ESTIMATION OF PROTEOLYTIC ACTIVITY AT MOUSE IMPLANTATION SITES BY THE GELATIN DIGESTION METHOD

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Summary. A faint proteolysis without any appreciable peak intensity on Days 5 to 9 of normal pregnancy surrounds the implantation sites in mice. Neither the antimesometrial trophoblastic zone nor the ectoplacental cone showed more activity than the rest of the tissue. Uterine epithelium during this period showed a slightly stronger and more distinctly localized proteolysis. No epithelial activity was found during early pregnancy. Day-4 blastocysts, studied in vitro, showed no activity. It is concluded that the uterine epithelium at the time of implantation has an intrinsic ability to undergo autolysis and that the autolysis can be elicited by the trophoblast.

INTRODUCTION

The mechanisms by which the blastocyst adheres to and finally invades the uterine wall are unknown. The model of invasion is thought to be phagocytic (Finn & Lawn, 1968), since the trophoblast cells appear to contain remains of degenerating uterine epithelial cells. The supposed phagocytosis by the trophoblast cells implies a possible proteolytic activity during the invasive stage of trophoblastic growth.

Biochemically, the presence of peptidases has been taken as a sign of proteolytic activity by a number of workers using rat uteri under different hormonal influences (Shelesnyak & Kraicer, 1960; Albers, Bedford & Chang, 1961; Schmidt, Walther & Voigt, 1966). Shelesnyak & Kraicer (1960) concluded that a similar degree of proteolysis occurs during both implantation and pseudopregnancy. Hence, no measurable proteolytic activity should occur in the trophoblast tissue during implantation in the rat if the sensitivity of their method is sufficient to exclude trophoblastic activity.

The presence of proteolytic enzymes has been demonstrated histochemically on the surface of free guinea-pig blastocysts but not on that of rat blastocysts by incubation of such blastocysts on gelatin films (Blandau, 1949). Owers & Blandau (1968) reported proteolytic activity also in the implanted 8-day guinea-pig blastocyst after its removal from the animal on to a gelatin film. However, no sections have been examined histochemically for proteolytic activity at the
implantation sites. The gelatin digestion method is an appropriate technique for this purpose.

This method as originally described by Adams & Tuqan (1961) and evaluated by Daoust (1965, 1968) and Cunningham (1967) is convenient for general histochemical localization of proteolytic activity. Since gelatin contains a lot of different proteins, it is heterogeneous as to peptide specificity and hence it is convenient as a screening method before undertaking detailed investigation with defined substrates. Fratello (1968) obtained enhanced sensitivity by using colour film emulsion. This method was used in the present investigation.

The purpose of the present study was to determine whether or not a proteolytic activity exists in pre-implantation mouse blastocysts and at mouse implantation sites during the early invasive stage of trophoblastic growth.

**MATERIAL AND METHODS**

Virgin albino mice of N.M.R.I. (Bethesda) strain, aged 7 to 8 weeks and weighing 20 to 27 g, were examined on Day 3 and on Days 5 to 9 of pregnancy (the day on which a vaginal plug was detected was designated as Day 1). On each of these days, all the implantation sites of two to three animals were examined. After the injection of Nembutal intraperitoneally the animals were perfused through the abdominal aorta with 4% formaldehyde (adjusted with phosphate buffer to pH 7-4) for 5 to 7 min. The implantation sites were then removed for immediate rapid freezing in isopentane previously cooled in liquid nitrogen using the method described by Fuxe & Nilsson (1962) and longitudinal serial sections of 10 µ were cut in a cryostat. Simultaneously, strips of unexposed Ferrania 3M colour film, which had been processed earlier, were dampened in 0·1 M-phosphate buffer and allowed to undergo partial drying at room temperature. The cryostat sections were mounted as soon as possible on to the film strips, where the sections melted. Immediately after incubation for 30 min at 38° C in an atmosphere saturated with water vapour, the strips were dried at room temperature and subsequently examined uncovered.

At first, the film strips were allowed to freeze in the cryostat before mounting the frozen sections, but this resulted in too much fluid on the emulsion when the film was thawed: the diffusion of proteolytic enzymes was greater on the gelatin when the gelatin was frozen and thawed than when it was not frozen before use.

Adhesion of the tissue sections on to the gelatin surface of the film was so firm that the sections could neither be removed nor washed off before or after drying. Prolonged washing dissolved the proteolytic enzymes in the mounted sections and, in the case of ileum, rendered the film colourless by complete digestion.

The possible effect of the fixative used (formaldehyde) was tested by mounting non-fixed sections on the film in the same way as above.

