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Extracellular breakdown of collagen by mice decidual cells. A cytochemical and ultrastructural study

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ABSTRACT: The interaction of antimesometrial decidual cells and collagen fibrils was studied by light microscopy and ultrastructural cytochemistry in fed and acutely fasted mice on days 9-11 of pregnancy.

Fibrillar elements in the extracellular space consisted of collagen fibrils and filamentous aggregates (disintegrating collagen fibrils). Intracellular vacuoles exhibited typical collagen immersed in electron-translucent material (clear vacuoles) and faint cross-banded collagen immersed in electron-opaque material (dark vacuoles).

Fibrillar elements showed extracellular acid phosphatase activity which was stronger in the region of mature decidua than in predecidual cells region in all animals; it was conspicuous in mature decidua of fasted animals. Intracellular acid phosphatase activity was observed in dark vacuoles and lysosomes, and was absent in clear vacuoles in all cells studied.

Since acid phosphatase activity reflects the presence of lysosomal hydrolases in general, the results indicate that breakdown of extracellular collagen occurs by release of lysosomal enzymes by decidual cells and also by internalization of collagen for intracellular degradation in fed and fasted mice. Collagen breakdown may be part of the process of tissue remodeling in mature and predecidual regions, however, in mature decidua, collagen breakdown is enhanced and may therefore contribute to nutrition of the fetus, specially in acutely fasted mice.

Introduction

Remodeling of the extracellular matrix of the endometrium, specially collagen, is a normal and complex process which occurs during pregnancy development (Fainstat, 1963; Martello and Abrahamsohn, 1986; Zorn *et al*., 1986) and involution (Luse and Hutton, 1964; Woessner, 1965; Parakkal, 1969; Okamura *et al*., 1976).

In the mouse decidua, from the fifth day of pregnancy onwards, the extracellular matrix surrounding decidual cells exhibits thick collagen fibrils. The region of mature decidual cells shows the thickest fibrils, reaching 370nm in diameter (Zorn *et al*., 1986; Alberto-Rincon *et al*., 1989). The large decidual collagen fibrils are thought to arise by lateral association of smaller diameter fibrils. Biglican is associated with the presence of thick collagen fibrils in mouse decidua (San Martin and Zorn, 2003).

On days 9-11 of pregnancy, mouse antimesometrial decidua exhibits mature decidual cells situated near the embryo, surrounded by predecidual cells (immature decidual cells) (Katz and Abrahamsohn, 1987).

During decidual involution, in the period of 9-11 days of pregnancy, the antimesometrial decidual tissue undergoes remodeling in the region where mature decidual cells are involuting and in the region where predecidual cells are differentiating. Synthesis and deg-

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radation of collagen fibrils are part of these processes. Mouse decidual cells synthesise (Oliveira *et al*., 1991) and also engulf thick collagen fibrils for intracellular degradation (Zorn *et al*., 1989). However, if decidual cells internalize entire fibrils or fibrils previously submitted to enzyme activity and what are the functions of collagen fibrils in the decidual tissue, besides their mechanical role, this is still unclear.

Cell-extracellular matrix interactions are fundamental to the success of pregnancy. Changes in the extracellular environment, as in the case of withheld of maternal food, alters maternal-fetal exchange (Rosso, 1981) and extracellular lysosomal enzymes activity is more conspicuous in antimesometrial mature decidua of acute fasted mice than in fed mice (Katz, 1998).

Lysosomal enzymes (Katz, 1998) and metalloproteinases (Alexander *et al*., 1996) show activity in the extracellular matrix during decidual involution. However, extracellular degradation of decidual collagen fibrils has not yet been demonstrated.

We have used acid phosphatase reaction as a marker of lysosomes and hence for lysosomal hydrolitic enzymes which may be active on collagen fibrils in fed and fasted pregnant animals.

In the present study we have examined acid phosphatase activity on collagen fibrils from the antimesometrial decidua in fed mice and in acutely fasted mice, during days 9-11 of pregnancy, by electron microscopy.

Materials and Methods

Female, ten-week-old Swiss virgin mice were mated with males of the same strain and observed each morning for the presence of vaginal plugs. The first day of occurrence of a vaginal plug was taken as the first day of pregnancy.

The study was approved by the Institutional Ethics Committee on Research.

Nine pregnant mice, respectively three on each day, 9th, 10th and 11th of pregnancy, were fed *ad litibum* and were used as controls. Eighteen animals were submitted to a 24 h fasting period beginning, each group of six mice, respectively, on the morning of day 8, day 9 or day 10 of pregnancy. Water was freely available. The animals were isolated in cages containing a wire-mesh grid so that the mouse could not reach the sawdust at the bottom of the cage.

In the uteri from fed pregnant mice the implantation sites appeared, macroscopicaly, as spherical dilatations of similar dimensions, distributed regularly along the axis of the uterine horns.

