Protein synthesis and degradation in non-cultured and in-vitro cultured rabbit blastocysts

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Summary. In 'pulse-chase' experiments synthesis and half-lives of leucine-labelled proteins were determined in rabbit blastocysts. Embryos were either non-cultured controls or were cultured for 24 h or 48 h in Ham's F-10 medium supplemented with homologous serum or uterine flushings. In control blastocysts protein synthesis increased by a factor of 10 between Day 4 and Day 5. Half-lives of newly synthesized proteins were 32 h in Day-4 and 99 h in Day-5 control blastocysts. In-vitro culture of Day-4 blastocysts led to dramatically shortened half-lives, amounting to 6–10 h. Blastocysts developing in uterine flushing-supplemented media differed significantly from those cultured in serum-supplemented media. Protein synthesis was enhanced and protein degradation was normal for culture times up to 24 h. These results demonstrate (1) that half-lives of proteins in rabbit blastocysts increase with advancing embryonic age, and (2) that a characteristic feature of the altered metabolism of cultured blastocysts is a dramatically accelerated protein degradation, which (3) can be prevented for some time by supplementation of the culture medium with uterine secretions.

Keywords: rabbit; blastocysts; protein synthesis; protein degradation; in-vitro culture; uterine secretions

Introduction

Protein synthesis in rabbit embryos has been studied at various developmental stages *in vivo* as well as *in vitro* (Manes & Daniel, 1969; Klinger *et al.*, 1971; Karp *et al.*, 1974; Lambert & Hamner, 1978; Matheson & Schultz, 1980; Jung *et al.*, 1987; Jung & Fischer, 1988). However, less information is available on protein degradation by preimplantation embryos (Epstein & Smith, 1974; Brinster *et al.*, 1976, 1979; Merz *et al.*, 1981; Wiebold & Anderson, 1985).

The present study was performed to investigate protein synthesis and degradation in cultured and non-cultured rabbit blastocysts.

Materials and Methods

Embryos and in-vitro culture. Rabbit blastocysts were obtained from superovulated cross-bred females 4 or 5 days after mating (Jung *et al.*, 1987; Fischer & Meuser-Odenkirchen, 1988). Uteri were flushed with medium BSM II containing 0.1% BSA (Maurer, 1978). Embryos were washed free of genital tract fluid, examined under a stereomicroscope, pooled, and allocated randomly to the various experimental groups. In-vitro culture was performed in 1 ml Ham's F-10 medium (Ham, 1963) supplemented with 10% (v/v) heat-inactivated blood serum from Day-4 pregnant females or with uterine flushings derived from Day-4 pseudopregnant does (Jung *et al.*, 1987). Uterine flushings were obtained by instillation of 3 ml protein-free Ham's F-10 medium into excised, double-ligated uteri.

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After 30–45 min storage at 37°C the medium was recovered and the cellular components were removed by centrifugation. The protein concentrations amounted to 7–8 mg/ml in serum-supplemented media and ranged from 2.5 to 5.0 mg/ml in media supplemented with uterine secretions.

Amino acid incorporation. Blastocysts were pulse labelled for 2 h in 1 ml Ham's F-10 medium containing L-[4,5-³H]leucine (sp. act. 1.91 TBq/mmol; Amersham Buchler, Braunschweig, FRG) at 1.85 MBq/ml, then rinsed 5 times in medium BSM II containing 0.1% BSA and randomly allocated to 3 groups. Embryos in Groups 2 and 3 were chase cultured for 2.5 h or 5 h, respectively, in Ham's F-10 medium supplemented with 10% (v/v) rabbit serum and enriched with unlabelled leucine (final concentration: 100 mM). The chase medium contained a 1000-fold greater concentration of leucine than the pulse medium in order to prevent the re-incorporation of the labelled amino acid. After the chase periods or immediately after pulsing (Group 1) the embryos were rinsed 5 times in PBS (phosphate-buffered saline, Dulbecco & Vogt, 1954) and processed individually for scintillation counting. Processing involved lysis in a buffer (1% SDS, 0.05% DTT and 8 M-urea in H₂O), freezing, precipitation of embryonic proteins with TCA, centrifugation, dissolution of the pellet in a tissue homogenizer, and liquid scintillation counting (for further methodological details see Jung *et al.*, 1987).

