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Cause of Low Embryogenesis-promoting Ability of Bovine and Swine Sera for 9.5-day Rat Embryo in Culture

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Abstract

Were bovine and swine sera to have the same embryogenesis-promoting ability as rat serum, they might be able to replace rat serum as a medium for whole rat embryo culture, given their availability at any butchery. When 9.5-day rat embryos were cultured in 50% and 100% of bovine and swine sera, respectively, the embryogenesis-promoting abilities were compared with those of rat serum. Development of rat embryos cultured in 50% and 100% of these sera was significantly poorer than that of embryos cultured in 50% rat sera. Therefore, these sera probably have low embryogenesis-promoting ability for 9.5-day rat embryos in culture. Furthermore, the bovine and swine sera contained a 180 kDa serum component in place of the 190 kDa component in rat serum, and DEAE-eluate in these sera also contained most of the 180 kDa serum component. In addition, development of 9.5-day rat embryos that were cultured in serum-free medium, containing rat transferrin, supplemented with DEAE-eluate in these sera, also was poorer than that of embryos cultured in medium with rat serum. The difference in presence of the defined serum components seemed to reflect the limited embryogenesis-promoting abilities in these sera

KeyWords: *whole rat-embryo culture, culture medium, domestic-animal's sera, rodent sera*

Introduction

In vitro whole embryo cultures have been found to be useful as a developmental toxicity assay system. The sera from rodent animals as the culture medium have a finite embryogenesis-promoting ability for post-implantation rat embryos cultured *in vitro*. On the other hand, it is said that non-rodent sera have a low embryogenesis-promoting ability for them. In an early study, New (1966) showed that rat serum was superior to sera of rabbit, chicken, horse, calf and sheep in supporting rat embryo growth and development. Reti et al. (1982) reported that there was no normal growth in 9.5-day rat embryos cultured in human serum.

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Chatot et al. (1980) suggested that head-fold-stage rat embryos cultured in human serum did not indicate optimal growth in vitro without glucose supplementation. Other workers have also reported that 9.5-day rat embryos did not normally grow in human serum (Tanimura and Shepard, 1970; Gupta and Beck, 1983). Flynn et al. (1987) observed that development of heads was retarded in 9.5-day rat embryos cultured in canine serum. Coelho and Klein (1990) found a delay of development and failure of neural tube closure in 9.5-day rat embryos cultured in cow serum. We recently demonstrated that 50% 3-h-DC (delayed-centrifuged) sera from rat have the same embryogenesis-promoting ability for 9.5-day rat embryos as 50% IC (immediately-centrifuged) sera from rat (Katoh et al., 1998a). In addition, we found that serum components (P-8, P-20, P-35 and albumin) from rat serum have embryogenesis-promoting ability for 9.5-day rat embryos and we called them embryogenesis-promoting factors (EGPFs) (Katoh et al., 1998b). P-8, P-20 and P-35 were identified as α_1-inhibitor 3, transferrin and HDL(high density lipoprotein), respectively. Furthermore, bovine transferrin was found to be replacable to the rat transferrin. Then we focused sera largely obtained from bovine and swine, and attempted to culture 9.5-day rat embryos in bovine or swine serum to compare with the development when cultured in rat serum. Therefore, we examined for possible differences of serum components among bovine, swine and rat sera.

Materials and Methods

Materials

Chemicals: Dulbecco's Modified Eagle (DME) medium and L-glutamine were obtained from Nissui Pharmaceutical Co., Ltd., Tokyo. Glycine, sodium bromide, ammonium persulfate, sodium bicarbonate, penicillin and streptomycin were from Nakarai Tesque Chemicals (Osaka, Japan). Filter units (0.22 μm) were from Millipore Products Division, Bedford, MA. The gels of Sephacryl S-300 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The DEAE Bio-Gel was purchased from Bio-Rad Laboratories (Richmond, CA). Trizma-base was from Sigma Chemical Co. (St. Louis, MO). Acrylamide, N,N'-methylen bis acrylamide and N,N,N',N'-tetramethylethylene diamine were from Wako Pure Chemicals (Osaka). Other chemicals used were of the purest grade available from regular commercial sources.