In the uteri from the eighteen mice that were submitted to a 24 h fasting period we observed many uteri with the same gross morphology as the unfasted control mice described above (5 on day 9 of pregnancy, 4 on day 10 and 5 on day 11). These uteri were used in the experiment. The other fasted animals showed uteri with little spherical dilatations, irregularly distributed along its long

FIGURE 1. Region of mature decidual cells of fasted mouse (day 9 of pregnancy) showing collagen fibrils in a small compartment in the extracellular matrix. Thick fibrils are transversely (arrowheads) and longitudinally (double arrows) sectioned. Thin fibrils are longitudinally sectioned (arrow). Magnification X 35,600. Bar = 0.5 μm.

FIGURE 2. Thick collagen fibrils in the extracellular matrix between decidual cells. The fibril longitudinally sectioned is close to the cellular surfaces (arrows) and the fibrils transversely sectioned are surrounded by superficial projections of the cell cytoplasm (arrowhead). Fasted animal, on day 11 of pregnancy. Magnification X 35,600. Bar = 0.5 μm.

FIGURE 3. Detail showing typical collagen cross-banding of longitudinally sectioned thick (long arrow) and thin (short arrow) collagen fibrils. Fasted animal, on day 10 of pregnancy. Magnification X 89,000. Bar = 0.1 μm.

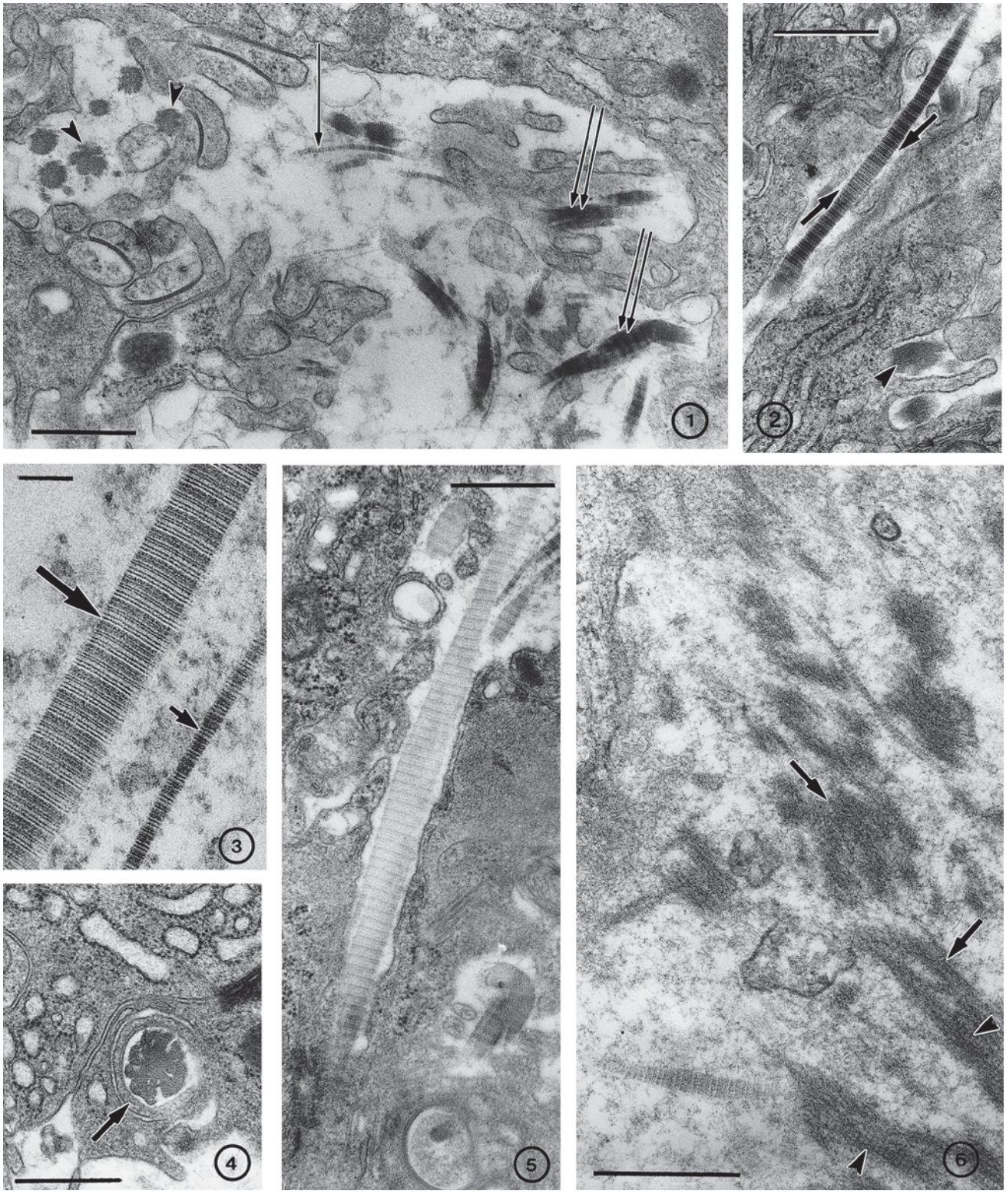
FIGURE 4. Thick collagen fibril completely encircled by a long and narrow projection (arrow) of the decidual cell cytoplasm.Fasted mouse, on day 11 of pregnancy. Magnification X 35,600. Bar = 0.5 μm.

