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Acid phosphatase and cathepsin D are active expressed enzymes in the placenta of the cat

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Abstract

Enzymes are crucial for the metabolism of macromolecular substrates. In the great majority of cells, most enzymes are constitutive. Nevertheless, inducible enzymes can predominate, determining specialized cell functions. Within this context, histochemistry/immunohistochemistry and biochemistry were used to investigate expression of peroxidase and reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase, as well as the expression and activity of cathepsin D and acid phosphatase, in trophoblast cells within the endotheliochorial labyrinth and marginal hematoma of the term cat placenta. In the marginal hematoma, elevated Cathepsin D expression and activity was accompanied by erythrophagocytosis. In contrast, acid phosphatase activity was much more intense in the labyrinth, where metabolic exchanges occur. Peroxidase and NAD(P)H-oxidase were predominantly active in trophoblast cells within endosomal vesicles of different placental compartments, indicating that, although reactive oxygen species might participate in endosomal/lysosomal processes, they are not territorially specific or functional markers. These findings highlight differential characteristics of cathepsin D and acid phosphatase activity within each placental compartment, thereby contributing to the comprehension of the territorial role played by the placenta and facilitating future metabolic studies.

Keywords: Placenta; Chorionic villi; Phagocytosis; Lysosomes; Trophoblasts; Cats

1. Introduction

The placenta of the domestic cat is zonary, lamellar, endotheliochorial and deciduate. It is subdivided into two distinct areas designated as the main placenta and the paraplacenta (Mossman, 1987; Leiser and Koob, 1993; Leiser and Kaufmann, 1994). The main placenta is responsible for pronounced metabolic and molecular exchanges, whereas the paraplacenta contains auxiliary areas such as the marginal hematoma, which is a specialized hematophagous region characterized by intense erythrophagocytosis. Iron uptake for fetal hemopoiesis has been considered one of the main roles of this placental region, in which the columnar phagocytic cytotrophoblasts are bathed in extravasated maternal blood (Malassiné, 1977, 1982, 2001; Leiser and Enders, 1980a,b; Mossman, 1987; Leiser and Koob, 1993).

Although the carnivore placenta, and especially the cat placenta, has been extensively described, the cellular physiology of placental compartments has not been completely

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explored (Malassiné, 1977; Malassiné, 1982; Mossman, 1987; Leiser and Koob, 1993; Leiser and Kaufmann, 1994). Within this context, enzymes that are crucial for the metabolism might provide valuable information on cell specializations in topographically distinct tissue regions. Various studies have shown that acid phosphatase, which is an important marker of phagocytic activity in professional phagocytes (activated macrophages and neutrophils, Rodman et al., 1990) and other cell types (Araki et al., 1995), is widely distributed among the different compartments of the maternal–fetal interface of the cat placenta (Christie, 1968; Malassiné, 1976; Miyoshi and Sawamukai, 2004). The enzyme cathepsin D is another marker of phagocytic activity in macrophages and neutrophils. It is the most significant aspartic-protease, and has also been found in uterine macrophages and stromal cells in the placentas of macaques (Blankenship and Enders, 1997) and horses (Green et al., 1998). Recently, cathepsin D has been reported to be correlated with invasion and phagocytic activity in the rodent trophoblast (Elangovan and Moulton, 1980; Afonso et al., 1999). Peroxidase is a hemoprotein present in all aerobic organisms, containing an iron porphyrin complex that takes part in substrate-specific oxidative process, as well as in the formation and degradation of oxygen peroxide (Nelson and Kulkarni, 1986). Finally, reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase is a plasma membrane-bound enzyme complex present in a wide variety of cells of mesodermal origin. Acting as an electron transporter, NADPH-oxidase is intrinsically associated with the generation of reactive oxygen species (ROS) in neutrophils and macrophages (Gagioti et al., 1996; Segal and Shatwell, 1997; Babior, 1999; Cui et al., 2006). According to Matsubara and Tamada (1991), NADPH-oxidase might also play an important role in the molecular transport between mother and fetus.

Thus, in the present study, we investigated the expression and activity of the lysosomal enzymes cathepsin D and acid phosphatase, and the activity of peroxidase and nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase in an attempt to better comprehend the biology of the cat placenta, correlating our findings with specific trophoblast functions.

2. Materials and methods

The cat placentas were kindly provided by the Veterinary Hospital of the University Santo Amaro, obtained from females undergoing elective cesareans performed for ovariohysterectomy procedures on a routine basis for animal population control. The entire procedure was performed in an operating room under aseptic conditions. All experimental procedures were performed in accordance with the guidelines established by the Brazilian School of Animal Experimentation and were approved by the Ethics Committee for Animal Experimentation of the Institute of Biomedical Sciences of the University of São Paulo.

Ten adult females of domestic cat (*Felis catus*) of undefined race, from 1.5 to 3 years of age were selected for this study. On average, 3 placentas were obtained from each female. After delivery the females were clinically evaluated. Only were experimentally processed the placental fragments from females that did not exhibit infections or systemic diseases and, were in term gestation phase (50–60 days post-coitus). For the biochemical assays, 12 placentas were used, and 18 were used for the remaining assays.