Animals: SD-rats were used throughout this work. They have been housed under standard conditions at the animal breeding laboratory in our Institute for Developmental Research, Aichi Human Service Center, and have been maintained on feed and water *adlibitum.*

Isolation of sera

All rats were anesthetized with ether, and blood was drawn from the abdominal aorta. Rat serum was obtained from the freshly drawn blood of adult male. We separated the 3-h-DC serum from clotted blood by centrifuging and decanting 2 h after leaving blood stand at 15℃. Bovine and swine sera were speedily separated from blood drawn from the jugular veins of animals in an abattoir in Nagoya, after clotting. It took 3 h to prepare these sera. Separation of all sera was carried out at 4℃. These pooled sera were immediately chilled and filtrated through a filter unit (0.22 μm) after inactivating at 56℃ for 30 min and then stored at -80℃ until use. The dilution of serum was undergone with DME medium. These sera were used as culture medium for whole rat embryo rolling culture. All fractions isolated from these sera by biochemical techniques were then filtrated onto Sephadex G-25 column equilibrated with DME medium before they were supplemented to the culture medium.

Isolation of fractions from sera by gel filtration and ion exchange chromatographies

The fractions with the embryogenesis-promoting ability from rat serum were isolated by the methods reported by Katoh et al. (1998b). The fraction with a molecular size range of 100-500 kDa and which was isolated from rat serum by Sephacryl S-300 gel chromatography, had the embryogenesis-promoting ability. The DEAE-eluate (containing D-1 and D-2) from the fraction with the molecular size range of 100-500 kDa was isolated by ion exchange chromatography with DEAE Bio-Gel, also had this ability. The fractions corresponding to rat DEAE-eluate from bovine and swine sera were isolated by the same procedures.

Whole embryo rolling culture

SD-rats were mated overnight and pregnancy was confirmed by the presence of the vaginal plugs. If the plugs were found the following morning, they were considered to be 0.5 days pregnant at 12:00 noon that day. Whole rat embryos were dissected at day 9.5 of gestation. The 9.5-day rat embryos were at head-fold stage and had the neural plate and the somite of 0-1. The total lengths and diameters of their conceptuses were 2.1-2.2 mm and 0.90-0.95 mm, respectively. Two embryos were immediately transferred in each roller bottle containing 4 mL of culture medium. Their bottles were closed with a rubber sleeve-type stopper after their space were filled with an initial gas mixture. They were cultured for 48 h according to the method developed by New (1978). The initial composition of gas mixture was 5% O_2, 5% CO_2 and 90% N_2. The next composition of gas mixture, which was 20% O_2, 5% CO_2 and 75% N_2, was changed at 20 h of culture. Final composition of 40% O_2, 5% CO_2 and 55% N_2, was changed at 40 h of culture. Culture media were 50% and 100% sera, and DME medium supplemented with D-2 from each serum. D-2 is a subfraction of DEAE-eluate (from a DEAE Bio-Gel column) isolated from serum (Katoh et al., 1998b). Supplemental penicillin and streptomycin were added at 31.2 μg/mL and 100 μg/mL to sera and DME medium, respectively. Serum fractions were filtrated onto a Sephadex G-25 column equilibrated with DME medium before they were added to the serum-free culture medium. Embryonic development was assessed by the morphological scoring system of Brown and Fabro (1981). In addition, the yolk-sac diameter, crown-rump length and head length of the embryos were measured, and the numbers of somite pairs were noted.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) using 8% and 10% gels under reducing condition. Each protein sample of 25 μg was applied to a gel, and proteins were stained with Coomassie Brilliant Blue R-250.

Results

Development of rat embryos cultured in sera of domestic mammals

In swine, bovine and rat, each serum was isolated from blood by centrifugating after clotting. Rat embryos cultured in bovine and swine sera were compared for development with those cultured in rat serum (Table 1, Fig.1). Although the number of somites of rat embryos cultured in 50% and 100% bovine sera was not significantly different from that in 50% rat serum, their morphological scores were lower than in 50% rat serum. In particular, development of their brains was retarded. Rat embryos cultured in 100% swine serum were small and retarded considerably, though their yolk-sac diameters grew to 94% of those in 50% rat serum. Furthermore, their yolk sacs had no visible color in the circulation. However, development of rat embryos cultured in 50% swine serum was slightly better than in the 100% one (Fig. 1). From these findings, it was considered that organogenesis-promoting abilities of bovine and swine sera for rat embryos might be significantly lower than

