Vol. 98, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1981 Pages 562-568

CHYMOTRYPSIN STIMULATED DEVELOPMENT OF DELAYED IMPLANTATION MOUSE BLASTOCYSTS

Lon J. Van Winkle

Biochemistry Dept., Chicago College of Osteopathic Medicine

1122 East 53rd Street, Chicago, Illinois, 60615

Received December 20,1980

SUMMARY

Chymotrypsin-like enzyme activity increases transiently in the uterine lumen of ovariectomized mice upon administration of progesterone and estrogen (1). This is one of the few known macromolecular changes associated with conditions which result in activation of delayed implantation blastocysts *in utero*. *In vitro*, α-chymotrypsin (100 μg/ml) was found to shorten the time required for these embryos to attach to the glass culture dish and then form outgrowths in fetal calf serum-supplemented medium. Higher concentrations of the enzyme (250 μg/ml) prevented embryo attachment probably by digesting the fetuin present in fetal calf serum. Nevertheless, 250 μg/ml α-chymotrypsin could apparently replace fetal calf serum as a stimulator of development during the first 24 hours of culture. In contrast, bovine serum albumin (3.0 mg/ml) seemed to slow development of blastocysts *in vitro*. It is suggested that chymotrypsin-like enzyme activity may stimulate development of delayed implantation blastocysts *in utero* (a) indirectly by removing inhibitory proteins such as albumin and (b) by directly affecting these embryos in a manner yet to be determined.

INTRODUCTION

Delayed implantation blastocysts undergo activation and peri-implantation developmental changes when cultured in Dulbecco's modified Eagle medium (MEM) containing fetal calf serum (FCS) (2). Similarly, these embryos implant in the uterus of ovariectomized mice more than 24 hours after administration of estrogen and progesterone (2). The latter hormone treatment changes the nature of uterine macromolecules during the pre-implantation period of activation (3,4) although most of these macromolecules have not been identified. Thus, it is difficult to theorize about the similarities and differences in the mechanism of activation and implantation of delayed implantation blastocysts *in utero* vs *in vitro*.

0006-291X/81/020562-07$01.00/0

Hoversland and Weitlauf (1) have reported that a chymotrypsin-like enzyme in uterine flushings from ovariectomized mice is transiently elevated to a peak activity 18 hours after administration of estrogen and progesterone. Moreover, some proteases stimulate the growth of cells in culture (5,6,7). Furthermore, epidermal growth factor has been shown to have proteolytic activity (8). For these reasons we examined the effect of α-chymotrypsin on peri-implantation development of delayed implantation mouse blastocysts *in vitro*.

MATERIALS AND METHODS

Delayed implantation mouse blastocysts were flushed from the uteri of mated, ovariectomized mice with Dulbecco's modified Eagle medium (MEM; GIBCO) on days 9 through 13 post coitus (p.c.). The mice had been induced to ovulate and mate by administering gonadotropins (9). The day of copulatory plug detection was designated day one of pregnancy. Ovariectomies were performed before noon on day 4 p.c. Embryos were washed twice in MEM, placed in the concavity of a Maximov slide containing 1.6 ml of one of the media described below, and cultured in a humidified 5% CO_2:95% air environment at 37°C. The Maximov slide was placed in a Falcon plastic petri dish containing deionized water during all incubations to help maintain 100% humidity and avoid temperature fluctuations while the cultures were being examined at room temperature with a dissecting microscope.

Blastocysts were incubated for several days in MEM plus 10% fetal calf serum (FCS; GIBCO) or this medium plus 100 or 250 μg/ml of α-chymotrypsin (Sigma Type I-S or Type II). After two or three days of incubation, trypsin inhibitor (TI) from turkey egg white (5.0 mg/ml where 1.0 mg of TI can inhibit 0.66 mg of α-chymotrypsin; Sigma), fetuin (2.0 mg/ml; Calbiochem), bovine serum albumin (3.0 mg/ml; crystallized and lyophilized; Sigma), glycoprotein (2.0 mg/ml; bovine, Fraction VI; Sigma) and/or mucin (2.0 mg/ml; Type I, from bovine submaxillary gland; Sigma) were added to some cultures which contained 250 μg/ml α-chymotrypsin. In other experiments, embryos were incubated for the first 22-26 hours in (a) MEM; (b) MEM plus α-chymotrypsin (250 μg/ml), BSA (3.0 mg/ml) or fetuin (2.0 mg/ml); (c) MEM plus 10% FCS or (d) MEM plus 10% FCS and α-chymotrypsin (250 μg/ml), Table II. Following the 22-26 hour incubation, embryos were washed in and then transferred to MEM plus 10% FCS and observed for the onset of attachment and the formation of outgrowths. The concentrations of BSA (3.0 mg/ml) and fetuin (2.0 mg/ml) were chosen to reflect the concentrations of these macromolecules in 10% serum (10) and 10% FCS (11) respectively. The numbers of embryos which had formed outgrowths following various treatments were compared statistically employing two-by-two contingency tables (12). Attached and unattached blastocysts were grouped together for the statistical analysis.

