ANIMAL EXPERIMENTATION

Culture with Matrigel Inhibits Development of Mouse Zygotes

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Purpose: It was reported that Matrigel improved hatching of mouse blastocysts produced in vitro from F₁ hybrid-derived zygotes. We investigated whether Matrigel would be similarly beneficial with outbred strain-derived embryos, which exhibit a "two-cell" block similar to the developmental blocks of other species.

Methods: Mouse embryo development was assessed with or without Matrigel in KSOM medium, which supports the development of blocking strain zygotes in vitro, and in human tubal fluid (HTF) medium, which normally does not but which is used for human IVF.

Results: Matrigel severely inhibited the development of zygotes to blastocysts in KSOM and did not improve culture in HTF. There was no effect on development from the two-cell stage. We were not able to replicate the previous finding of Matrigel's beneficial effect on hatching of F₁-derived zygotes.

Conclusions: Matrigel may be a deleterious addition to embryo culture or coculture systems.

KEY WORDS: culture; embryo; in vitro fertilization; Matrigel; media.

INTRODUCTION

One approach that has been taken to improving the success of embryo culture has been to coculture preimplantation embryos with other cells, in order to mimic the in vivo environment of the oviduct, where embryos grow in the presence of, e.g., oviductal epithelial and fibroblast cells, and are exposed to products of their secretion (1,2). During an investigation of a coculture system for mouse embryos, Carnegie *et al.* (3) found that embryos grown on a layer of Vero cells would hatch from the zona pellucida at a greater rate than embryos cultured in a simple serum-free medium. The Vero cells were grown on a commercially available extracellular matrix material, Matrigel. In control experiments, however, Matrigel was found to be equally effective with or without Vero cells, implying that any positive effect could be attributed to the presence of Matrigel alone. Thus, they proposed that Matrigel may be a beneficial addition to embryo culture systems without resorting to the need for coculturing embryos with epithelial cells.

The original work (3) was done using F₁ hybrid-derived mouse embryos, instead of random-bred or inbred-derived embryos, which exhibit a phenomenon known as the two-cell block. The two-cell block is characterized by most zygotes failing to develop in culture past the two-cell stage, which is not a problem with hybrid-derived embryos. Recently, however, defined media such as CZB and KSOM have been developed which allow even blocking-strain mouse embryos to be cultured successfully from zygotes to blastocysts in vitro (4,5).

Because Matrigel had exhibited a beneficial effect upon nonblocking strain embryo development, we wondered if a similar effect would be found for

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blocking strain embryos in media which support their development. In addition, we wondered whether Matrigel might improve the development of blocking-strain embryos in media which would otherwise not support their development.

Therefore, we undertook experiments to determine whether Matrigel improves the development or hatching rate of blocking strain zygotes in KSOM medium, which generally supports their development to the blastocyst stage (5). We also investigated whether Matrigel could relieve the two-cell block of these same embryos when cultured in HTF (human tubal fluid) medium, which normally does not support development to the blastocyst stage (6).

MATERIALS AND METHODS

Media

KSOM medium was prepared from stocks as described by Lawitts and Biggers (5). KSOM–Hepes medium, used for flushing and handling embryos, is identical to KSOM except that the NaHCO_3 is reduced from 25 to 4 mM, 21 mM Hepes-free acid is added, and the pH is adjusted to 7.4 at room temperature using NaOH. All chemicals used in KSOM and KSOM–Hepes preparations were embryo culture-tested grade or tissue culture grade and were obtained from Sigma (St. Louis) or BDH (Toronto). HTF medium (7) was obtained from Pharmasciences (Montréal). Bovine serum albumin (1.0 mg/ml; Sigma) was added to each medium before use. The compositions of the media used are shown in Table I.

Table I. Composition of Media^a

Component (mM)	KSOM	KSOM–Hepes ^b	HTF
NaCl	95	95	102
KCl	2.5	2.5	4.7
KH_2PO_4	0.35	0.35	0.37
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.20	0.20	0.2
Na-lactate	10	10	21
Na-pyruvate	0.20	0.20	0.33
Glucose	0.20	0.20	2.8
NaHCO_3	25	4.0	25
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.7	1.7	2.0
Glutamine	1.0	1.0	0
EDTA	0.010	0.010	0
Hepes	0	21	0

^a All media contain 1.0 mg/ml BSA, 100 U/ml Na penicillin G, and 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate.