Table 1. Development of 9.5-day rat embryos cultured in sera of mammals

Serum	n	YS [a] (mm)	HL [b] (mm)	CR [c] (mm)	Somite	Score [d]
Rat 50%	8	3.74 ± 0.04	1.79 ± 0.05	3.42 ± 0.06	25.0 ± 0.71	40.6± 1.51
Bovine 50%	8	3.29 ± 0.10	1.10 ± 0.06	2.30± 0.12	18.5 ± 1.00	16.0 ± 1.22
100%	8	3.54 ± 0.09	1.20 ± 0.16	2.66 ± 0.22	19.5 ± 2.60	22.9 ± 3.30
Swine 50%	8	3.48 ± 0.08	0.94 ± 0.05	1.73 ± 0.04	11.8 ± 0.83	9.0 ± 1.00
100%	8	3.40 ± 0.11	0.67 ± 0.51	1.36 ± 0.05	6.8 ± 0.66	7.8± 1.00

Notes:
a: Diameter of yolk sac.
b: Head length of embryo.
c: Crown-rump length of embryo.
d: Average score of embryos by morphological scoring system of Brown and Fabro (1981).
Values are mean ± SD. The 9.5-day rat embryos had the neural plate and somite of 0-1.

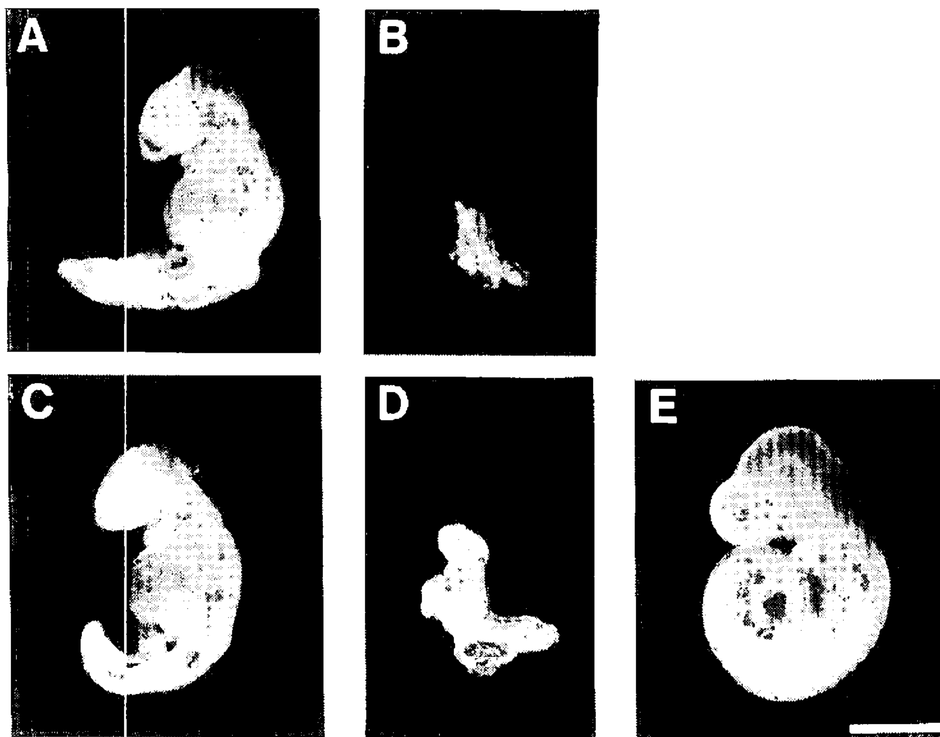

Fig. 1. Lateral view of 9.5-day rat embryos cultured for 48 h in 50% and 100% sera isolated from bleed of various animals. A, B, C, D and E: results with 50% bovine serum, 50% swine serum, 100% bovine serum, 100% swine serum and 50% rat serum, respectively. Bar, 1 mm.

those of rat serum, or their sera might have some inhibitors for embryogenesis. It was also found that both sera of bovine and swine could not be used intact as medium in the whole rat embryo culture after all.

