated aspect due to the presence of cotyledons covered by a thin layer of basal decidua) and fetal sides (covered by amniotic and chorionic membranes), frozen in liquid nitrogen, and stored at −80 °C. Gestational age at delivery was calculated according to the last menstrual period and confirmed by first trimester ultrasonography.

An oral glucose tolerance test (OGTT) was used for the diagnosis of GDM (World Health Organization, 1985). In our setting, an OGTT was arranged for all antenatal women with risk factors, such as relevant family and/or past obstetric history, maternal age > 34 years, weight ≥ 75 kg, or abnormal findings during antenatal visits (recurrent glycosuria, suspected polyhydramnios, and macrosomia). A routine random glucose screening was performed at 24–28 weeks of gestation for low-risk women. If the non-fasting venous plasma glucose concentration was ≥ 7.0 mmol/l, patients were referred for a 75 g OGTT after overnight fasting. GDM was defined as a fasting blood glucose ≥ 7.0 mmol/l and/or a venous plasma glucose concentration ≥ 11.1 mmol/l 2 h after OGTT in pregnant women without prior known diabetes. Women who were diagnosed with GDM were referred to a dietitian and started on a calorie-controlled diet within 1 week. Women with GDM were assessed with a 2 h postprandial blood glucose profile 3 days after they had been on the new diet. The dietary therapy was followed until the end of gestation. Patients in whom it was necessary to initiate insulin therapy were excluded from the study. The current study was approved by the Ethical Committee of Clinical Investigation of Asturias (Spain).

2.1.1. Western blot analysis

Placental samples were washed with ice cold phosphate-buffered saline (PBS) and homogenized immediately in 3 ml of lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% nonidet P40 [Roche Diagnostic, Barcelona, Spain], 0.05% Sodium deoxycholate, and 0.1% ortovanadate 1 M) at 4 °C using a Polytron (PT MR 3000; Kinematica AG, Lucerne, Switzerland) operated at maximum speed for 30 s.

Extracts from each sample were centrifuged at 12,000 g at 4 °C for 10 min to remove insoluble material. After centrifugation, the protein content was determined by the Bradford dye binding method [21], using Bio-Rad (Madrid, Spain) reagents and bovine serum albumin (BSA) as a standard. To ensure that the proteins were in a linear range of detection, preliminary experiments were conducted to determine that the amount of homogenate protein load was within a range that resulted in a proportionate change in signal intensity as the amount of protein load was varied.

To determine the levels of total apo D and 4-HNE, similar size aliquots (20 μg of total protein) were subjected to 7% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a Bio-Rad miniature slab gel apparatus. A pre-stained SDS molecular weight marker with a range from 10 to 250 kDa (Bio-Rad) was used. Electrotransfer of proteins from the gel to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotechnology, Barcelona, Spain) was performed for 60 min at 50 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described previously [22]. Non-specific protein binding to the nitrocellulose membranes was reduced by pre-incubating the filter for 2 h at room temperature (RT) in blocking buffer (TNT [100 mM NaCl, 100 mM Tris–HCl {pH 8}, and 0.2% Tween-20] and 7% BSA) and probe, using a 1:10,000 dilution of polyclonal antibody against human apo D (a gift from Dr. Carlos López Otín, Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo [23,24]) or a 1:2,000 dilution of polyclonal antibody against 4-HNE (HNE11-S; Alpha Diagnostic, San Antonio, TX, USA), and was carried out overnight at 4 °C diluted in blocking buffer. The membranes were rinsed several times with blocking buffer without BSA and incubated with anti-rabbit IgG-horseradish peroxidase conjugate secondary antibody (1:40,000 dilution in TNT) for 1 h at 4 °C. The membranes were washed again as above and the proteins were detected with the ECL reagent (Amersham Pharmacia Biotechnology) according to the manufacturer's instructions using autoluminography on Kodak X-Omat film. All membranes were stripped and probed with a monoclonal anti-β-actin antibody (sc1615, dilution 1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to ensure equal protein loading. To facility integral comparisons during analysis, standards prepared from pooled adult human skeletal muscle protein were used as a positive control and were run in each gel. The specificity of the inmunoreactive proteins was verified by loss of sample immunoreactivity when incubated directly with the secondary antibody. The total value of lipoxidation were determined by densitometry of all bands present in the Western blot for 4-HNE. The bands were quantified using a digital scanner (Nikon AX-110; Nikon, Madrid, Spain) and NIH Image 1.57 software (Scion Corp., MD, USA).