FIGURE 5. A deep and narrow invagination of the decidual cell surface contains a thick collagen fibril which seems to be partly inserted in the cell cytoplasm and show pale cross-banding. Fasted mouse, on day 9 of pregnancy. Magnification X 35,600. Bar = 0.5 μm.

FIGURE 6. Filamentous aggregates show bundles of periodic (arrowheads) and aperiodic parallel-arranged filaments (arrows).The periodic filaments exhibit repeating cross-bands. Fed animal, on day 11 of pregnancy. Magnification X 50,000. Bar = 0.5 μm.

axis or swollen tubular horns without apparent dilatations. These uteri undergoing regression were not examined.

Animals were anesthetized with ether and killed by cervical dislocation; uteri were dissected and immediately immersed in a fixation mixture. Implantation sites were removed under a stereomicroscope and cut transversely along the axis of the uterus. Segments were obtained from the antimesometrial region of each decidual swelling. Segments from three animals of each group were used for light microscopy and ultrastructural localization of acid phosphatase activity. For morphological studies segments from two animals of each group were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate, pH



7.2, for 3 h at 4°C, postfixed in osmium tetroxide, dehydrated in ethanol and embedded in Araldite (EMS). Ultrathin sections were cut on a Sorvall MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM9A electron microscope (Carl Zeiss). For light microscopy and ultrastructural localization of acid phosphatase activity, segments from decidual antimesometrial region from fed and fasted animals, on the same day of pregnancy, were processed concomitantly. For histochemistry the specimens were fixed in calcium formalin with sucrose (1% calcium chloride, 7% sucrose, in 10% neutral formalin) for 12 h, at 4°C and were sectioned at 8 μm on a microtome cryostat (Microm HM500 OM). Free-floating frozen sections were washed in saline and transferred, simultaneously, into the same incubation medium prepared according to Holt (1959) containig sodium ß- glycerophosphate as a substrate and lead salt in acetate buffer, final pH 5.0, and optimum incubation time of 60 min, at 37°C. Control sections were incubated in medium free of substrate. For ultrastructural cytochemistry the specimens were fixed in a mixture of 1% paraformaldehyde and 1% glutaraldehyde in 0.1M sodium cacodylate, pH 7.2, for 2 h at 4°C. The tissues were subsequently rinsed with buffer and transversely sliced (70 μm-thick slices) with a tissue sectioner (TC2, Sorvall) and kept in buffer overnight at 4°C. Incubation at 37°C was performed in a medium containing 1 mM sodium ß-glycerophosphate and 2 mM cerium chloride in 0.1M acetate buffer, pH 5.0, for 2 h (Robinson and Karnovsky, 1983). Control specimens were incubated in substrate-free medium or in a complete incubation medium containing 0.01M sodium fluoride. After incubation, the slices were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, for 2 h at 4°C, followed by 1% osmium tetroxide. Subsequently, the specimens were dehydrated and embedded in Araldite. Stained and unstained ultrathin sections were observed in a Zeiss EM9A electron microscope.

Results

In both fed and fasted mice, the extracellular space between decidual cells showed fibrillar elements consisting of collagen fibrils (Figs. 1-5) and some bundles of filaments (Fig. 6).

Collagen fibrils consisted of thick fibrils (60-330 nm) (Figs. 1-5) which predominated over thin fibrils (15-30 nm) (Figs. 1 and 3). Longitudinal sections of thick and thin collagen fibrils showed typical collagen cross-banding (Figs. 1 and 3). Cross-sections of thick collagen fibrils exhibited an irregular profile, with many indentations (Figs. 1, 2 and 4).

Collagen fibrils were observed in small matrix compartments located between decidual cells surfaces and cytoplasmic processes of decidual cells and in recesses of cellular surfaces (Figs. 1 and 2). Cytoplasmic processes were noted surrounding completely the collagen fibrils for one (Fig. 2) or more times (Fig. 4) and thick fibrils with faint cross-banding appeared partly within the decidual cell and partly without (Fig. 5).