^b pH adjusted to 7.4 with NaOH.

Embryos

Embryos were obtained from Crl:CF₁ BR (“CF1”) outbred female mice or C57B16 × Balb/c F₁ (“CB6F1”) F₁ hybrid mice (5–6 weeks old) mated with B6D2F₁/CrlBR (“BDF”) males (Charles River Canada, Montréal). Females were superovulated by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG; 5 IU; Sigma) followed 48 hr later by intraperitoneal injection of human chorionic gonadotropin (hCG; 5 IU; Sigma). Zygotes were removed 23–24 hr post-hCG from excised oviducts by flushing the oviducts with KSOM–Hepes medium containing 300 $\mu\text{g}/\text{ml}$ hyaluronidase (Sigma), and the zygotes from four to six females pooled. The zygotes, free of cumulus cells, were washed in KSOM–Hepes and distributed into the appropriate treatment groups. Two-cell-stage embryos were flushed from the oviducts 47–48 hr post-hCG, washed in KSOM–Hepes, and distributed into the appropriate treatment groups.

Culture

Embryos were cultured in tissue culture dishes (Falcon No. 3001) for the KSOM vs HTF comparison experiments or in organ culture dishes (Falcon No. 3037) for experiments where presence or absence of Matrigel was compared. Single 50- μl drops of medium were placed in each dish or organ culture dish well and covered with medium-equilibrated mineral oil (Sigma). The dishes were equilibrated overnight in 5% CO_2/air at 37°C. Fifteen embryos were placed in each drop. Culture was carried out at 37°C in 5% CO_2/air .

Matrigel

Matrigel (Collaborative Biomedical Products, Becton Dickinson)-coated organ culture dishes were prepared according to the manufacturer's directions and the procedure of Carnegie *et al.* (3): Matrigel aliquots were stored at -20°C until needed, then thawed for several hours at 4°C . They were diluted eight fold with sterile embryo culture-grade water, and 230 μl was placed in the center well of each organ culture dish. Each dish was then swirled to ensure an even coating of Matrigel. The Matrigel was then allowed to gel overnight at room temperature. Two lots (Nos. 902155 and 903025) of Matrigel were used; no difference was observed between lots.

Experimental Design

Each replicate consisted of a control group of 15 embryos cultured in parallel with one or more treatment groups of 15 embryos.

Zygotes. Development was assessed by recording the number of embryos which reached the morula stage after 3 days (72 hr) in culture, the number which reached the blastocyst stage after 4 days (96 hr) in culture, and the number of blastocysts after 5 days (120 hr) in culture. Hatching was assessed after 5 days (120 hr) and 6 days (144 hr) in culture.

Two-Cell-Stage Embryos. Development was assessed by recording the number of embryos which had reached the morula stage after 2 days (48 hr) in culture, the number of blastocysts after 3 days (72 hr) in culture, and the number of hatched blastocysts after 4 days (96 hr) in culture.

The data from all replicates were pooled for analysis. Thus, the data from each experiment consisted of several sets, taken on successive days, recording the number of embryos which reached the appropriate stage, or hatched, and those which did not. Each set of data forms a 2×2 contingency table, which was analyzed using Fisher's exact test. Because there were often responses near-zero, the usually employed asymptotic approximation methods of probability calculation fail. Therefore, we used exact calculations of probability (StatXact Turbo, Cytel Software Corp., Cambridge, MA).

Two experiments were performed with blocking strain-derived zygotes. The first tested the effect of Matrigel presence or absence on embryo development in KSOM medium. The second tested the effect of Matrigel on embryo development in HTF medium, to determine whether the usual block to development past the two-cell stage could be released. One further experiment was performed with blocking strain-derived two-cell-stage embryos, in which the effect of Matrigel on culture from the two-cell stage in KSOM was tested.

We also performed a set of experiments utilizing CB6F1 hybrid-derived zygotes, replicating the experiments reported by Carnegie *et al.* (3). Zygotes were cultured in HTF medium with or without Matrigel, and the rate of blastocyst formation and of hatching was determined as described above. Two separate experiments were performed. One was with HTF + 1 mg/ml BSA (same as above). The other was with HTF + 5 mg/ml BSA (as in Ref. 3). Statistical analysis was carried out as described above.