2.1.2. Immunohistochemistry

Placental samples were fixed by immersion in 10% buffered formalin. After fixation, pieces were dehydrated, cleared in butyl acetate, and embedded in paraffin. Sections, 10 μm thick, were obtained and attached to gelatin-covered slides.

Sections were deparaffined in xylene and rehydrated. The immunostaining was carried out according to the following protocol. The sections were treated sequentially with Triton X-100 (0.1%) for 5 min at RT, washed in distilled water, treated with H₂O₂ (3%) for 5 min in a wet chamber at RT, washed in distilled water, and treated with PBS for 2 min. Non-specific binding was blocked by incubation with BSA. Incubation with the specific antibody against apo D (1:2,000 dilution) and 4-HNE (1:2,000 dilution) was carried out overnight at 4 °C. Immunoreactivity was detected using the extravidin–biotin–peroxidase staining kit (Sigma Extra-3; Sigma Chemical Corp., St. Louis, MO, USA). Peroxidase activity was shown by incubation with Sigma Fast DAB (Sigma D 4168). The sections were counterstained with a modification of the formaldehyde–thionine method [25], dehydrated, cleared in eucalyptol, and mounted with Eukitt (Eukitt TM Mounting Medium; Electron Microscopy Sciences, Hatfield, PA, USA). The usual specificity control test was carried out. Control sections were incubated with buffer for antibody dilution or specific antibody, and pre-absorbed with each protein-immunizing peptide; this process showed an absence of staining.

2.1.3. Data presentation and statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Previously, we evaluated the Gaussian distribution of each variable. After this, analysis of variance followed by a Student–Newman–Keul's test was used to evaluate differences between groups statistically. A paired-samples' t test was used to evaluate differences between the maternal and fetal sides. Statistical significance was considered at p values ≤ 0.05. Statistical analysis was performed using SPSS, version 6.01 for Windows (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Study group characterization

The clinical data of women with uncomplicated pregnancies and GDM included in the present study have been previously reported [22]. The maternal age and placental weight were significantly

higher in the women GDM. However, gestational age and maternal and fetal weight did not differ significantly between the normal and GDM pregnancies. A significantly lower fetal/placental weight ratio existed in the GDM group.

3.2. Protein expression

Western blot analysis of the maternal and fetal sides isolated from the placentas of normal and GDM pregnancies showed that the relative density of apo D (Fig. 1) was significantly higher in GDM pregnancies.

Products of lipoxidation were present in homogenates of the maternal and fetal sides isolated from placentas of normal and GDM pregnancies on Western blot analysis of 4-HNE. Multiple bands were recognized with anti-4-HNE antibody, ranging from 33 to 170 kDa. Western blots did not revealed significant differences in the levels of 4-HNE-modified proteins in the GDM group when compared to the normal group, or the maternal side when compared with the fetal side in both groups (Fig. 2).