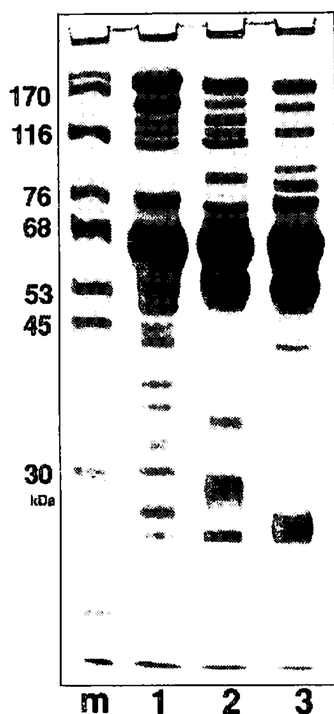

Fig. 2. SDS-PAGE of sera of various animals. Lane m, marker proteins (170 kDa for α-macroglobulin; 116 for β-galactosidase; 76 for human transferrin; 68 for bovine serum albumin; 53 for glutamine-dehydrogenase; 45 for ovalbumin; 30 for carbonic anhydrase); lane 1, rat serum; lane 2, bovine serum; lane 3, swine serum. The samples were applied to SDS gel (8%).

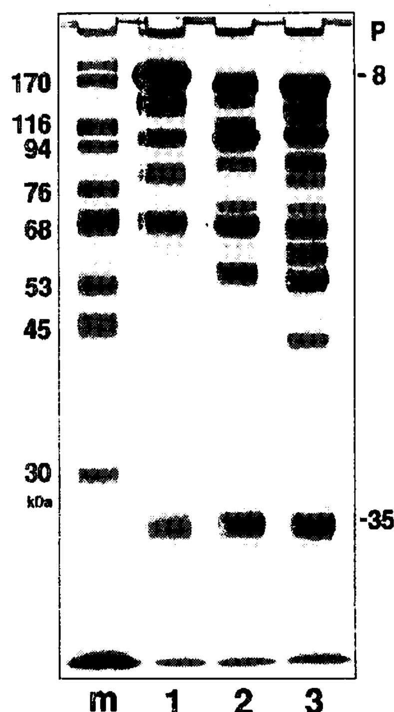

Fig. 3. SDS-PAGE of D-2 isolated from sera in various animals. Lane m, marker proteins (170 kDa for α-macroglobulin; 116 for β-galactosidase; 94 for phosphorylase b; 76 for human transferrin; 68 for bovine serum albumin; 53 for glutamine-dehydrogenase; 45 for ovalbumin; 30 for carbonic anhydrase); lane 1, rat D-2; lane 2, bovine D-2; lane 3, swine D-2. The samples were applied to SDS gel (8%).

EGPF-like components in bovineand swine sera

In bovine and swine sera, the components expected to have promoting ability in embryogenesis were compared with those in rat serum on SDS-PAGE (Fig. 2). The 190 kDa component (identified as α_1-inhibitor 3 by Katoh et al., 1998), which is one of the EGPFs from rat serum, was absent in bovine and swine sera. Another component was present in them, and its molecular size was 180 kDa. In addition, bovine and swine sera had a larger amount of 27 kDa component than rat serum. The 27 kDa component in rat serum is apoprotein AI of HDL which is also one of EGPFs. The 76 kDa component (transferrin) present in both sera of bovine and swine was the same as in rat serum. Then serum components among rat D-2, bovine D-2 and swine D-2 were compared (cf. Methods, Isolation of fractions). The 180 kDa component also was present in bovine D-2 and swine D-2, but not in rat D-2, while the 190 kDa one was present in rat D-2, but not in bovine D-2 and swine D-2 (Fig. 3). The 180 kDa component contained in both sera bovine and swine seemed to be a subunit of α_2-macroglobulin. A significant difference of both sera from rat serum was the presence of the 190 kDa- or 180 kDa-component.

Development of 9.5-day rat embryos with D-2 of bovine and swine sera

Table 2. Development of 9.5-day rat embryos cultured in media supplemented with D-2 from rat, bovine and swine sera

Medium	n	YS [a] (mm)	HL [b] (mm)	CR [c] (mm)	Somite	Score [d]
Rat D-2	8	3.48±7 0.03	1.55 ± 0.04	3.29 ± 0.04	23 ± 0.71	40.0 ± 0.50
Bovine D-2	8	3.13 ± 0.20	1.21± 0.06	2.33 ± 0.04	16 ± 1.23	22.0 ± 1.23
Swine D-2	8	3.10 ± 0.10	1.17 ± 0.06	2.38 ± 0.07	17 ± 1.41	21.0 ± 1.23
DC-50 [e]	8	3.65 ± 0.04	1.69 ± 0.05	3.40 ± 0.05	24 ± 0.71	40.5 ± 0.71

Notes
a Diameter of yolk sac.
b Head length of embryo.
c Crown-rump length of embryo.
d Average score of embryos by morphological scoring system of Brown and Fabro (1981).
e Control.
Values are mean ± SD. Rat transferrin was added to all of D-2 fractions.