2.1. Collection of samples

2.1.1. For morphological analysis

The placentas were immediately washed in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), and immersed in a fixative solution of 4% paraformaldehyde in PBS. The paraplacenta (marginal hematoma) and the main placenta (labyrinth) were cut into small fragments. The samples were stored in the fixative solution for an additional 4–24 h. Due to the bilateral localization of the paraplacenta in relation to the main placenta, both regions were dissected and processed as individual units: the proximal and distal, having the ovary as the anatomical reference.

After 1 h of fixation, samples for histochemical identification of acid phosphatase and peroxidase were incubated in a cryoprotective solution (1% Arabic gum and 0.88 M sucrose in 0.1 M PBS) and rapidly frozen in chilled isopentane in a dry-ice/acetone bath. Five-micrometer sections were then obtained on a cryostat (IEC Minotome, Needham Heights, MA, USA) and mounted onto slides pretreated with 1% gelatin and 1% formaldehyde in 0.1 M PBS.

2.1.2. For hydrogen peroxide-formation sites (NAD(P)H-oxidase activity)

To evaluate areas with NAD(P)H-oxidase activity, fragments of the paraplacenta and the main placenta were sequentially washed in 0.1 M PBS/0.1 M Tris–maleate buffer (pH 7.5), and incubated in 0.1 M PBS at 4 °C to be taken to the laboratory.

2.1.3. For biochemical analysis

The placentas were first washed in PBS, and then the main placenta, as well as the distal and proximal paraplacenta (marginal hematoma), were identified and kept for at least 1 h on ice until starting the biochemical procedures. To that, samples were cut into tiny pieces.

2.2. Immunohistochemistry

Immunohistochemical reactions were performed to identify the enzyme cathepsin D, as well as the mesenchymal and trophoblastic components of the placenta. To identify the mesenchymal and trophoblastic components, the intermediate filaments vimentin and cytokeratin, respectively, were used as markers.

Deparaffinized sections were irradiated with microwaving (at 800 MHz) in 10 mM citrate buffer (pH 6.0), followed by treatment with 0.3% H_2O_2 in 0.05 M PBS. After blocking with 1% bovine serum albumin (BSA) type V (Sigma, St. Louis, MO, USA) in 0.1 M PBS (pH 7.4), the sections were incubated in a DAKO Autostainer Universal Stainer System (DAKO Corp., Carpenteria, CA, USA) with mouse antibodies against cathepsin D (anti-human monoclonal clone DB2000, DAKO), vimentin (goat anti-human, polyclonal antibody, Sigma) and cytokeratin (anti-human monoclonal clone AE1/AE3, DAKO) respectively diluted at 1:250, 1:50 and 1:50 in Tris-buffered saline (TBS), for 30 min at 37 °C. Biotinylated secondary antibodies were applied, and immunoreactivity was visualized using the avidin–biotin peroxidase complex method (LSAB and DAB Plus Kits; DAKO) followed by a light nuclear counterstaining with Mayer's hematoxylin.

2.3. Histochemistry

Histochemical reactions were used to identify acid phosphatase, peroxidase and NADPH-oxidase activities.

For acid phosphatase, 5-μm thick sections were incubated at 37 °C for 15–45 min in Gomori incubation solution (Gomori, 1936), 3.2 mg/mL sodium β-glycerophosphate, 2 mg/mL lead nitrate in 0.2 M acetate buffer (pH 5.0). After rinses in 0.2 M acetate buffer containing 0.25 M sucrose, samples were immersed in 1% ammonium