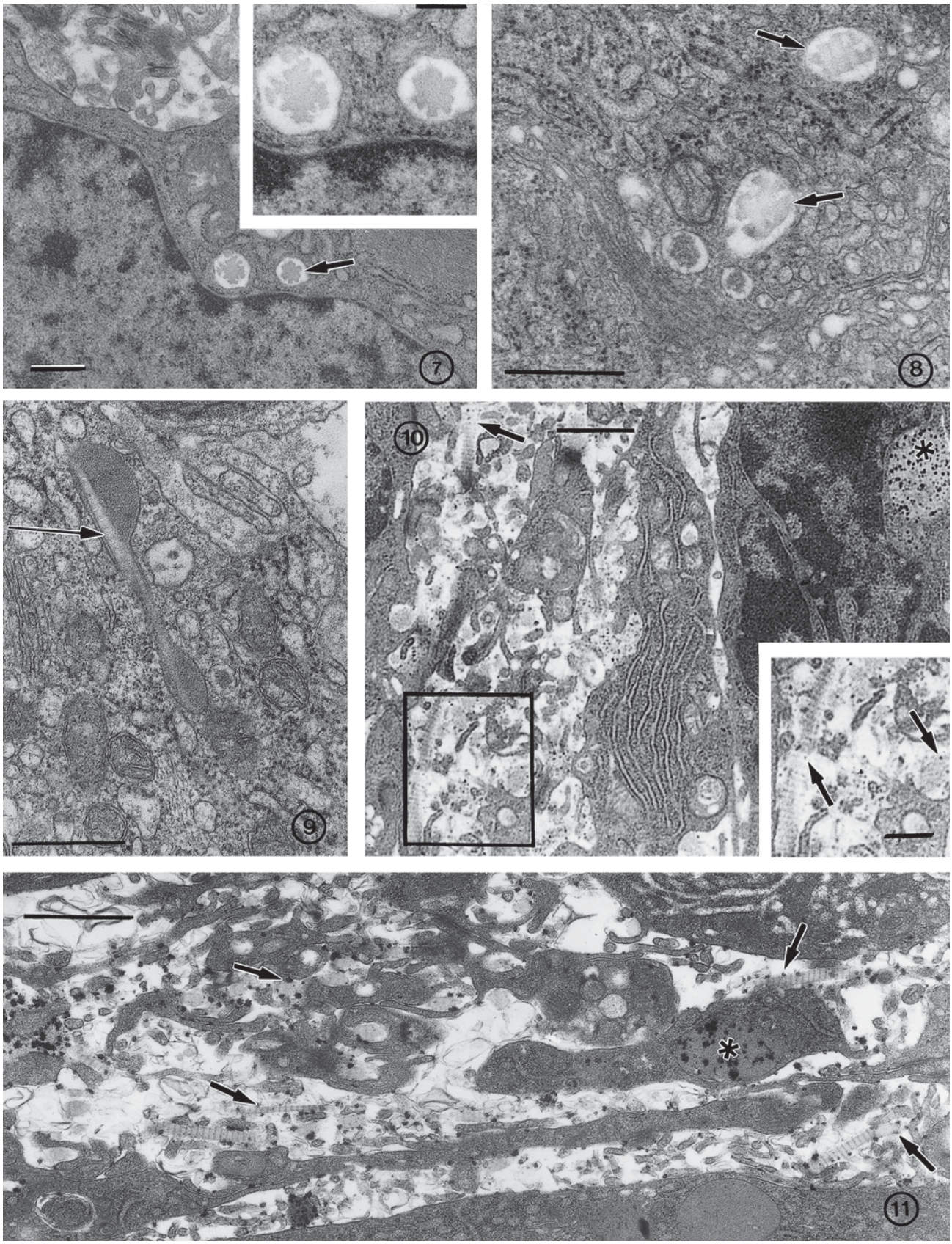
Bundles of filaments consisted of parallel-arranged filaments showing alternating dark and clear transverse bands intermingled with bundles of parallel-arranged filaments devoid of cross-banding and with similar electron-density (Fig. 6). These bundles of filaments are

FIGURES 7-8. Decidual cells of fasted mouse on the 10th and the 9th day of pregnancy, respectively, showing intracellular collagen containing vacuoles with transversely sectioned collagen fibrils surrounded by electron-translucent material (arrows). The vacuoles are present near the nucleus (Fig. 7; see detail of vacuoles in the inset) and in the region of Golgi complex (Fig. 8). Magnification Figure 7: X 18,800; X 44,500 (inset); Bar = 0.5 μm. Figure 8: X 42,720; Bars = 0.2 μm (inset); 0.5 μm.

FIGURE 9. Intracellular collagen containing vacuole showing a longitudinally sectioned thick collagen fibril, with faint cross-banding (arrow) immersed in electron-opaque material. Fasted mouse, on day 9 of pregnancy. Magnification X 40,050. Bar = 0.5 μm.

FIGURE 10. Region of mature decidual cells of fed animal (day 11 of pregnancy) incubated for acid phosphatase reaction. Granular electron-dense deposits due to acid phosphatase activity (arrows) are present in extracellular collagen fibrils which are located close to decidual cell surfaces and in recesses of the cellular surfaces (see detail in the inset). A lysosome exhibits dark deposits of reaction product (*). Magnification X 13,500 (Fig. 10); x19,500 (inset). Bar=1 μm (Fig.10); 0.5 μm (inset).