Since bovine and swine D-2 did not have the important serum component (190 kDa component) contained in rat D-2, the embryogenesis-promoting ability of D-2 of bovine and swine sera was examined. Table 2 shows a comparison of the development of rat embryos cultured in D-2 of bovine, swine or rat serum. Development of 9.5-day rat embryos cultured in serum-free medium (containing 12 mg of rat transferrin) supplemented with D-2 of bovine or swine serum was poorer than that with D-2 of rat serum after all. Development of 9.5-day rat embryos cultured with bovine D-2 was similar to that with bovine serum. On the other hand, development of 9.5-day rat embryos cultured with swine D-2 became marginally better than that with 50% swine serum; its morphological score was about 53% of the control (morphological score with 50% swine serum was 22%). These results suggested that neither bovine nor swine sera can be used to replace rat serum in the whole embryo culture medium. Serum components except transferrin in non-rodent sera might not serve for rat EGPFs.

Discussion

It has been reported that sera from cow, human and dog are not suitable as medium for whole rat embryo culture (Chatot et al., 1980; Reti et al., 1982; Gupta and Beck,1983; Flynn et al., 1987; Coelho and Klein, 1990). Delay of development and failure of neural tube closure of 9.5-day rat embryos cultured in cow serum were avoided by supplementing with methionine (Coelho and Klein, 1990). Flynn et al. (1987) reported that abnormalities of rat embryos cultured in canine serum were improved by addition of methionine and FePIH, since the levels of free methionine and iron in canine serum were lower than those in rat serum. On the other hand, we had the result that development of 9.5-day rat embryos cultured in serum-free medium supplemented with bovine D-2 was poor whereas development of 9.5-day rat embryos cultured in serum-free medium supplemented with rat D-2 was similar to control. In addition, the concentration of methionine and iron in its serum-free medium is mostly high. Therefore, the low

level of methionine and iron does not seem to be the cause of retardation in development of rat embryos cultured in bovine serum, and its cause seems to depend on some component in bovine D-2. The α-macroglobulin families in sera from many mammal account for about 20% of serum protein and have a function as inhibitor of trypsin-like protease. α_2-macroglobulin is a main member of the α-macroglobulin family in sera from dog, pig and cow. The main member of the α-macroglobulin family in human serum also was α_2-macroglobulin, which had the concentration of 2.5 mg/ml and was not classified as an acute phase reactant (Travis and Salvesen, 1983). In this work, D-2 in bovine and swine sera also seemed to contain a large amount of α_2-macroglobulin. Moreover, rat and mouse sera differ considerably in α-macroglobulin family composition from human and bovine sera which is rich in α_2-macroglobulin. The α-macroglobulin family of rat serum consists mainly of α_1-macroglobulin and α_1-inhibitor 3, and few of α_2-macroglobulin and pregnancy associated protein, as mentioned by Geiger et al. (1987). Saito and Shinohara (1985) have isolated α_1-macroglobulin and murino-globulin (the same as α_1-inhibitor 3) from rat serum and have characterized them. In the previous study, we found that rat D-2 contained α_1-inhibitor 3, which had high embryogenesis-promoting ability for 9.5-day rat embryos in culture (Katoh et al., 1998b). Therefore, the absence of α_1-inhibitor 3 in bovine and swine sera may relate to their poor embryonic development. In the field of whole embryo culture, the reports on functions of α-macroglobulin family are few. In addition, we have demonstrated that commercial bovine transferrin has the same embryogenesis-promoting ability as rat transferrin (Katoh et al., 1998b).

In conclusion, the results of this study indicated that a difference between compositions α-macroglobulin family was probably the cause of the low embryogenesis-promoting ability of bovine and swine sera for 9.5-day rat embryos in culture.

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