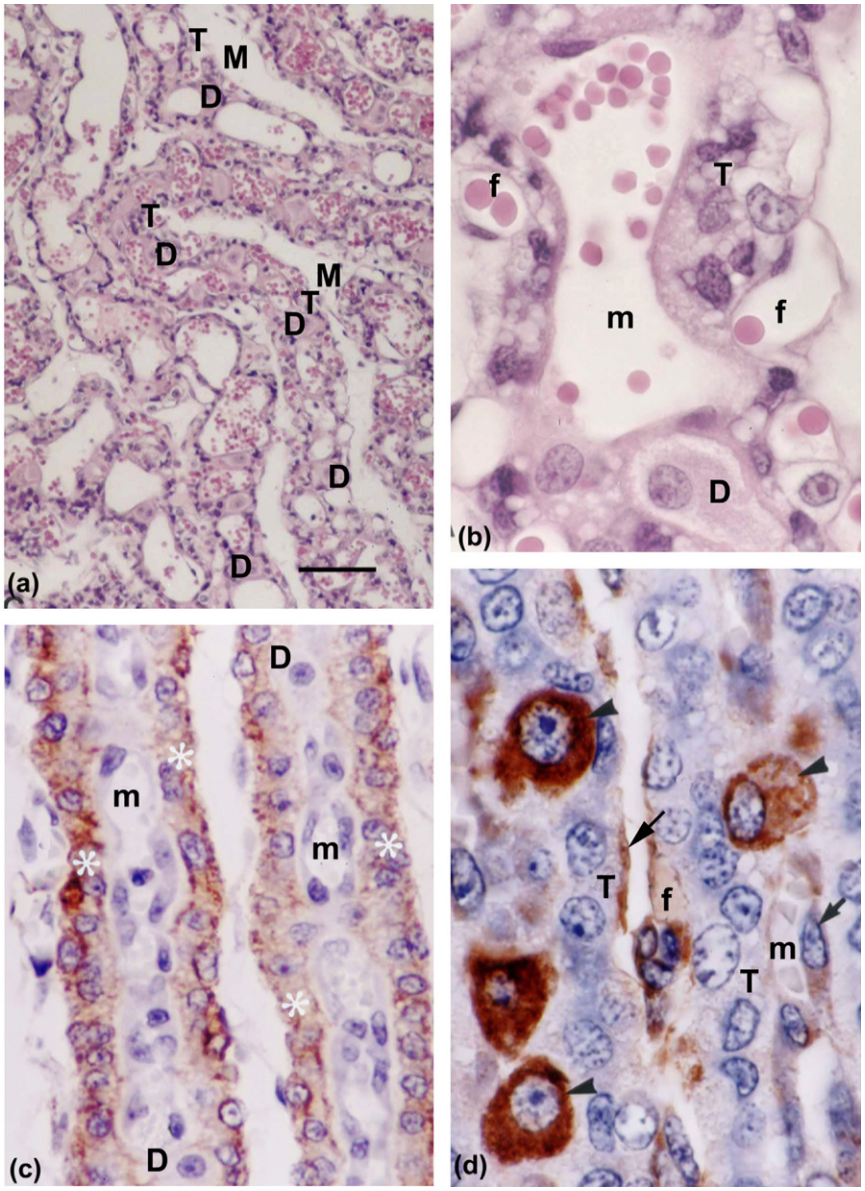

Fig. 1. Photomicrographs of the labyrinth zone of the endotheliochorial cat placenta stained with hematoxylin and eosin (a, b) and counterstained with Mayer hematoxylin (c, d), comprising decidual cells (D), trophoblast layer (T) and mesenchyme (M). In higher magnifications (b–d), fetal (f) and maternal (m) capillaries are better visualized. In (c) white asterisks show immunolocalization of intermediate cytokeratin filaments in trophoblast cell layer; and in (d) arrowheads and arrow respectively highlight vimentin-reactive decidual and endothelial cells. Bar in (a) = (a) 75 μm; (b) 20 μm; (c) 30 μm; (d) 10 μm.

sulfide, washed in distilled water and counterstained with Mayer's hematoxylin.

For peroxidase, the sections were incubated in 0.5 mg/mL diaminobenzidine–0.02% H_2O_2 (Sigma) in 0.05 M Tris-buffered saline, pH 7.6, at room temperature. After being washed in distilled water, samples were counterstained with Mayer's hematoxylin.

Sites of hydrogen peroxide production and, consequently, of NADPH-oxidase activity were determined according to Briggs et al. (1975). Freshly collected placenta samples were sequentially processed as follows: incubation in 0.1 M Tris–maleate buffer (pH 7.5), containing 0.71 M NADH and 1 mM cerium chloride ($CeCl_3$), for 20 min at 37 °C; washing in 0.1 M Tris–maleate buffer; and fixation in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), containing 0.05 M sucrose, at 4 °C for 24 h. The fragments were then washed in 0.1 M sodium cacodylate, pH 6.0 and pH 7.3, both at 4 °C, for 1 h. The samples were postfixed in 2% osmium tetroxide in the sodium cacodylate buffer at room temperature for 1 h, and usual procedures for Spurr's resin embedding and ultrastructural observation were followed (JEOL CX-100-II transmission electron microscope).

As reaction controls, additional samples were processed following the procedures detailed above but in the absence of: (i) NADH; (ii) $CeCl_3$; (iii) NADH and $CeCl_3$.

In all experimental groups, some of the samples were pretreated for 10 min at 37 °C with 1 mM of potassium cyanide in Tris–maleate buffer to inhibit site III of the mitochondrial respiratory chain (Briggs et al., 1975).

To assure the specificity of the positive reaction, some of the samples from each experimental group were preincubated with 1 U of catalase (an H_2O_2 scavenger) and 1.5 U of superoxide dismutase (an O_2^- scavenger).

2.4. Biochemical assays

Parts of the main placenta and paraplacenta were thawed, homogenized in 20 mM acetate buffer, pH 4.0, using an Omni-Mixer (Sorvall Inc., Wilmington, DE, USA) and centrifuged at 15,000 rpm and 4 °C for 30 min. Some of the samples were homogenized in 20 mM acetate buffer containing the following proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM *p*-polyhexamethylene biguanide and 10 mM ethylenediaminetetraacetic acid. The protein quantification was performed according to Morton and Evans (1992).