FIGURE 11. Region of mature decidual cells of fasted animal (day 11 of pregnancy) showing acid phosphatase activity. Dark deposits of reaction product are present in both extracellular collagen fibrils (arrows) and lysosomes (*); many collagen fibrils are surrounded by projections of decidual cells cytoplasm. Magnification X 19,360. Bar = 1 μm.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referred to as filamentous aggregates; they were observed in the region of mature decidual cells.

Decidual cells showed two types of vacuolar structures containing collagen fibrils: one of them exhibited typical collagen fibrils, more commonly one thick fibril, immersed in a large region of finely granular electron-translucent material (clear vacuole) (Figs. 7 and 8) and the other showed thick fibrils with a faint cross-banding surrounded by electron-opaque material (dark vacuole) (Fig. 9).

Clear vacuoles were seen in the periphery of the cell or deep inside the cell, near the nucleus (Fig. 7) or adjacent to the Golgi complex (Fig. 8).

Acid Phosphatase

Acid phosphatase activity was present both extracellularly and intracellularly in the antimesometrial involuting decidua from fed and fasted mice.

Enzyme activity was observed in the extracellular matrix including collagen fibrils that were observed in small compartments between decidual cell surfaces and projections of decidual cells cytoplasm or surrounded by cytoplasmic processes of the decidual cells (Figs. 10 and 11), located in very narrow spaces between decidual cell surfaces (Fig. 12) or in recesses or deep invaginations of the cell surface (Fig. 13). Filamentous aggregates, some with cross-banding (Fig. 14) also showed reaction product.

Decidual cells from fed and fasted mice showed acid phosphatase activity in lysosomes (Fig. 10) and in dark vacuoles (Fig. 15); activity was absent in the clear vacuoles.

In the region of mature decidual cells, fully transformed cells including many disintegrating cells, which surround the embryo, acid phosphatase activity was stronger than in the region of predecidual cells, which are located farther away from the embryo (Fig. 16). Enzyme activity in mature decidua was conspicuous in fasted mice (Fig. 11).

In extracellular collagen fibrils and filamentous aggregates, acid phosphatase activity was stronger in the region of mature decidual cells, chiefly in fasted mice (Fig. 13) than in predecidua (Fig. 12). Acid phosphatase activity in lysosomes was similar in all decidual cells in both fed (Fig. 10) and fasted (Fig. 11) mice. Control sections, without substrate (Fig. 17) or in complete medium containing sodium fluoride, were negative.

Discussion

We observed acid phosphatase activity in extracellular collagen fibrils and in filamentous aggregates in the mature region and predecidual region of antimesometrial decidua of fed and fasted mice; activity was also present in lysosomes and vacuoles of decidual cells.

Our morphological results showed that collagen fibrils were found in recesses or deep invaginations of the surface of decidual cells as reported by Welsh and Enders (1985) in the rat; a few fibrils inside deep invaginations showed signs suggestive of initial degradation and were apparently partly within the cell and partly without; some collagen fibrils were apparently enclosed by processes of decidual cells. Similarly morphological findings suggested extracellular degradation of collagen fibrils in periodontal ligament (Ten Cate and Deporter, 1975; Svoboda *et al*., 1979) in mouse and monkey, in the primary decidua (Parr *et al*., 1986) and in the antimesometrial deciduoma (O' Shea *et al*., 1983)

FIGURE 12. Portions of two closely packed predecidual cells from a fasted mouse (day 9 of pregnancy) that were incubated for acid phosphatase. Interdigitations between the cells are clearly delineated by the granular electron-dense deposits of reaction product (arrowheads) and an extracellular thick collagen fibril, with pale cross-banding, exhibits the granular electron-dense deposits (arrows). Unstained section. Magnification X 48,000. Bar = 0.5 μm.

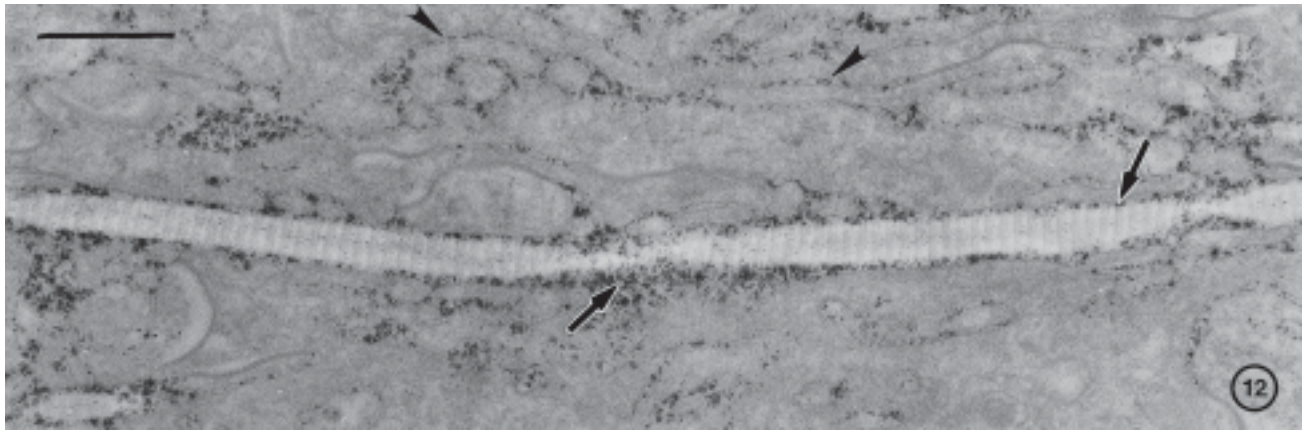
FIGURE 13. Extracellular collagen fibrils inside deep invaginations of mature decidual cell surface show dark deposits due to acid phosphatase positive reaction (arrows). Fasted mouse on the 11th day of pregnancy. Magnification X 48,000. Bar = 0.5 μm.