Statistical analysis. Differences were assessed by the Kruskal–Wallis non-parametric test with multiple comparisons (synthesis studies) or by analysis of variance (degradation studies). Pair comparisons were made by the two sided Mann–Whitney–Wilcoxon test or by Duncan's multiple *t* test. Least squares regression analysis was used to analyse the logarithmically transformed recovery rates after the chase periods of pulse incorporated [³H]leucine (pulse period = 100% recovery rate). The mean half-life (*t*₁) of labelled proteins was calculated from the equation of regression (Sachs, 1984).

Results

Control blastocysts

Embryonic protein synthesis increased by a factor of 10 from Day 4 to Day 5 (Table 1: see incorporation data after pulse period). Half-lives of the labelled proteins amounted to 32 h and 99 h in Day-4 and Day-5 blastocysts, respectively (Table 1). Because of the short chase periods in comparison to the calculated half-lives, the coefficients of regression for protein degradation were not statistically significantly different, indicating only the tendency towards longer half-lives with advancing age (Table 3).

Table 1. Protein synthesis and degradation in non-cultured Day-4 and Day-5 rabbit blastocysts

Embryonic age (Day <i>p.c.</i>)	Pulse period (2 h)	Chase period		Coefficient of regression* (1/h)	Half-life (h)
	(pmol incorporated leucine per blastocyst)	2.5 h	5 h		
4	6.6 (0.4) 100% (6%)† N = 9; n = 61	6.0 (0.4) 92% (7%) N = 3; n = 23	5.9 (0.5) 90% (8%) N = 3; n = 25	−0.021 (0.010)	32
5	65.6 (3.9) 100% (6%) N = 7; n = 91	61.3 (3.9) 93% (6%) N = 3; n = 58	64.0 (6.0) 98% (9%) N = 3; n = 38	−0.007 (0.008)	99

Values given are weighted means (s.e.m.) for N = number of independent experiments and n = number of blastocysts used.

*Calculated after logarithmic transformation of the incorporation data.

†% Recovery of incorporated leucine.

Cultured blastocysts

After 24 h in culture leucine incorporation was approximately doubled compared with Day-4 controls (*P* < 0.001), irrespective of the medium supplement involved (Table 2, *P* > 0.05).

Blastocysts cultured for 24 h in serum-supplemented Ham's F-10 medium showed a statistically significantly enhanced protein degradation as demonstrated by reduced recovery rates ($P < 0.01$) and a mean half-life of labelled proteins of 7 h ($P < 0.001$, Table 2). Protein degradation in blastocysts grown in media supplemented with uterine flushings for up to 24 h was comparable to that of Day-4 control blastocysts ($P > 0.05$, Table 2).

Table 2. Protein synthesis and degradation in in-vitro cultured Day-4 rabbit blastocysts

Ham's F-10 medium supplemented with:	Pulse period (2 h)	Chase period		Coefficient of regression* (1/h)	Half- life (h)
		2.5 h	5 h		
		(pmol incorporated leucine per blastocyst)			
24 h serum (24 S)	13.3 (1.2) 100% (9%)† N = 7; n = 46	11.3 (1.4) 85% (11%) N = 3; n = 24	7.7 (0.4) 58% (3%) N = 3; n = 25	-0.107 (0.012)	7
24 h uterine flushing (24 U)	13.0 (0.9) 100% (7%) N = 4; n = 36	12.4 (1.1) 95% (8%) N = 3; n = 19	11.5 (1.2) 88% (9%) N = 3; n = 19	-0.027 (0.016)	26
48 h serum (48 S)	19.8 (1.6) 100% (8%) N = 6; n = 41	12.8 (1.2) 65% (8%) N = 4; n = 28	12.4 (0.9) 63% (5%) N = 3; n = 22	-0.100 (0.014)	6
48 h uterine flushing (48 U)	28.7 (2.5) 100% (9%) N = 5; n = 42	23.5 (2.7) 85% (9%) N = 3; n = 19	17.1 (1.5) 59% (5%) N = 3; n = 21	-0.102 (0.019)	7
24 h serum + 24 h uterine flushing (24 S + 24 U)	32.0 (1.6) 100% (5%) N = 4; n = 35	21.1 (1.7) 66% (5%) N = 2; n = 20	18.2 (1.3) 57% (4%) N = 3; n = 31	-0.113 (0.014)	6
24 h uterine flushing + 24 h serum (24 U + 24 S)	30.9 (2.2) 100% (7%) N = 2; n = 23	23.0 (2.1) 74% (7%) N = 2; n = 22	21.1 (2.3) 68% (8%) N = 2; n = 22	-0.076 (0.019)	9
24 h uterine flushing + 24 h uterine flushing (24 U + 24 U)	29.7 (2.5) 100% (8%) N = 2; n = 25	22.4 (2.7) 76% (9%) N = 2; n = 24	18.5 (2.0) 62% (7%) N = 2; n = 24	-0.062 (0.017)	10