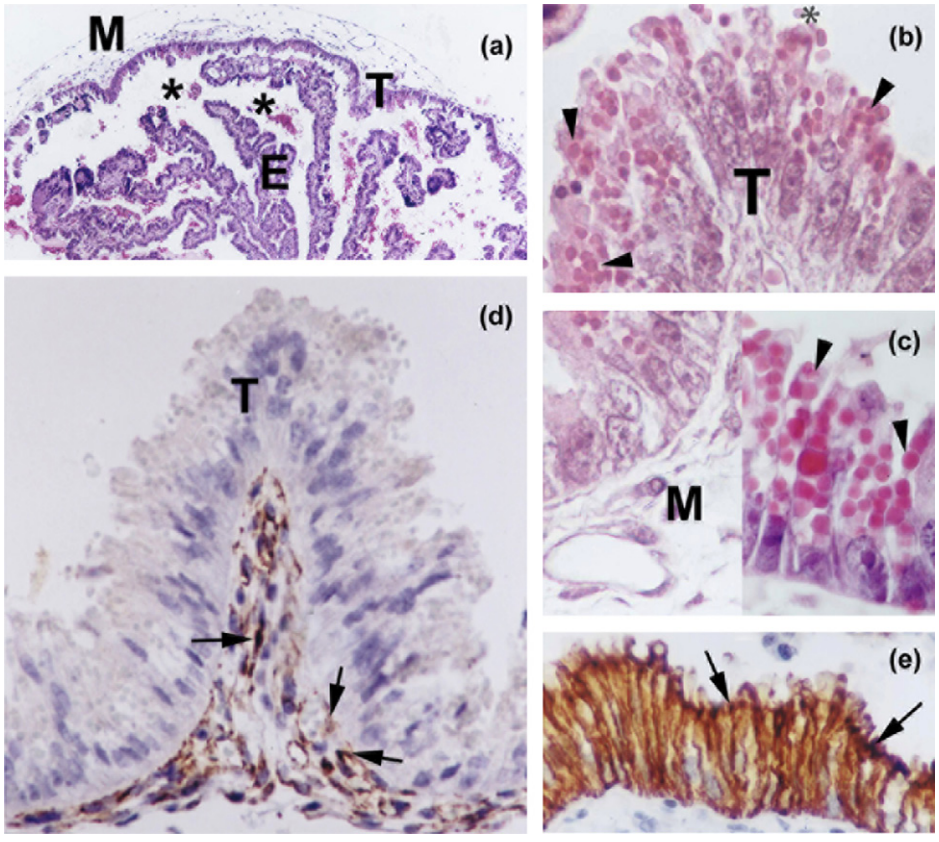

Fig. 2. Photomicrographs of the marginal hematoma on the cat placenta stained with hematoxylin and eosin (a–c) and counterstained with Mayer hematoxylin (d, e). (a) Observe that the trophoblast layer (T) and endometrium (E) are not closely apposed, but are interposed by extravasated maternal erythrocytes (*). (b, c) Numerous erythrophagosomes (arrowheads) can be seen in the cytotrophoblast cells (T). (d, e) The arrows reveal immunolocalization of intermediate (d) vimentin filaments and (e) cytokeratin respectively in the mesenchyme and in the trophoblast cell layer. M, mesenchyme. Bar in (a) = (a) 250 μm; (b) 20 μm; (c) 35 μm; (d) 40 μm; (e) 25 μm.

For detection of acid phosphatase enzyme activity, the supernatant of the samples was incubated in 4 ng/mL *p*-nitrophenyl phosphate in acetate buffer (1:1 vol/vol) (pH 5.0), at 30 °C for 15–60 min. The reaction was stopped by the addition of 1 mL of 0.25 M bicarbonate (0.25 M carbonate containing 1% sodium dodecyl sulfate). This concentration of sodium dodecyl sulfate is sufficient to completely denature all of the enzymes assayed. The absorbance was measured using a spectrophotometer (Ultrospec Plus; Pharmacia LKB, Uppsala, Sweden) at 420 nm, and the values obtained were compared to a standard curve prepared with *p*-nitrophenol. The enzyme activity was calculated in mU/mg according to Terra et al. (1979) based on the following parameters: (i) 1 enzyme unit (1 U) producing 1 M *p*-nitrophenol/min at 30 °C; (ii) total protein contained in each sample; and, (iii) total *p*-nitrophenol produced/min.

For determination of cathepsin D activity, 2% bovine hemoglobin in 100 mM citrate phosphate buffer (pH 3.5), was used as a substrate. The samples and substrate were mixed (20 μL, 1:1) and incubated at 30 °C for 10, 20, 30 or 40 min. The reaction was interrupted with a solution of 50 mM phosphate buffer (pH 7.3), and 0.03% fluorescamine in acetone (1:1, vol/vol). The sample fluorescence (excitation to 375 nm, emission to 475 nm) was measured with a spectrofluorometer (F-2000; Hitachi Ltd., Tokyo, Japan) according to Garesse et al. (1979). For calculating the specific activity of cathepsin D (in U/μg), it was assumed that 1 enzyme unit (1 U) produces 1 μM of amino groups per minute at 30 °C, and a standard curve was prepared with ovalbumin.