3.3. Immunohistochemistry

The pattern of staining was similar in tissue blocks obtained from different situations and at different levels from the chorionic plate to the decidua basalis (fetal and maternal sides) of the same placenta. Consistently strong expression of apo D was found in the decidua and chorionic villi of all placentas evaluated (Fig. 3A–H). The immunoreactivity was mainly localized in the adventitia tunica around large blood vessels of stem and branch villi (Fig. 3A and B). In normal pregnancies, the expression of apo D in decidual cells and blood vessels of decidua basalis was low, but increased in GDM placentas. Positive staining was found in the cytoplasm of decidua cells located in a perinuclear distribution (Fig. 3C and D). The amniotic membranes were also positive in all samples. Small branches of villi had apo D immunoreactivity in syncytio-trophoblasts and cytotrophoblasts in which numerous apo D-positive granules were noted in the cytoplasm (Fig. 3E–H). Positive staining was diffuse in the connective tissues around big blood
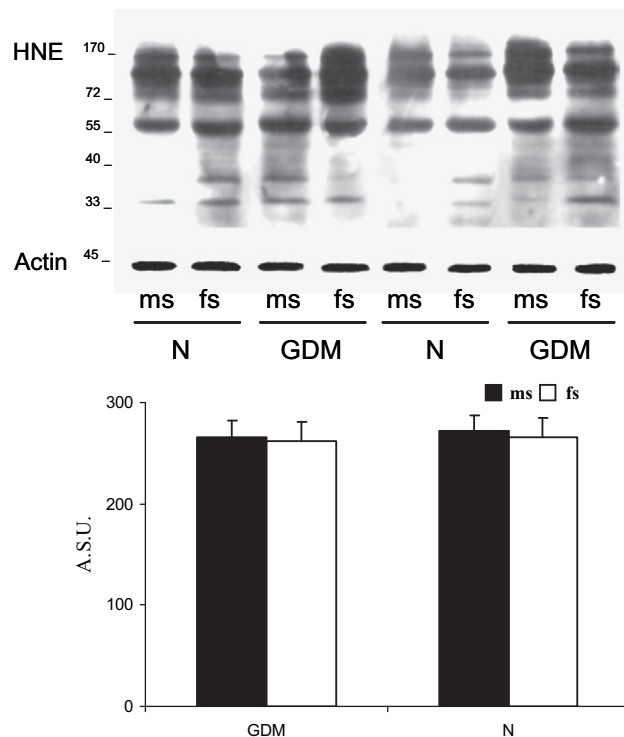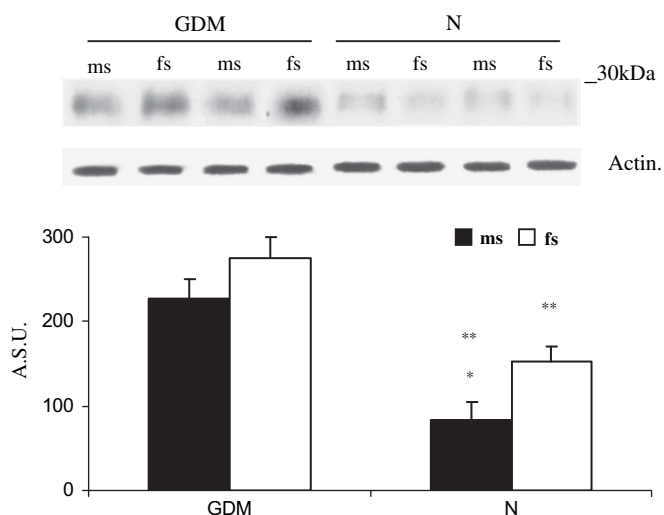

Fig. 2. Western blot analysis in maternal side (ms) and fetal side (fs) of the 4-HNE in placental samples from 30 women with uncomplicated pregnancies (N) and 20 women who developed gestational diabetes mellitus (GDM). Bottom: Relative amount of 4-HNE (ASU, arbitrary scanning units) in ms and fs for the two groups of a representative experiment data presented as means with standard error of the mean shown by vertical bars.

vessels in which strongly stained macrophage-like cells were noted (Fig. 3F–G). The signal was faint in villous endothelium and mesenchymal cells.

In comparing the degree of expression of apo D by staining intensity, all placental specimens of the GDM pregnancies showed the strongest staining (Fig. 3B, D, F, H). Differences in apo D expression were detected in villous stroma. In GDM term placentas, strong signals for apo D were shown in mesenchymal cells and fetal capillaries of chorionic villi (Fig. 3H).