RESULTS

Chymotrypsin (100 μg/ml) shortened the time required for delayed implantation blastocysts to begin forming outgrowths *in vitro* ($p < 0.01$)

TABLE I

Effect of α-chymotrypsin on attachment and the formation of outgrowths by delayed implantation blastocysts 49 hours after the onset of *in vitro* culture.[1]

Concentration of α-chymotrypsin in MEM + 10% FCS	Number of Embryos Which Have		
	Formed Outgrowths	Attached	Remained Unattached
none[a]	14	11	0
100 µg/ml[b]	24	2	0
250 µg/ml[c]	0	0	26

[1] In a given experiment some embryos were incubated under all three culture conditions. Presented are results from three pooled experiments since independent experiments gave similar results. Groups with different superscripts had significantly different fractions of embryos which formed outgrowths ($p < 0.01$).

but higher concentrations of this enzyme (250 µg/ml) completely prevented attachment of these embryos to the culture dish, Table 1. When embryos were incubated with 250 µg/ml α-chymotrypsin, addition of 0.18 ml of fresh FCS after two or three days of culture resulted in attachment of most embryos within four hours. If no additions were made, the embryos remained unattached. It has been suggested that fetuin, a glycoprotein found in FCS, is required for mouse blastocyst attachment *in vitro* (13). In support of this suggestion, added fetuin also induced attachment within 4 hours but the embryos were again unattached after 24 hours. Attachment may have been permanent when FCS but not fetuin was added because FCS contains protease inhibitors. Addition of more FCS may have been sufficient to prevent fetuin digestion just as a lower level of added α-chymotrypsin may have been insufficient to overcome protease inhibitors in the medium, Table 1. Addition of fetuin plus trypsin inhibitor (TI) induced permanent attachment of embryos probably because the proteolytic activity of α-chymotrypsin was inhibited. Fetuin seems to have some specificity in facilitating attachment since added BSA, glycoprotein or mucin, with or without TI, did not induce attachment.

TABLE II

Effect of α-chymotrypsin, bovine serum albumin, and fetuin on development of delayed implantation blastocysts.[1]

Additions to MEM during the first 22-26 h of culture[2]	Number of Experiments (total number of embryos)	Percentage of embryos forming outgrowths, attaching or remaining unattached, respectively, after various times in culture			
		22 - 26 h	44 - 48 h	52 - 54 h	67 - 75 h
None[a]	7 (138)	0.0, 44.9, 55.1	8.7, 37.7, 53.6	25.4, 57.2, 17.4	89.9, 5.8, 4.3
α-chymotrypsin(α-C) (250 μg/ml)[b]	4 (65)	0.0, 23.1, 76.9	49.2, 35.4, 15.4	70.8, 27.7, 1.5	96.9, 1.5, 1.5
FCS (10%)[b]	11 (180)	0.6, 7.2, 92.2	45.6, 45.6, 8.9	71.3, 22.8, 5.9[3]	95.0, 3.9, 1.1
FCS (10%) + α-C (250 μg/ml)[b]	3 (50)	0.0, 0.0,100.0	64.0, 28.0, 8.0	———	100.0, 0.0, 0.0
Fetuin (2.0 mg/ml)[a]	3 (71)	0.0, 7.0, 93.0	14.1, 36.6, 49.3	35.2, 47.9, 16.9	85.9, 9.9, 4.2
BSA (3.0 mg/ml)[c]	3 (72)	0.0, 56.9, 43.1	5.6, 23.6, 70.8	6.9, 41.7, 51.4	50.0, 26.4,23.6

[1] Embryos were incubated 22-26 h in the medium indicated, washed, then transferred to MEM + 10% FCS. In cases where one treatment group is compared statistically to another, embryos were taken from a common pool and placed in the different culture conditions. The results of individual experiments were similar so the data were pooled.

[2] Groups with different superscripts have significantly different fractions of embryos which had formed outgrowths after 44-48, 52-54, and/or 67-75 h of culture ($p < 0.01$).

[3] Only 8 of 11 cultures in this group were examined after 52-54 h of culture (total of 136 embryos).

Embryos prevented from attaching by a high α-chymotrypsin concentration appeared collapsed after three days and tended to clump together after 3-5 days. Nevertheless, these embryos attached and formed outgrowths upon transfer to fresh medium containing 10% FCS.

A high concentration of α-chymotrypsin (250 μg/ml), as the only macromolecule in the medium during the first day of culture, reduced the time required before delayed implantation blastocysts began forming outgrowths *in vitro* ($p < 0.01$), Table II. The enzyme apparently stimulated development to the same extent as 10% FCS during the first 24 hours of culture, Table II. BSA, but not fetuin, slowed pre-attachment development ($p < 0.01$), Table II.