3. Results

3.1. Morphology of the labyrinthine and paraplacental areas of the feline placenta

Under light microscopy, a peculiar interaction was observed between the chorion and the endometrial stroma, both organized as parallel lamellae, characteristic of an endotheliochorial placenta (Fig. 1a, c and d). In the chorion, two distinct layers of trophoblasts – the cytotrophoblast layer and the syncytiotrophoblast layer (Fig. 1c) – were seen lining the axis of mesenchymal tissue (Fig. 1d), which is rich in fetal vessels. In the maternal tissue, the decidual cells were found to be interposed among the numerous capillaries (Fig. 1a and b). Interestingly, syncytiotrophoblast shows membrane-bound vesicles of heterogeneous diameters and content throughout the cytoplasm (Fig. 5a). Fig. 2 shows that, in the marginal hematoma (paraplacenta), a single layer of cytotrophoblast covers the fetal tissues and uterine epithelium on the maternal side, constituting the maternal–fetal interface. The space between the fetal and maternal surfaces was found to contain extravasated maternal blood cells (Fig. 2a) and trophoblast cells exhibiting marked erythrophagocytosis were often observed (Fig. 2b and c).

3.2. Enzyme localization

The immunolocalization of intermediate filaments of vimentin and cytokeratin, respectively allowed the clear characterization of decidual cells, endothelial cells (Fig. 2d) and trophoblasts (Fig. 2e).

Immunoreactivity to cathepsin D was observed in the main placenta and in the marginal hematoma (Fig. 3). In the main placenta (Fig. 3a), reactivity was found in the syncytiotrophoblast layer, whereas, in the marginal hematoma (Fig. 3c and d), a stronger reaction was seen

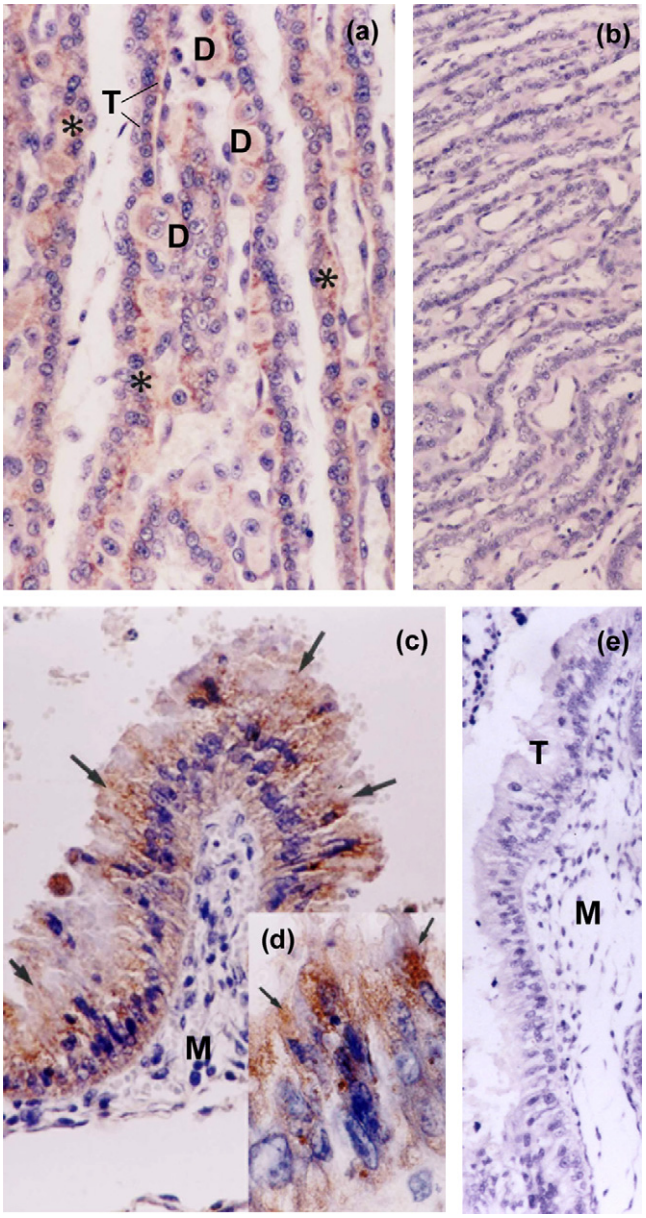

Fig. 3. Photomicrographs show the immunolocalization of cathepsin D in (a) main placenta and (c, d) paraplacenta. (b, e) Negative controls in which the primary antibody was omitted: Observe reactive cells (arrows) in: (a) main placenta–at the trophoblast layer (T) and decidua (D) and, (d) paraplacenta–in cytotrophoblast cells. M, mesenchyme. Bar in (a) = (a) 30 μm; (b, e) 50 μm; (c) 25 μm; (d) 40 μm.