Regarding 4-HNE, specific immunoreactivity was also demonstrated in all examined placentas (Fig. 4A–F). The villous stroma, mesenchymal cells, and fetal capillaries were usually stained (Fig. 4C and D). The immunolabeling appeared finely granular within the cytoplasm of trophoblast cells. Endothelial cells and villous trophoblast showed more positive signals for 4-HNE in GDM pregnancies (Fig. 4D). Granular 4-HNE immunoreactivity was seen in the epithelium of the amnion in normal and GDM placentas (Fig. 4E). Decidual cells were not immunopositive for 4-HNE in normal placentas, but weakly in diabetics (Fig. 4F). Direct visual comparisons of normal and diabetic placentas show obvious differences in the intensity of the signals. These differences were not observed in Western blots.

A similar pattern of positive immunostaining for apo D and 4-HNE was observed. In the placentas of normal pregnancies, positive staining for apo D and 4-HNE was weakly observed within the cytoplasm of the syncitiotrophoblast. Extracellular immuno-labeling for both apo D and 4-HNE was very strong in connective tissues around large vessels of stem villi in GDM pregnancies. In these vessels, the signals were primarily located in the adventitia tunica and no signals were in the media tunica. Neither apo D nor 4-HNE staining was observed in negative control sections.

Fig. 1. Top: Western blot analysis in maternal side (ms) and fetal side (fs) of the apo D in placental samples from 30 women with uncomplicated pregnancies (N) and 20 women who developed gestational diabetes mellitus (GDM). Bottom: Relative amount of apo D (ASU, arbitrary scanning units) in ms and fs for the two groups of representative experimental data presented as means with standard error of the mean shown by vertical bars. Only significant differences are show. *ms vs fs; **N vs GDM.
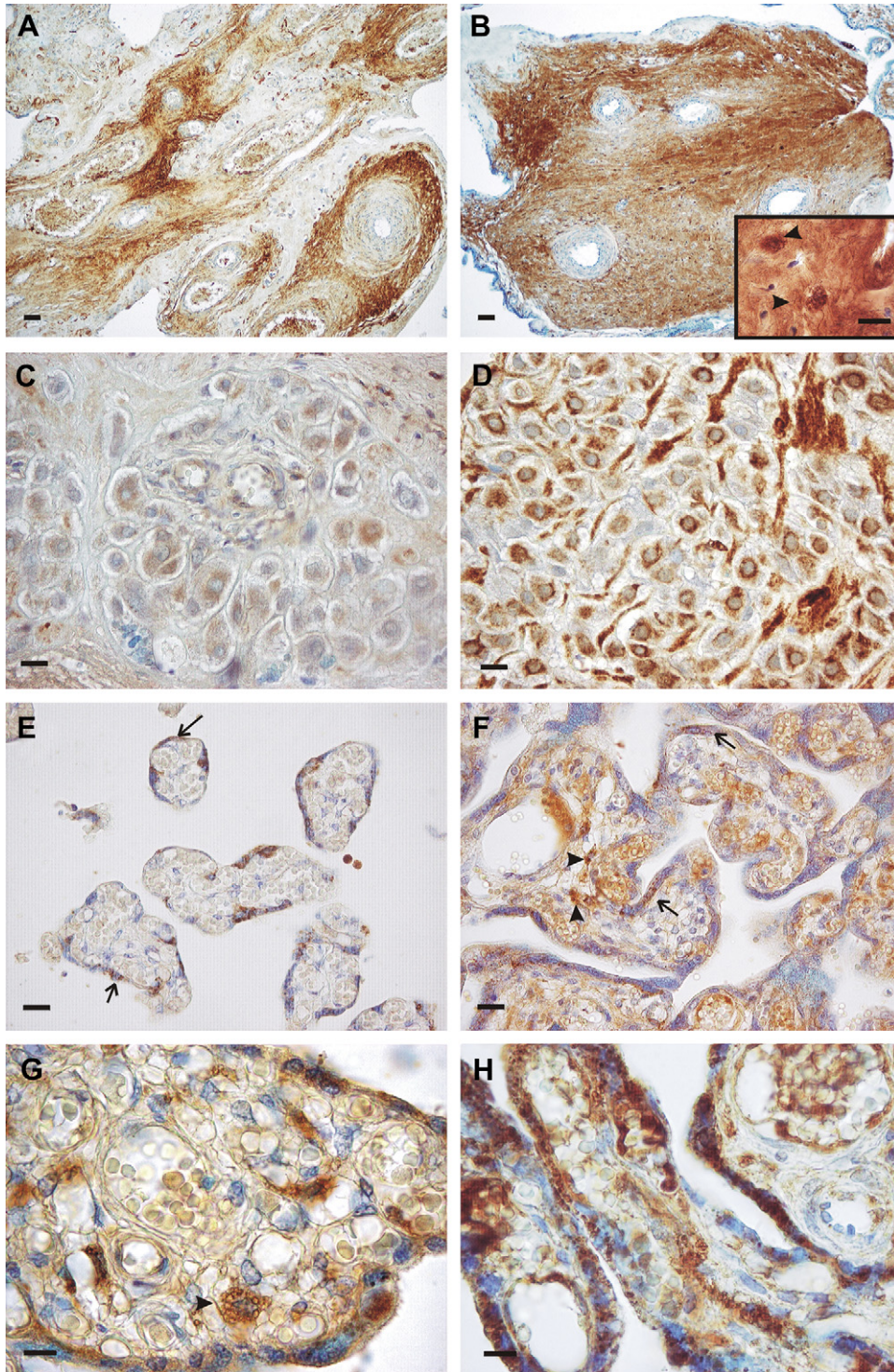
Fig. 3. Immunohistochemical localization of apo D in term placentas of normal pregnancies (A, C, E, G) and GDM pregnancies (B, D, F, H). Note the strong signal in connective tissue around blood vessels (A, B) of the biggest villous branches. Positive macrophagic-like cells are very numerous in this tissue (insert). Decidual cells are slightly positive for apo D in normal placentas (C) but the signal increases in GDM samples. Immunostaining is also detectable in syncytiotrophoblast (arrows) of both placentas (E, F, G, H) but is more intense in GDM placentas, increasing besides trophoblast the number of positive mesenchymal cells (arrow heads). Scale bar, 50 μm (A, B); 20 μm (C–F); 10 μm (G, H).