Pre-implantation blastocysts were recovered on Day 4 of pregnancy and subsequently transferred to tissue chambers using the colour film as a 'floor'. Incubation was carried out for 30 min at 37° C. The 'floor' was then examined for digestion-'prints'. The medium described by Whitten & Biggers (1968) was used throughout.
Reference control tissue of known proteolytic activity was taken from rat ileum (Daoust, 1965) after washing the lumen with saline. Uterine tissue of Day 3 of pregnancy served as control to that of Days 5 to 9 of pregnancy.

RESULTS

Progestational uterine tissue (Day 3 of pregnancy) did not demonstrate any proteolytic activity, either in the stroma or the epithelium.

The uterine epithelium taken from between the implantation sites showed a low and distinctly localized level of activity while the implantation sites showed a still lower but more diffuse proteolysis. Neither the anti-mesometrial trophoblastic zone nor the ectoplacental cone situated mesometrially showed more activity than the rest of the tissue.

The pre-implantation blastocysts showed no proteolytic activity \textit{in vitro}.

In general, unfixed sections showed a more pronounced activity than fixed ones, but diffusion artifacts made localization difficult.

The villi in control sections of the ileum showed a high level of activity, distinctly localized in the epithelium.

DISCUSSION

Tissue proteolytic activity is reported to be only slightly decreased by formaldehyde fixation and histochemical results are said to be improved by using fixed tissues instead of unfixed ones (Adams & Tuqan, 1961). With the present technique, however, there seems to be an advantage in using unfixed fresh-frozen tissue to judge by the extent of digestion of the emulsion by the sections of rat ileum. Unfortunately, unfixed tissues gave a rather bad preservation of the structure on the film. Glutaraldehyde (2.5\% buffered to pH 7.4) was also tried, but it coloured the emulsion yellow which interfered with examination for proteolysis.

The physical conditions for enzymatic digestion were kept constant as far as possible. The incubation time of the implantation sites was extended from 30 min to 24 hr but no further activity was noted. In this investigation, the pH was not altered deliberately. The emulsion dampened with buffer was found to keep a constant pH throughout the dampening and incubation processes as seen from indicator strips.

The degree of proteolysis was estimated by observation of the colours left in the emulsion after digestion. Since the most superficial film layer is yellow and the deepest is blue, a faint activity will dissolve only the yellow layer leaving a bluish colour. The yellow pigment, however, can sometimes be absorbed by the tissue sections, which then turn yellow. If, on the other hand, there is an intense proteolysis, all layers are digested and the remaining emulsion turns pale or slightly bluish.

The pitfalls of gelatin digestion methods were discussed in detail by Daoust (1968). As the colour film is about ten times more sensitive than conventional panchromatic film due to thinness of the layers (Fratello, 1968), the need for control tissue sections is obvious. The technical handling of the cut sections and
their placing on the dampered film are critical preparatory steps and the requisite degree of dampering of the film must be found out experimentally in order to get maximum digestion and minimum diffusion. The conditions for optimum dampering are obtained by using a reference tissue with known proteolytic activity (here, rat ileum).

Earlier studies of proteolytic enzymes in the rat uterus have shown an increased activity during implantation but they have not revealed the site of the activity. The present experiments demonstrate that an increased capacity for proteolytic digestion is present in the uterine epithelium. This capacity is also observed diffusely at the implantation sites and is not limited to the uterine epithelium in these regions.

The faint and somewhat diffuse proteolysis found at the implantation sites is probably not a diffusion artifact since adjacent epithelium between the implantation sites showed a distinctly localized activity. Rather, this diffuse activity may reflect the changed properties of the uterine cells at the sites of trophoblastic invasion and may indicate an initial autolysis at these sites. The experiments do not exclude the possibility of proteolytic activity by the trophoblast cells but suggest that such activity is weak.

The uterine epithelium during early pregnancy is influenced by oestrogen. Since lysosome-like bodies in the uterine epithelium of the mouse (Nilsson, 1962) and the rat (Ljungkvist, personal communication) increase in number in response to oestrogen, there may be a relation between the appearance of proteolytic activity and the presence of lysosome-like bodies in the uterine epithelium in a hormonal condition compatible with that of implantation.

The results of these experiments suggest that the uterine luminal epithelium at the time of implantation has an intrinsic ability to undergo autolysis and that the autolysis can be elicited by the trophoblast tissue and other agents, such as a deciduoma (Finn, 1966).

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REFERENCES


Proteolytic activity at mouse implantation sites