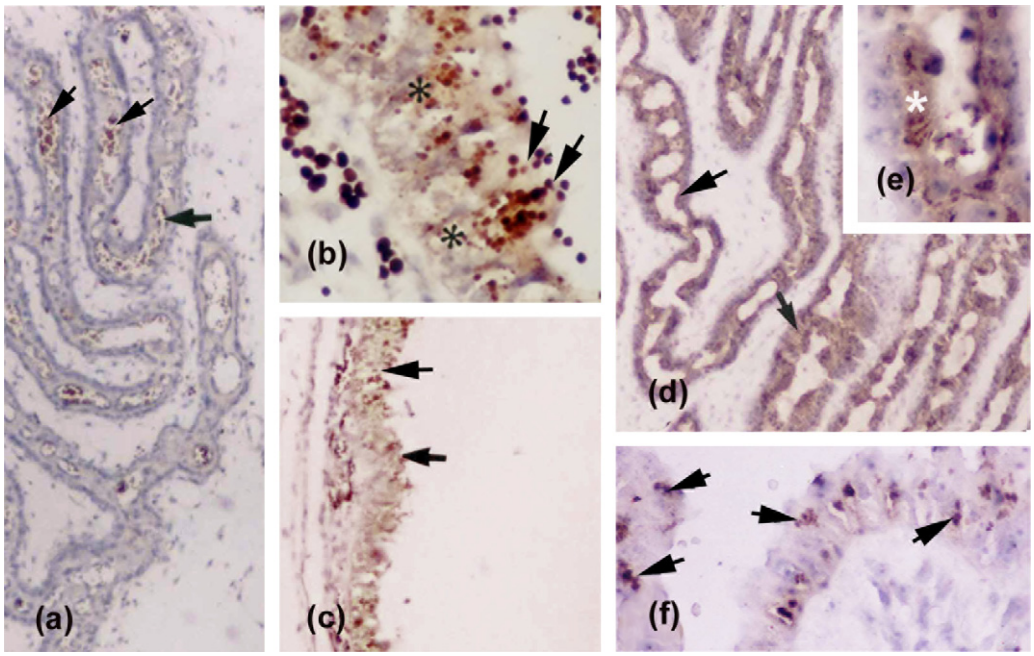

Fig. 4. Photomicrographs show histochemical reactions for peroxidase a–c and acid phosphatase (d–f). Arrows indicate reactive cells. a–c Observe peroxidase activity in the main placenta (a) and in paraplacenta (b, c) mostly in erythrocytes. Black asterisks identify cytotrophoblast cells. d–e Strong acid phosphatase activity is observed at the main placenta, where not only trophoblast layers are reactive, but also maternal components (white asterisks). (f) In the paraplacenta, the enzymatic activity is located in cytotrophoblast cells. Bar in (a) = (a, c) 60 μm; (b, e) 35 μm; (d) 30 μm.

in cytotrophoblast cells exhibiting erythrophagocytic activity. In the negative controls, in which the primary antibody was omitted, no reaction was observed (Fig. 3b and e).

In the main placenta, peroxidase activity was only observed in maternal and fetal erythrocytes, which were the only reactive cellular components (Fig. 4a). In the marginal hematoma, reaction was also seen in maternal and fetal erythrocytes, extravasated or within phagosomal vacuoles in the cytoplasm of the cytotrophoblast cells (Fig. 4b and c).

Acid phosphatase-positive granules were seen in both placental regions, but, they were much more expressive in the main placenta (Fig. 4d and e) than in the paraplacenta (Fig. 4f). In the main placenta the enzyme activity was seen scattered throughout the cytoplasm of trophoblast, decidual and endothelial cells, whereas, in the paraplacenta it was mostly restricted to the cytoplasm of cytotrophoblast cells (Fig. 4f).

The cytochemical reaction designed to identify sites that produce H_2O_2 is also able to indirectly localize NADPH-oxidase. This reaction revealed precipitation of cerium perhydroxide in the main placenta and paraplacenta and was apparently associated to phagocytic activity. Discrete reactivity was seen in heterogeneous population of heterophagosomes-like dense bodies of the syncytiotrophoblast in the main placenta (Fig. 5a, insert). In syncytiotrophoblast cytoplasm abundant large electron lucid vesicles could be seen (Fig. 5a). Occasionally reaction could be also observed at peripheral areas of such structures. In the cytotrophoblast of the paraplacental layer the reaction was stronger than in the main placenta and associated with erythroheterophagosomes. Only part of this vesicles containing electrondense material exhibited reaction (Fig. 5b–d). The reactivity could not be associated with size or any other peculiar characteristic of the vesicles. Potassium cyanide, which is used as an inhibitor of site III of the mitochondrial respiratory chain, prevented the mitochondrial reaction in all samples (data not shown). In the control group samples in which the substrate NADPH-oxidase, $CeCl_3$ or both were omitted, no enzyme activity was observed. Reactions were not even observed in the samples in which catalase and superoxide dismutase were introduced as ROS scavengers (data not shown).