4. Discussion

In the present study we showed that apo D, a lipocalin previously reported in placental tissues [2], is enhanced in tissue samples from patients with GDM. This fact is not surprising because Baker et al. [26], in a study of the genes involved in lipoprotein metabolism as candidates for predisposition to non-insulin dependent diabetes mellitus (NIDDM), found a significant association with apo D. A similar association was demonstrated between apo D and the principal characteristics of syndrome X (obesity and hyperinsulinemia) [27]. Recently, human apo D overexpression in transgenic mice has been reported to induce insulin resistance and
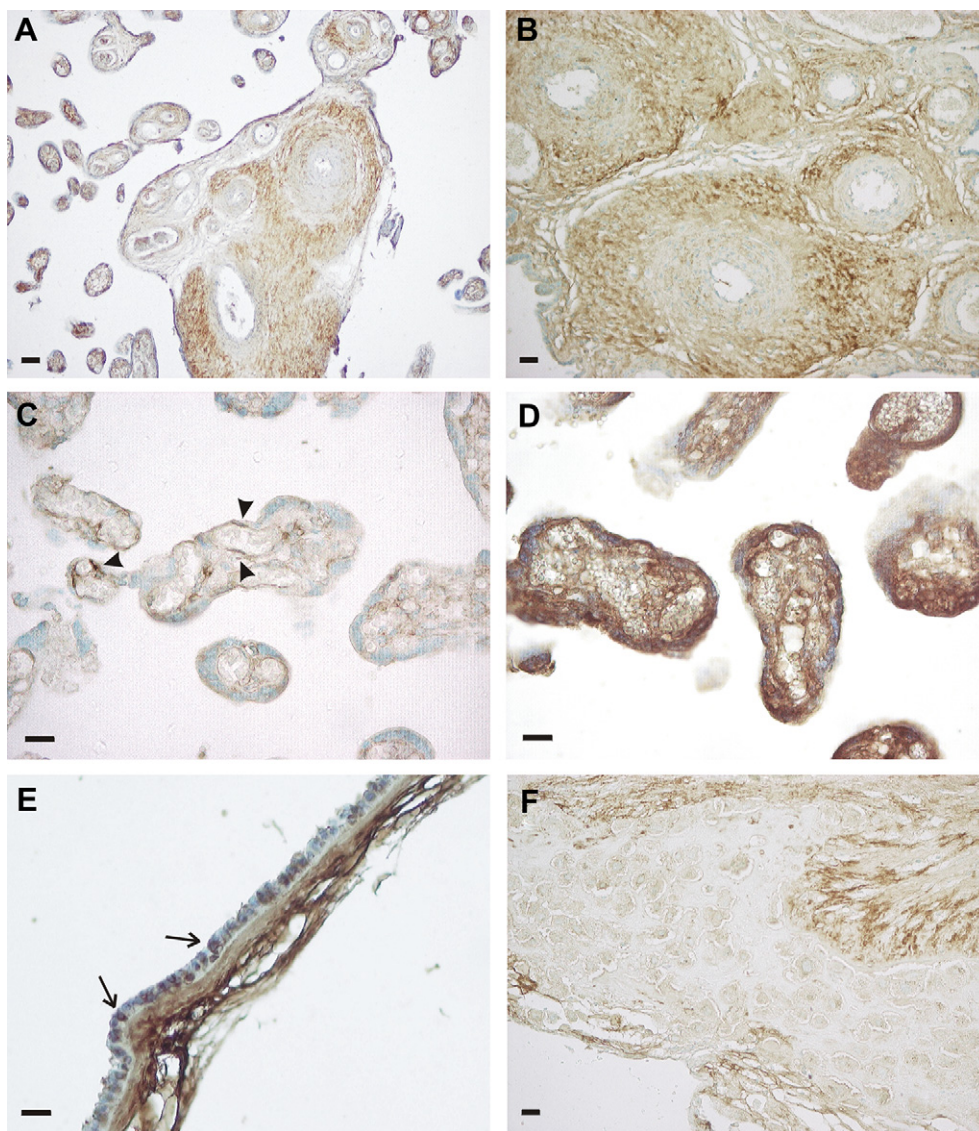
Fig. 4. Immunostainning for 4-hydroxynonenal (4-HNE) in placental tissue of normal (A, C) and GDM pregnancies (B, D-F). Low levels of immunoreactivity were observed in normal samples (A, C) when they were compared with GDM (B, D). Immunolabelling for 4-HNE presents similarities with apo D but syncytiotrophoblast displayed weak signal, whereas strong staining was present in endothelial cells of capillaries (arrow heads). Epithelial cells of amnion show a granular immunoreactivity in both samples (arrows) (E). Decidual cells showed low levels of immunoreactivity in GDM samples (F). Scale bar, 50 μm (A, B); 20 μm (C–E); 10 μm (F).

alter lipid metabolism [28]. Our results are in agreement with the hypothesis that apo D is a target for insulin resistance-related disorders.

Oxidative stress mechanisms have recently drawn special attention in relation with GDM pregnancy impairment. Lipid peroxidation is considered a harmful event that is difficult to revert and will lead as a chain reaction to the spread of oxidative stress. This process, which often develops during oxidative stress, will lead to the production of the "end products," malondialdehyde (MDA) and 4-HNE. Some studies have reported that both mechanisms (a decrease in antioxidant defenses and an increase free-radical production) are implicated in the pathogenesis of GDM [16]. The levels of MDA and/or HNE therefore represent a measure of the degree of oxidative damage in the tissues. Controversial data have been reported on the oxidative stress status in GDM. In some studies, no difference was found between MDA levels in pregnant women with GDM and healthy pregnant women [29]. Recently, a study has demonstrated an increase in MDA levels and impaired antioxidant defense in the plasma and placental tissues of women