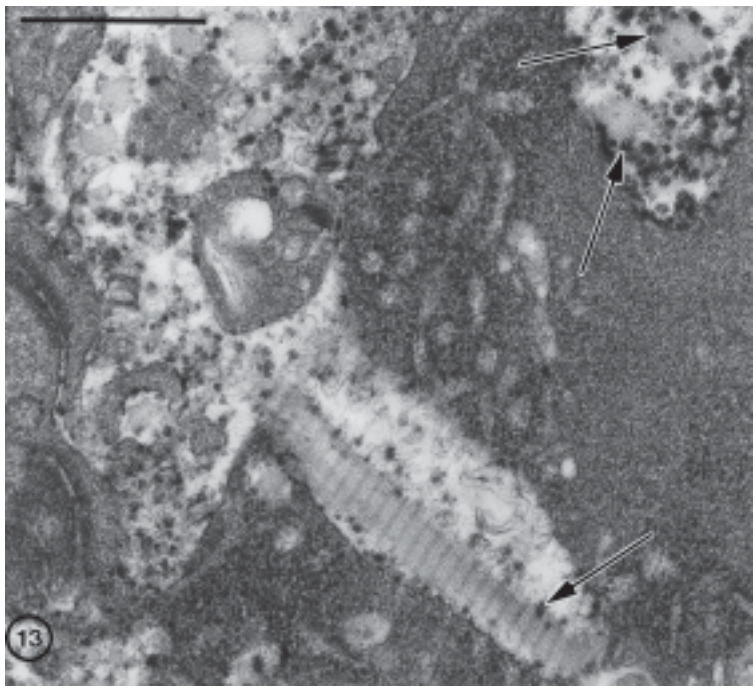
FIGURE 14. Filamentous aggregates of collagen show granular electron-dense deposits due to acid phosphatase activity (arrows). Fasted mouse on the 11th day of pregnancy. Magnification X 66,000. Bar = 0.5 μm.

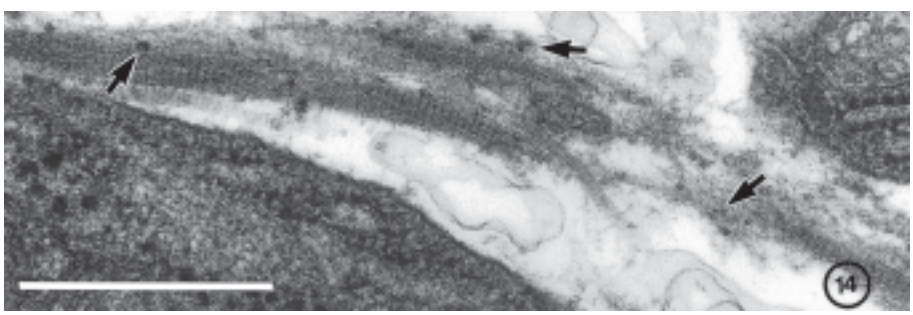
FIGURE 15. Decidual cell of fasted mouse (day 10 of pregnancy) showing intracellular collagen containing vacuole with acid phosphatase positive reaction. The collagen fibril show pale cross-banding. Arrows – vacuole delimiting membranes. Magnification X 44,000. Bar = 0.5 μm.

in rat. Thus, the small compartments between decidual cells processes and the recesses or deep invaginations of the surfaces of decidual cells may form microenvironments in which conditions may be suitable for extracellular degradation of collagen fibrils.

Acid phosphatase activity associated with extracellular collagen fibrils and filamentous aggregates chiefly in small compartments and recesses of the mature decidual region and predecidual region of fed and fasted mice suggest that lysosomal hydrolytic enzymes may be involved in extracellular collagen breakdown. Release of acid phosphatase into the extracellular space has been shown in bone matrix close to osteoclasts (Doty and Schofield, 1972; Baron *et al*., 1985), in involution of liver cirrhosis (Ryvniak, 1986), in the glomerular basement membrane of rats (Singh, 1993) and in small compartments of the matrix in mouse trophoblastic giant cells (Katz, 1995). Moreover acid phosphatase ac-







tivity was shown to take part in extracellular degradation of collagen fibrils during the involution of liver cirrhosis (Ryvniak, 1986) and also in mice trophoblastic giant cells region (Katz, 1995).