3.3. Biochemical analyses

Significant differences among placental regions were observed regarding specific activity of acid phosphatase and cathepsin D (Table 1). In the main placenta, the acid phosphatase activity was approximately twofold greater than that observed in any other region (distal or proximal) of the paraplacenta ($p < 0.05$). In contrast, significant higher values were obtained for cathepsin D in the paraplacenta (~threefold, $p < 0.05$) as compared to the main placenta.

4. Discussion

Communication between the fetal and maternal tissues is complex and is fundamental for fetal survival. It includes not only metabolic and gaseous exchanges, but also hormones and regulatory molecules secretion from both

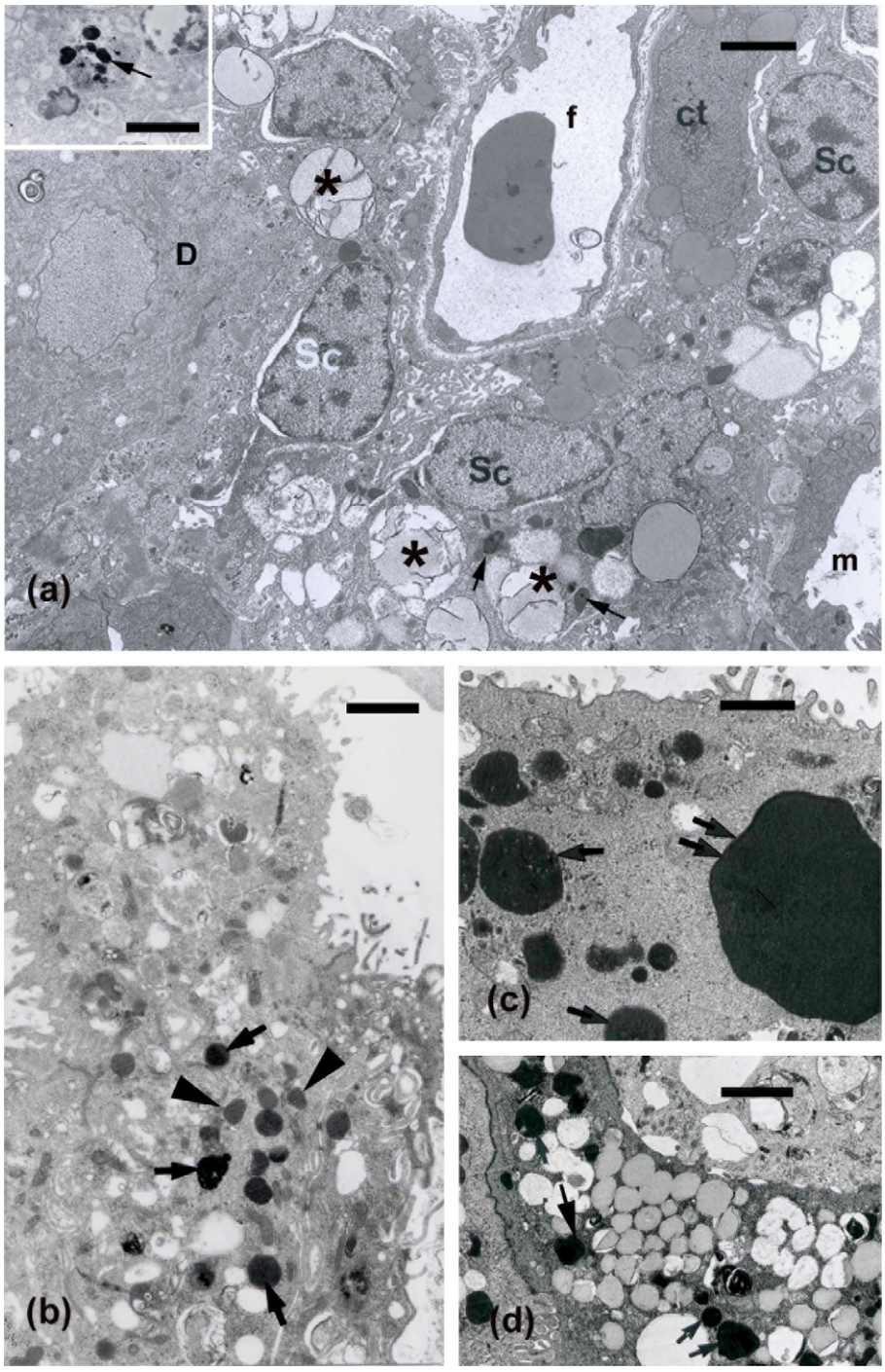

Fig. 5. Electromicrographs of NADH oxidase activity detected at the ultrastructural level using cerium ions to trap H_2O_2 generated by the enzyme. (a) Maternal fetal interface at the labyrinth zone in the main placenta. Note the fetal vessel (f) surrounded by cytotrophoblast cell (ct) and the syncytiotrophoblast (Sc). A decidual cell is also shown (D) as well as part of a maternal capillary (m). The asterisks highlight the membrane-bound vesicles of varying diameter and heterogeneous content into the syncytiotrophoblast cytoplasm. Arrows indicate heterophagosome-like structures in which discrete reaction is seen in stained and unstained (insert) sections. (b–d) In paraplacental cytotrophoblast NADPH-oxidase activity is indicated by arrows in heterogeneous in size erythrophagosomes. Observe that not all vesicles show NADPH-oxidase activity (b, arrowheads). Bars = 1.5 μm in (a, b, d and insert) and 0.5 μm in (c).