with GDM [16]. The Western blot for HNE, a major aldehydic product of lipid peroxidation used as a biomarker of oxidative stress in our study, revealed no differences in levels of HNE in GDM and normal placentas. When immunohistochemistry for HNE was applied, a positive signal was found in all placentas and the cellular location of HNE was similar to previously described by Casasco et al. [30]. This immunohistochemical study showed signal differences between GDM and normal placentas. The HNE signal was present in cytotrophoblast cells, syncytiotrophoblast cells of the villous mesenchyme, and endothelial cells. This intracellular detection, considering the fact that no significant degree of lipid oxidative modification takes place in the plasma, suggests that HNE is a local product of normal placental metabolism [30]. The immunostaining was more intense in the GDM group for HNE. This increase reflects local changes for oxidative stress that were masked when we studied tissue homogenates. These results are in agreement with previous work involving pregnancies complicated by pre-eclampsia in which immunohistochemical and Western blot data for HNE were conflicting [31].

It has been shown that oxidative stress, which mainly arises from hyperglycemia, is implicated in the development of diabetic complications and there are several pathways for the production of free oxygen radicals in the presence of high glucose concentrations [17]. These slight differences in HNE between GMD and normal placentas could indicated that the level of oxidative stress in GDM women is partially controlled by diet. Therefore, an impaired antioxidant system may also play a significant role in the occurrence of oxidant stress in GDM as it has been demonstrated [16,19].

Since its isolation and characterization, apo D has been reported to be linked by many different processes, but its precise function remains unknown. In human plasma, apo D and lecithin-cholesterol acyl transferase (LCAT) are found on high density lipoprotein particles (HDLs) and have been postulated to form part of a complex involved in the transport of cholesterol from peripheral tissues to the liver for excretion [32]. Then, one possibility to be considered is that apo D might exert in placenta a transport function that controls the supply of lipids across the placental barrier. The expression of apo D by placental cells can be related with some function in transport or regulation of lipid ligands. Apo D shows a strong binding affinity to AA, pregnenolone, progesterone, and bilirubin, while its affinity for estrogens and cholesterol is very low. Apo D binding to progesterone and AA plays a role in cancer development and neurologic diseases [1,4].

Several data suggest that apo D and its orthologs could play an evolutionarily-conserved role in response to stress, possibly managing or preventing lipid peroxidation [33,34]. Furthermore, the apo D promoter contains many potential regulatory elements as serum response elements, estrogens, progesterone, and stress response elements [10,35]. Recent reports have shown that loss of mouse apo D function increases the sensitivity to oxidation stress and the levels of brain lipid peroxidation and human apo D overexpression in the mouse brain produces opposite effects preventing the increase in brain lipid peroxides after oxidant treatment [14]. Apo D also provides resistance against coronavirus [36]. Moreover, recent studies have revealed a neuroprotective effect of apo D against kainite-induced excitotoxicity, and suggest that this effect may be due to the ability of apo D to sequester and reduce the levels of AA and cholesterol oxidation products, F2-isoprostanes and 7-ketocholesterol, respectively. Apo D appears to play a protective role in neuropathologic situations by controlling the level of peroxidated lipids [15,36–39].

The increased apo D expression in the placenta from diabetic pregnancies may indicate an increased need for the apo D function and an exit in its role. Hence, the enhanced expression of apo D in the villous chorion of GDM placentas may indicate an activation of an adaptive placental mechanism to maintenance of oxidative stress status. Since trophoblastic cells and villous macrophages are immunopositives for apo D, it is conceivable that these cells may play a scavenger role protecting the diffusion of lipoperoxidation products from the mother to the embryo. In this sense, enhanced levels of AA and docosahexaenoic acids in the placenta of the GDM women have reported [40].

In summary, we hypothesize that overexpression of placental apo D could represent a protective response to GDM as in several neuropathologies [11,39,41–44]. Apo D could have particular relevance in scavenger lipids preventing their oxidation and protecting the growth and viability of the fetus.

Acknowledgments

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