RESULTS

Effect of Matrigel on CF1-Derived Zygote Culture in KSOM

Zygotes were cultured in KSOM as described above. In each experiment, 15 zygotes were cultured in the presence of, and 15 in the absence of, Matrigel. This was repeated 12 times, for a total of 178 control embryos and 175 embryos cultured with Matrigel (one experiment had fewer than 15 embryos per group due to lower yield). After 3 days in culture, slightly fewer embryos had reached the morula stage in the presence of Matrigel compared to those cultured in the absence of Matrigel (Fig. 1; $P = 0.014$). Development to blastocyst was severely impaired by Matrigel, with only a very few embryos reaching the blastocyst stage on day 4 or 5 in the presence of Matrigel (Fig. 1). Although the fraction of hatched blastocysts was low in the presence or absence of Matrigel, the presence of Matrigel led to an even lower fraction of total embryos reaching the hatched blastocyst stage ($P = 0.0014$).

Fig. 1. Comparison of CF1-derived zygote development in KSOM with or without Matrigel. Embryos were cultured from the one-cell zygote stage. Development was scored as the percentage reaching the morula or greater stage by day 3 of culture, blastocysts by day 4 and 5, and hatching by day 5 and 6, as shown at the bottom. Development by each measure was significantly decreased in the presence of Matrigel vs. development in the absence of Matrigel (although only slightly on day 3). P values, determined by Fisher's exact test, are shown above the paired bars. The number of days shown corresponds to the number of days in culture. Experimental design is described in the text.

Effect of Matrigel on CF1-Derived Zygote Culture in HTF

We first confirmed that HTF medium did not support the development of these blocking-strain embryos. Embryos were cultured in groups of 15 in either KSOM or HTF medium alone. Nine replicates with KSOM and 11 replicates with HTF were done, for a total of 134 (one replicate had 14) and 165 embryos, respectively. We found that the two-cell block was not complete in HTF, with 39% of zygotes progressing to the four-cell or greater stage after 2 days of culture in HTF alone (Fig. 2); the majority of the remainder blocked at the two-cell stage. In contrast, 97% of zygotes progressed to the four-cell or greater stages after 2 days in culture in KSOM medium alone. Even those embryos which passed the two-cell block in HTF were mostly unable to progress to the blastocyst stage. Only 5% of zygotes in HTF developed to blastocysts after 4 days in culture, vs 81% in KSOM (Fig. 2).

We then tested whether the poor development in HTF medium could be reversed by the presence of Matrigel, since Carnegie *et al.* (3) had shown improved hatching of F₁ hybrid-derived embryos in this medium. Embryos were cultured in HTF in groups of 15, with or without Matrigel. Eight replicates were done, for a

Fig. 2. Comparison of CF1-derived zygote development in KSOM and HTF media. HTF medium was significantly worse at supporting development, with only about 40% passing the two-cell block (day 2 data). Development to blastocysts was severely and highly significantly inhibited in HTF as compared to KSOM. *P* values, from Fisher's exact test, are shown above paired bars. The number of days shown corresponds to the number of days in culture. Experimental design is described in the text.

Fig. 3. Comparison of CF1-derived zygote development in HTF with or without Matrigel. As also shown in Fig. 2, development in HTF is low. There is clearly no improvement in development in the presence of Matrigel. *P* values from Fisher's exact test, for the comparison at each stage with or without Matrigel, are shown above paired bars; there is either no significant difference (NS) or a significant decrease in development with Matrigel at any stage. The number of days shown corresponds to the number of days in culture. Experimental design is described in the text.

total of 119 embryos without, and 121 embryos with, Matrigel. Consistent with our results described above, HTF failed to support development to the blastocyst stage (3% at day 4; Fig. 3), and the addition of Matrigel clearly had no beneficial effect on development in HTF. Development was actually lower at each stage in the presence of Matrigel (Fig. 3).

Effect of Matrigel on CF1-Derived Two-Cell-Stage Embryo Culture in KSOM

In each experiment, 15 two-cell-stage embryos were cultured in the presence of, and 15 in the absence of, Matrigel. Three replicates, for a total of 45 embryos in each treatment, were performed. There was no significant effect of Matrigel on culture from the two-cell stage (Fig. 4). A high proportion of embryos progressed past the morula stage on day 2 of culture and to the blastocyst stage by day 3 of culture. Approximately 60% of embryos hatched by day 4 in culture, with or without Matrigel.