The origin and nature of the filamentous aggregates is not known but it is possible that they are part of the degradative process of collagen. Filamentous aggregates which appeared as bundles of parallel aperiodic and periodic filaments were similar to those described previously in our studies about mature decidua of food deprived mice (Spadacci-Morena and Katz, 2001) and trophoblastic giant cells region of fasted mice (Katz, 1995). Fainstat (1963) studied the disappearance of coarse collagen bundles in the early stages of pregnancy in the rat and concluded that these bundles broke up into finer filaments. Pérez-Tamayo (1970) in carrageenin granulomas and Kobayasi *et al*. (1985) in pathological dermis, showed similar filamentous aggregates due to collagen fibril desintegration. The striated pattern of filamentous aggregates is similar to that of fibrin (Hall and Slayter, 1959). However, in decidua, the filaments are not arranged in the configuration which is usual for fibrin. Acid phosphatase activity observed in filamentous aggregates suggested degradation by lysosomal enzymes similar to what has been shown in the trophoblast region of fasted mice (Katz, 1995).

Other enzymes, such as metalloproteinases and their inhibitors, have been shown to play a role in the extracellular space during decidualization and decidual involution; they are expressed by both decidual cells and trophoblastic giant cells (Alexander *et al*., 1996; Teesalu *et al*., 1999). During decidual involution, lysosomal enzymes are secreted to the extracellular space by both, the invasive trophoblast (Katz, 1995; Afonso *et al*., 1999) as well as by decidual cells (Katz, 1998). As suggested by Pérez-Tamayo (1978) it is probable that collagen degradation needs several enzymatic steps.

From 9-11th days of pregnancy in rodents, embryonic structures expand dramatically in the uterine antimesometrial direction. Antimesometrial decidua

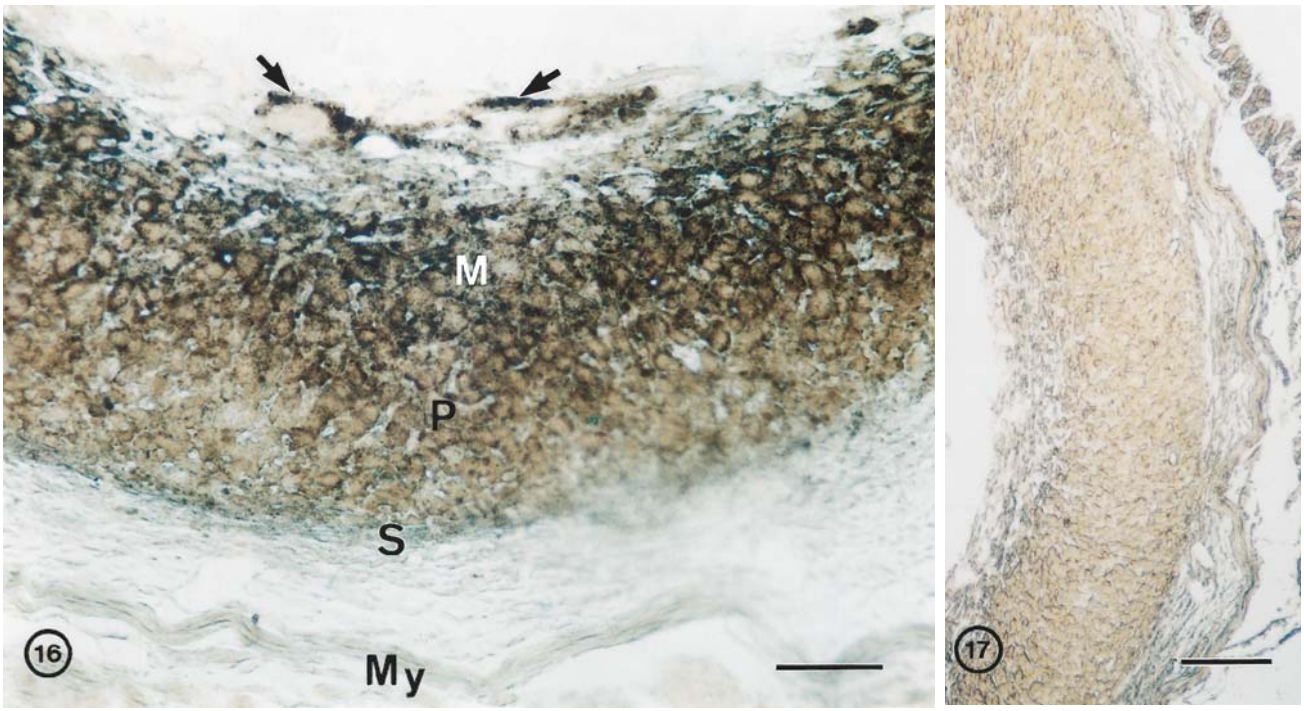


FIGURE 16. Light photomicrograph of a transverse section of the antimesometrial decidua of a fed mouse on day 9 of pregnancy showing acid phosphatase activity. Dark deposits of reaction product are present in the region of mature decidua (M) and in the region of trophoblastic giant cells (arrows); a weak reaction for acid phosphatase is observed in the region of predecidua (P). S, nondecidualized stromal cells; My, myometrium. Magnification X 354. Bar = 10 μm.

