Concanavalin A binding capacity of preimplantation mouse embryos

J. T. Wu

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, U.S.A.

Summary. Mouse embryos collected on Days 2 (2-cell) to 4 (early blastocyst) were treated with pronase to digest the zona pellucida and cultured at 37°C for 3 h. These embryos and untreated Day-5 late blastocysts were exposed to [³H]Con A at 25, 50, 100 or 200 µg/ml, 4°C for 30 min, washed and counted for radioactivity. Other groups of embryos were incubated with [³H]Con A in the presence of 0.06 M- α -methyl-D-mannoside for the detection of non-specific binding. Scatchard plot analysis showed that the total number of Con A molecules bound/embryo increased with development, from 12×10^7 in 2-cell embryos to 19×10^7 in early blastocysts and to 150×10^7 in late blastocysts. The association constants decreased from $3 \times 10^6 \text{ M}^{-1}$ in 2-cell embryos to $0.83 \times 10^6 \text{ M}^{-1}$ in late blastocysts. The results suggest that the Con A binding sites (probably glycoproteins) on the embryo surface change quantitatively and qualitatively during preimplantation development.

Introduction

Changes in the cell surface, as reflected in differential lectin-induced agglutinability, occur during embryonic development and differentiation (Moscona, 1971; Kleinschuster & Moscona, 1972; Krach, Green, Nicolson & Oppenheimer, 1974). Studies with rats, mice, hamsters and rabbits have shown that the spermatozoa, zona pellucida and plasma membranes of eggs and embryos contain binding sites for several lectins (see Oppenheimer, 1977). Yanagimachi & Nicolson (1976) found that fertilized hamster eggs were slightly more agglutinable by concanavalin A (Con A), *Lens culinaris* agglutinin and wheat germ agglutinin (WGA) than were unfertilized eggs, and that binding of fluorescent Con A, WGA and *Ricinus communis* agglutinin I (RCA_I) was maximal when the oocyte reached full maturity but it decreased at the blastocyst stage.

The agglutinability of mouse eggs by Con A was increased after fertilization (Pienkowski, 1974); it was reduced at the 2-cell stage, recovered at the 4-cell stage, then again reduced at the morula stage and lost at the blastocyst stage (Rowinski, Solter & Koprowski, 1976; Sobel & Nebel, 1976). Compaction at the 8-cell stage greatly decreased the agglutinability (Rector & Granholm, 1978). Pienkowski (1974) further reported that unfertilized mouse eggs and 1–8-cell embryos had the same binding capacity for Con A when they were incubated at a concentration of 50 µg/ml. Brownell (1977) showed that morulae and blastocysts bound the same amount of fluorescent Con A, WGA, RCA_I and RCA_{II}.

The lectins, due to their specific binding to oligosaccharides (Sharon & Lis, 1972; Kabat, 1978), are useful tools for localizing and measuring the sugars on cell surfaces. Con A binds specifically to α -D-mannose and α -D-glucose residues. The present study was designed to determine whether or not there is a change in the number of Con A binding sites at various stages of preimplantation development in the mouse. An earlier study had shown a great increase in Con A binding sites in the implanting mouse blastocyst (Wu & Chang, 1978).

Materials and Methods

Mature CD-1 mice purchased from Charles River Breeding Laboratories were maintained in animal quarters under controlled lighting (07:00–19:00 h, EST) and temperature (20–23°C), with free access to food and water. For mating, the females were caged with males overnight and examined in the morning for signs of mating. The day of finding spermatozoa or a copulatory plug in the vagina was considered Day 1 of pregnancy.

Pregnant animals were killed between 09:00 and 11:00 h on Days 2–5 and the reproductive tracts were trimmed and flushed for embryos with a medium which has been used for the production of mouse chimaeras in the laboratory of Dr W. K. Whitten. It differed from the medium of Mullen & Whitten (1971) in that NaHCO₃ and sodium pyruvate were 190 and 2.5 mg/l instead of 2106 and 36 mg/l, respectively. The medium also contained 4 mg bovine serum albumin (BSA) (Sigma Chemicals)/ml, and was used for flushing and culture of embryos after pronase treatment. Zona-encased embryos were treated with 0.5% pronase in Hanks' balanced salt solution without bicarbonate to digest the zona pellucida (Mintz, 1971). Soon after the zonae showed signs of thinning, the embryos were removed and washed in 2 changes of medium; zona digestion was usually completed by the end of washing. The embryos were then incubated in the medium in an atmosphere of 5% CO₂ in air at 37°C for 3 h to allow for recovery from possible injuries to the cell surface during the pronase treatment. The Con A binding after 1, 2, 3 or 4 h of culture was compared in groups of Day-4 blastocysts, and found to be no different. For late blastocysts without a zona, the pronase treatment and subsequent culture at 37°C were omitted.