Values given are weighted means (s.e.m.) for N = number of independent experiments and n = number of blastocysts used.

*Calculated after logarithmic transformation of the incorporation data.

†% Recovery of incorporated leucine.

Table 3. Coefficients of regression for protein degradation

Experimental groups†	Day 4	Day 5	24 S	24 U	48 S	48 U	24 S + 24 U	24 U + 24 S
Day 5	n.s.							
24 S	***	***						
24 U	n.s.	n.s.	***					
48 S	***	***	n.s.	***				
48 U	***	***	n.s.	***	n.s.			
24 S + 24 U	***	***	n.s.	***	n.s.	n.s.		
24 U + 24 S	**	***	n.s.	*	n.s.	n.s.	*	
24 U + 24 U	*	**	*	*	*	*	*	n.s.

†See Tables 1 and 2.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. = $P > 0.05$.

After 48 h *in vitro*, blastocysts in serum-supplemented Ham's F-10 medium incorporated leucine at only a slightly higher level than after a 24-h culture period (Table 2, $P < 0.01$), while supplementation with uterine flushings resulted in a marked increase (Table 2, $P < 0.001$). This stimulating effect of uterine secretions after 48 h *in vitro* was independent from duration (24 h or 48 h, $P > 0.05$) and time (1st or 2nd 24-h period, $P > 0.05$) of supplementation, or of renewal of the medium after 24 h ($P > 0.05$, Table 2).

Culture for 48 h in serum- or uterine flushing-supplemented media resulted in a marked enhancement of protein degradation, as indicated by half-lives of 6–10 h (Tables 2 and 3). Supplementation with serum during the first 24 h of the 2-day culture led to a more pronounced protein breakdown ($t_{1/2} = \sim 6$ h, Table 2) than did supplementation with uterine secretions ($t_{1/2} = 7$ –10 h, Table 2). The recovery rates after the 2.5-h chase period were clearly reduced in blastocysts grown in serum-supplemented medium during the first 24 h (Table 2, $P < 0.05$), indicating a larger proportion of proteins with short half-lives in these embryos.

Discussion

There is little information available concerning protein degradation in preimplantation embryos. Half-lives of newly synthesized proteins in ovulated unfertilized oocytes and zygotes of the mouse are approximately 17–18 h (Merz *et al.*, 1981). For cleavage-stage mouse embryos slightly different results are reported, mainly caused by methodological differences such as the durations of the chase periods. Brinster *et al.* (1976) and Wiebold & Anderson (1985) calculated half-lives of 18–20 h (chase periods of up to 24 h), while Merz *et al.* (1981) found 12–13 h (chase periods of up to 5 h). Half-lives of 11–14 h are reported for mouse morulae and blastocysts (Brinster *et al.*, 1976, 1979; Merz *et al.*, 1981; Wiebold & Anderson, 1985).

In comparison to mouse embryos, proteins synthesized by rabbit blastocysts clearly have longer half-lives, which differ considerably between blastocysts of different ages (29–99 h; Table 1). This finding is in good agreement with changes in the protein content during the preimplantation development in these species. In the mouse the protein content diminishes from the oocyte up to the morula stage to increase with blastocyst formation (Brinster, 1967; Schiffner & Spielmann, 1976). In the rabbit the protein content remains constant up to the morula stage and increases afterwards significantly faster than in the mouse (Brinster, 1971), probably correlated to the higher cell proliferation.