DISCUSSION

The present results suggest that the transient rise in chymotrypsin-like enzyme activity in the uterine lumen of ovariectomized mice after administration of progesterone and estrogen (1) could hasten pre-implantation development of delayed implantation blastocysts (a) indirectly by removing inhibitory proteins, such as albumin, and (b) directly by affecting blastocysts in a yet to be determined manner, Figure 1. Psychoyos (14) suggested that the "estrogen surge", which occurs in rats near the time of nidation, causes transudation of serum proteins, including albumin, into uterine tissue. Furthermore, protein with an electrophoretic mobility the same as albumin has been detected in mouse uterine flushings (3). It has also been reported that a brief pre-attachment exposure of normal blastocysts to pronase (two minutes) and trypsin (30 minutes) enhances development of these embryos after they form outgrowths *in vitro* (15).

Fetuin, which facilitates attachment *in vitro*, is apparently α-chymotrypsin-labile. If similar protein molecules are required for attachment *in utero*, then the rise in uterine chymotrypsin-like enzyme activity following progesterone and estrogen administration may need to be transient. In this regard, uterine chymotrypsin-like enzyme also increases after admin-

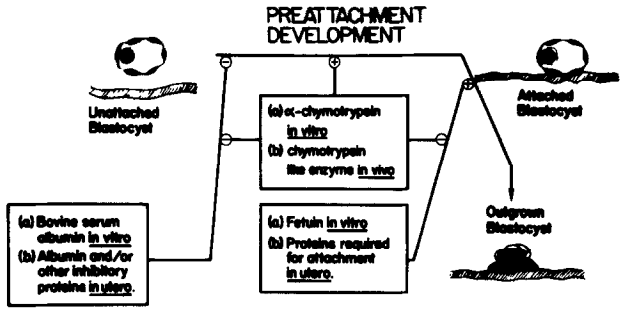

FIGURE 1: Possible correlation between factors (a) which affect activation of delayed implantation blastocysts in vitro and (b) those which may affect activation in utero.

istration of estrogen to ovariectomized mice, but the activity remains elevated (1). The fact that the enzyme activity remains high may help account for the lack of attachment and further development of delayed implantation blastocysts in utero after administration of estrogen alone (1), just as a high α-chymotrypsin level prevented attachment in vitro, Table I.

Some previously reported evidence is consistent with the suggestion that proteins secreted into the uterine lumen may be digested more readily 15 to 20 hours after administration of estrogen and progesterone to ovariectomized mice. Newly synthesized proteins appear to be secreted into the uterine lumen (a) in relatively large amounts 2 hours and 30 hours after injecting estrogen and progesterone but (b) in small amounts between 15 and 20 hours after administration of these hormones (3). However, increased degradation of newly secreted proteins could also result in diminished detection of protein secretion between 15 and 20 hours.

ACKNOWLEDGMENTS

The author wishes to thank Drs. Wells Farnsworth and David Mann for reading the manuscript criticially and Ms. Barbara Le Breton for typing it. These studies were supported by the Chicago College of Osteopathic Medicine.

REFERENCES

1. Hoversland, R. C. and Weitlauf, H. M. (1978) Biol. of Reprod. 19, 908-912.
2. Sherman, M. I. and Wudl, L. R. (1976) The Cell Surface in Animal Embryogenesis and Development, pp. 81-125, Elsevier/North-Holland Biomedical Press.
3. Pratt, H. P. M. (1977) J. Reprod. Fert. 50, 1-8.
4. Fishel, S. B. (1979) J. Reprod. Fert. 55, 91-100.
5. Sefton, B. M. and Rubin, H. (1970) Nature 227, 843-845.
6. Chen, L. B. and Buchanan, J. M. (1975) Proc. Natl. Acad. Sci. 72, 131-135.
7. Noonan, K. D. (1976) Nature 259, 573-576.
8. Green, D. A. and Moore, J. B., Jr. (1980) Archives of Biochem. and Biophys. 202, 201-209.
9. Fowler, R. A. and Edwards, R. G. (1957) J. Endocrinol. 15, 374-384.
10. Melby, E. C. and Altman, N. H. (1976) A Handbook of Laboratory Animal Science 3, p. 498, CRC Press, Cleveland.
11. Pedersen, K. J. (1947) J. Phys. Colloid Chem. 51, 164.
12. Woolf, C. M. (1968) Principles of Biometry, pp. 302-307, D. Van Nostrand Company, London.
13. Rizzino, A. and Sherman, M. I. (1979) Exptl. Cell Res. 121, 221-233.
14. Psychoyos, A. (1973) Handbook of Physiology, Section 7, vol. 2, part 2, 187-215.
15. Pienkowski, M., Solter, D., and Koprowski, H. (1974) Exptl. Cell Res. 85, 424-428.