Effect of Matrigel on CB6F1-Derived Zygote Culture in HTF

The design was the same as used above, and two or three replicates were performed for each treatment. In the presence of 1 mg/ml BSA, there was no effect of Matrigel on blastocyst formation or on hatching rate (Fig. 5). In the presence of 5 mg/ml BSA, a higher proportion of zygotes developed to blastocysts and hatched, compared to 1 mg/ml BSA. Matrigel signifi-

Fig. 4. Comparison of CF1-derived two-cell-stage embryo development with or without Matrigel. No difference was observed in the development of two-cell embryos in the presence or absence of Matrigel, nor was there any effect on rate of hatching: *P* values from Fisher's exact test, for the comparison at each stage with or without Matrigel, are shown above paired bars. The number of days shown corresponds to the number of days in culture; thus, e.g., day 2 here corresponds to day 3 in a one-cell zygote culture. Experimental design is described in the text.

cantly inhibited both blastocyst formation and hatching in 5 mg/ml BSA (Fig. 5).

DISCUSSION

Previously, it had been reported that Matrigel significantly improved the hatching rate of F_1 hybrid-derived zygotes cultured in HTF medium (3). We could not, however, demonstrate any beneficial effect of Matrigel for zygotes from a strain of random-bred (CF1) female-derived embryos which exhibit the two-cell block in vitro. In our study, we find that in KSOM medium, which supports blocking strain development, Matrigel severely inhibits zygote development and progression to blastocysts. We confirm that HTF medium does not support the development of CF1 zygotes to blastocysts, while KSOM does, consistent with the findings reported by Quinn (6).

In Carnegie and co-workers' previous work with nonblocking embryos, the beneficial effect of Matrigel was reported to be an increased rate of hatching. In the present study, we find that for blocking strain-derived embryos in KSOM medium, 16% of blasto-

Fig. 5. Comparison of CB6F1 (F_1 hybrid)-derived zygote development in HTF with or without Matrigel. F_1 hybrid-derived zygotes, which do not exhibit the two-cell block, were cultured in HTF medium at two levels of BSA. A higher BSA concentration was clearly beneficial. Matrigel, however, was either without effect or detrimental. *P* values from Fisher's exact test, for comparisons of with or without Matrigel, are shown above paired bars. The number of days corresponds to the number of days in culture. Experimental design is described in the text.

cysts went on to hatch. With Matrigel added, 10% of blastocysts hatched (NS; $P = 0.33$ by Fisher's test). A similar result is obtained after an additional day in culture (see Fig. 1). Therefore, we are unable to demonstrate a beneficial effect of Matrigel on the rate of hatching of CF1-derived blastocysts.

Our results with culture from the two-cell stage show that Matrigel is not generally toxic to these embryos, since two-cell-stage embryos develop and hatch equally well in the presence or absence of Matrigel (Fig. 4). Thus, the detrimental effect of Matrigel appears to be restricted to the one-cell zygote stage (Fig. 1).

The detrimental effect of Matrigel on zygote development has led us to revisit the question of whether it indeed has a beneficial effect on hatching of F_1 hybrid-derived blastocysts cultured from zygotes, as reported by Carnegie *et al.* (3). We have not detected such an effect. We used two concentrations of BSA—1 mg/ml, to match the concentration which is standard in KSOM and which was used in all other experiments reported here, and 5 mg/ml, to match the concentration previously used by Carnegie *et al.* Depending on the

level of BSA in the medium, Matrigel either was without effect or was inhibitory to development. Clearly, we found very different results from those of Carnegie *et al.* (3). The simplest explanation is that Matrigel varies greatly in its embryo toxicity. Although we tried two lots of Matrigel, lot-to-lot variation may have led to the toxic effects we observed in contrast to the beneficial effects reported by Carnegie *et al.*

CONCLUSIONS

Matrigel may be a risky additive to embryo culture or coculture systems and should not be employed in human or animal embryo culture without rigorous testing. Improved culture media are still needed, however, to maximize the potential for embryonic development in vitro.

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