organisms. Although cat placenta explants have recently gained importance in studies of the mechanisms of transplacental nutrient transport (Champion et al., 2004, 2005; Jones et al., 2005), the *in situ* characterization of the molecular repertoire of each specific compartment is still necessary, and can contribute to the development of *in vivo* models for understanding the complex maternal–fetal relationship.

Table 1
Cat placenta: specific activity of the acid phosphatase (μU/mg) and cathepsin D (U/μg)

	Proximal paraplacenta	Main placenta	Distal paraplacenta
Acid phosphatase (μU/ mg)	22.93 ± 10.75	50.08 ± 21.09*	26.35 ± 6.43
Cathepsin D (U/ μg)	17 ± 5*	6 ± 1	18 ± 2*

The values represent the mean ± S.D. of four different experiments.
* Significant variations from values obtained in the proximal and distal paraplacenta (ANOVA, *U*-Mann Whitney, $p \leqslant 0.05$).

In the current study, histochemical and biochemical procedures showed that acid phosphatase (ap), universally used as a lysosomal marker for phagocytic cells (Manyonda and Choy, 1999; Miyoshi and Sawamukai, 2004), does not correlate proportionally with the phagocytic activity in the placenta regions. Acid phosphatase was twofold greater in the main placenta, in which transport processes are extremely important, but where phagocytosis was faintly characterized by heterophagosome-like structures, when compared to the highly phagocytic cytotrophoblast layer of the marginal hematoma. These findings are in agreement with previous histochemical studies (Christie, 1968; Malassiné, 1976) and suggest that acid phosphatase may be associated with absorption mechanisms in the main placenta, counteracting the metabolic exchange between the foetus and the mother, as also indicated in intestinal mucosa (Miyoshi and Sawamukai, 2004).

Adding to that, our results show that the erythrophagocytosis in the paraplacenta region is strongly correlated with cathepsin D expression/activity rather than with that of acid phosphatase. Cathepsin D is efficient in the cleavage of long peptides such as hemoglobin (Rodman et al., 1990), which might explain its high expression in the cytotrophoblast layer of the marginal hematoma, a peculiar hematophagous area associated with iron uptake in the placenta (Enders and Carter, 2004) and, considered as an adaptive and functionally effective mechanism for supplying fetal iron requirement. However, other pathways might also be connected to this expression. Recent reports showed that metabolites derived from hemoglobin degradation, such as the alpha chain fragment PVNFKFLSH, designated hemopressin (Rioli et al., 2003) may act as vasoactive molecules. Therefore, since the activity of cathepsin D also involves the degradation of hemoglobin, it is possible that this enzyme might be indirectly involved in the changes in blood pressure caused by hemoglobin derivative metabolites. If this appears to be correct, the expression and activity of this enzyme might be part of placental physiology as a regulatory factor for local hypotension and control of the blood flow at the maternal–fetal interface. Furthermore, mechanistic evidences indicate that cathepsin D can be mitogenic, angiogenic, and inhibitor of apoptosis in tumour and endothelial cells (Glondu et al., 2001; Berchem et al., 2002; Laurent-Matha et al., 2005). In contrast, upon specific stimuli leading to lysosomal destabilization, the release of cathepsin D has been associated to Bax activation, mitochondrial membrane permeabilization and release of mitochondrial apoptosis-inducing proteins, responsible for early apoptotic events (Boya et al., 2003; Bidere et al., 2003). Particularly in placenta, this pathway might guarantee that damaged cells with hydrolytic enzymes can be safely eliminated through apoptosis, avoiding the extracellular release of such content and consequently, the extensive lost of local functions and inflammation.

In order to determine whether ROS-related enzyme complexes play a causal role in the functional compartmentalization of the placenta, as suggested for other species (Gagioti et al., 1996), we investigated the cytochemical identification of peroxidase and NADPH-oxidase enzymatic activities. However, the expression of both was very weak, almost incipient, distributed in some phagosomal vacuoles, suggesting that ROS generation is not associated with specific functional zones of the placenta.

In conclusion, this study has sought to describe correlations between the expression and activity of enzymes and the functional compartmentalization of the cat placenta. We added new data to the literature by measuring the biochemical activity of cathepsin D and acid phosphatase enzyme complexes, which appear to be the key in determining placental functional activities.

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