It is well known that, compared with non-cultured controls, cultured embryos show significant changes in metabolism such as reduced metabolic activity (Anderson & Foote, 1975a, b; Sellens *et al.*, 1981; Edirisinghe *et al.*, 1984), retarded cell proliferation (Streffer *et al.*, 1980; Fischer, 1987, 1988) and lower cell number (Anderson & Foote, 1975a; Kane, 1983, 1985). In a previous study we were able to demonstrate that the reduced amino acid incorporation of cultured rabbit blastocysts was dependent on length of the *in-vitro* culture and on the medium supplement involved (Jung *et al.*, 1987). The present findings demonstrate that *in-vitro* culture not only changes protein synthesis but also enhances protein degradation.

In cultured Day-3 and Day-4 rabbit embryos there is an increase in secondary lysosomes and in the number of degenerated cells (Fischer, 1988; Hegele-Hartung *et al.*, 1988). Similar morphological differences have been described for mouse blastocysts cultured *in vitro* (McReynolds & Hadek, 1972).

Under physiological conditions lysosomes play only a minor non-selective role in the turnover of cellular proteins, but the basal rates of proteolysis increase when mammalian cells *in vitro* are deprived of growth factors or essential nutrients. In this situation most of the enhanced proteolysis results from autophagy. Portions of the cytoplasm are randomly encapsulated in membrane vesicles that subsequently fuse with lysosomes (Waterlow *et al.*, 1978; Amenta & Brocher, 1981; Rechsteiner, 1987). The observed types of cell death in cultured Day-4 rabbit blastocysts are

necrosis and, mainly, apoptosis (Fischer, 1988). Apoptotic bodies are either extruded into an adjacent lumen (in embryos: blastocyst cavity and perivitelline space) or undergo phagocytosis by nearby cells (Wyllie, 1981; Fischer, 1988). In-vitro culture is therefore characterized not only by a developmental retardation, but also by clearly enhanced breakdown reactions, shown by morphological and protein-degradation studies.

DNA, RNA and protein synthesis function on significantly higher levels when cultured rabbit blastocysts have access to uterine flushing proteins or secretions *in vitro* (Gulyas *et al.*, 1969; El-Banna & Daniel, 1972a, b; Fischer, 1987, 1988; Jung *et al.*, 1987). In our previous and in the present study (Table 2) we were able to demonstrate that uterine secretions exert their favourable effects in a time-dependent fashion, i.e. on embryonic protein synthesis not before 48 h *in vitro*. In addition, stimulation of amino acid incorporation was generally found in experimental groups having access to uterine flushings for at least one 24-h period within 2 days of in-vitro culture. The beneficial effects of uterine secretions on blastocyst development *in vitro* become particularly clear from the protein-degradation studies. Up to 24 h *in vitro*, uterine secretions prevented enhanced proteolytic activity in cultured blastocysts (Table 2). Normal in-vitro development of rabbit embryos therefore seems at present to be limited to short culture times and to supplementation of the culture medium with uterine flushing. Thereafter and under suboptimal conditions embryonic development *in vitro* should not be characterized as retarded but as hindered. Apart from nutritional effects this suggests the presence of some uterine factors, which support embryo development and which are able to act differently on embryonic protein synthesis and degradation.

Embryo development *in vitro* cannot be evaluated by the morphological development of the blastocyst but rather by the maintenance of its subsequent developmental viability (Gandolfi & Moor, 1987). In the rabbit the rate of blastocyst formation *in vitro* is very high (Kane, 1975; Maurer & Beier, 1976), but the viability of blastocysts grown *in vitro* is limited (Maurer, 1978; Beier *et al.*, 1983; for review see Fischer, 1988). This failure in developmental capacity may reflect the decisive requirements for subtle uterine factors missing *in vitro* analogous to those known to influence embryonic development in sheep (Wilmot & Sales, 1981; Gandolfi & Moor, 1987).

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