FIGURE 17. Transverse section of the antimesometrial decidua of a fed mouse on day 9 of pregnancy incubated for the demonstration of acid phosphatase activity in a medium free of substrate. No reaction product is observed. Magnification X 140. Bar = 20 μm.

must remodel in a short time to accomodate the growing embryo (Welsh and Enders, 1985; Katz and Abrahamsohn, 1987). Kornfeld (1987) referred that "lysosomes participate in the remodeling of tissues and lysosomal enzymes are actually secreted by the cell into a localized acidic extracellular compartment". In involuting decidua, there are healthy decidual cells and a gradual death of many mature decidual cells which surround the embryo to vacate space for the growing embryo; hydrolytic enzymes secreted by healthy decidual cells are enhanced in mature decidua with release of the enzymes contained in cells with disintegration and it appears that breakdown of collagen and other matrix constituents are part of remodeling. Similar occurrence is related by Ten Cate (1971) in erupting teeth where the author had observed breakdown of extracellular collagen fibrils in connective tissue including disintegrating fibroblasts.

In our material there seems to be, in the decidua, partial degradation of collagen in the extracellular space, followed by phagocytosis of brokendown fragments of collagen fibrils by decidual cells. In addition our results show large pale vacuoles (clear vacuoles) containing banded collagen fibrils and electron-opaque vacuoles (dark vacuoles) filled with fibrils with very faint cross-banding.

Similarly as observed in fibroblasts (Deporter and Ten Cate, 1973; Ten Cate and Syrbu, 1974) and in mice trophoblastic giant cells (Katz, 1995), in decidual cells acid phosphatase activity was absent in clear collagen-filled vacuoles and was observed in electron-dense collagen-filled vacuoles. Our observations suggest that collagen-containing vacuoles (clear vacuoles) may migrate to regions close to the Golgi complex, or deep in the cytoplasm, where they eventually fuse with lysosomes, where their content are further degradaded by lysosomal enzymes thus becoming a dark vacuole. However, in mice decidual tissue only intracellular electron-dense collagen-containing vacuoles were observed and exhibited acid phosphatase reaction product; it was suggested that intracellular degradation of collagen was the only mechanism of collagen breakdown (Zorn *et al*., 1989).

Our results indicate that there is extra and intracellular degradation of collagen in decidua. It has been suggested (Fainstat, 1963) that decidual collagen fibril degradation and disappearance may be associated with lessened rigidity of the tissue, thus permitting the growth of trophoblast in the uterine region, while contributing to the supply of extracellular nutrient molecules used for the nourishment of the growing cells; small peptides derived from the collagen degradation may be rapidly taken up by the blood stream (Shimizu *et al*., 1986).

In acute food restricted mice (day 9 of pregnancy) extracellular collagen fibrils and filamentous aggregates are more conspicuous as food restriction becomes more severe; it is proposed that collagen breakdown is enhanced in food-restricted animals (Spadacci-Morena and Katz, 2001). Rao and Rao (1981) observed reduction in collagen synthesis in the uterus of rats submitted to protein malnutrition. In trophoblast giant cells region (days 9 – 11 of pregnancy), extracellular collagen fibrils showed acid phosphatase activity in acute fasted mice and it was absent in fed animals (Katz, 1995).

During 9-11 days of pregnancy in rodent embryos growth is fast and there is substantial organogenesis (Rugh, 1968); maternal fasting during this period results in embryos with retarded growth and differentiation and with incidence of deformities (Runner and Miller, 1956; Ellington, 1980). There is a prompt fall in fetal glucose concentrations and, to compensate, an increase in available amino acids (Simmons *et al*., 1974; Johnson *et al*., 1986; Liechty *et al*., 1991).

It is possible that extra and intracellular collagen degradation by lysosomal enzymes in the region of predecidual cells, where enzyme activity in both fed and fasted mice, is weaker than in mature decidual cells region, may be part of the process of predecidual cells remodeling and differentiation in this region. In the region of mature decidual cells, which surround the embryo, acid phosphatase activity on collagen fibrils was strong in fed and fasted mice and conspicuous in fasted animals, suggesting that in addition to remodeling, mature decidual cells are related to nutrition of the fetus and can provide nutrients as amino acids, monosaccaharides, for the developing fetus, also in acute maternal fasting.

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