Instead of pronase, manual dissection with microneedles or acidic Tyrode's solution, pH 2.5 (Nicolson, Yanagimachi & Yanagimachi, 1975), was employed to remove the zonae of some Day-4 blastocysts. In this case, the culture at 37°C was also omitted.

All embryos were then washed twice in cold Dulbecco's phosphate-buffered saline (PBS) containing 4 mg BSA/ml (PBSA) and exposed, with occasional stirring, at 4°C for 30 min, to 25, 50, 100 or 200 µg [³H]Con A (38.8 Ci/mmol: New England Nuclear, Boston, Massachusetts)/ml PBS containing 1.8 mg BSA/ml. Generally, up to 30 embryos were placed in 50 µl solution. Duplicate samples were incubated in [³H]Con A solutions containing 0.06 M- α -methyl-D-mannoside to measure non-specific binding.

After incubation the embryos were washed in 4 changes, 1 ml each, of cold PBSA, and 1–3 Day-5 blastocysts or 3–12 Day-2, -3 or -4 embryos were picked up in 2 µl of the final wash fluid and placed into a 20 ml glass scintillation vial which was further examined under a dissecting microscope ($\times 24$) for confirmation of the number of embryos collected. The whole washing process took about 15 min for each incubation of embryos. The control vials received the same amount of wash fluid without embryos. Each vial then received 0.2 ml deionized water,

Table 1. Effects of different methods of zona removal on the Con A binding in Day-4 mouse blastocysts

Treatment	No. of determinations (and embryos)	Mean $\pm$ s.e.m. no. of Con A molecules bound/embryo ($\times 10^{-6}$)
Pronase + culture		
1 h	3 (15)	151 $\pm$ 25
2 h	4 (19)	140 $\pm$ 7
3 h	7 (38)	142 $\pm$ 12
4 h	4 (20)	150 $\pm$ 14
Acidic Tyrode's (10 sec)	6 (30)	141 $\pm$ 16
	8 (14)*	572 $\pm$ 101*
Micro-needle dissection	3 (13)	145 $\pm$ 15

* Day 5 blastocysts.

and after being frozen and thawed twice, received 7.3 ml Aquasol (New England Nuclear). Each vial was vortexed and counted in liquid scintillation counters for 40–60 min.

The counting efficiency was estimated from sample channels ratio. The disintegrations per minute (d.p.m.) value in the control vial represented the background count and was subtracted from the count for each embryo-containing vial. The net Con A binding was defined as the difference between the Con A binding value in the presence and the absence of α -methyl-D-mannoside. The net binding values in d.p.m. were then converted into the number of Con A molecules according to $1 \text{ d.p.m.} = 7.05 \times 10^6 \text{ molecules}$, derived from the specific activity of 38.8 Ci/mmol. The data were analysed by Scatchard plot (Rosenthal, 1967) and the number of Con A binding sites/embryo was estimated. Each point consisted of 2 to 12 duplicates of experiments. The Con A binding data of various embryos at each concentration of Con A were compared by *t* test.

Text-fig. 1. Binding of Con A to mouse embryos (2C = 2-cell; 4C = 4-cell; 8C = 8–10-cell; 4B = Day-4 blastocyst; 5B = Day-5 blastocyst) at different Con A concentrations. The non-specific bindings for 2-cell to Day-4 embryos are simplified here and shown as an average, N1; that of Day-5 blastocysts is represented by N2.

Results

The effect of zona removal by 3 methods, namely pronase, acidic Tyrode's solution and dissection, on the Con A-binding capacity of the embryo was determined at 100 μg [^3H]Con A/ml. As clearly shown in Table 1, there were no significant differences between any two treatments. Nor was there any difference after 1, 2, 3 or 4 h of culture at 37°C following pronase removal of the zona. For convenience, 3 h culture after pronase treatment was used for subsequent experiments. Treatment of Day 5 blastocysts with acidic Tyrode's solution did not affect their Con A binding capacity compared to that for untreated blastocysts (Text-fig. 1).

As can be seen from the binding curves in Text-fig. 1, the amount of Con A bound appeared to increase with the stage of development and with the concentrations of Con A used. For Day-2 (2-cell) to Day-4 (early blastocyst) embryos, the Con A binding reached the plateau at about the concentration of 100 $\mu\text{g}/\text{ml}$, while for Day-5 embryos (implanting blastocysts), the Con A binding was still significantly increasing between the concentrations of 100 and 200 $\mu\text{g}/\text{ml}$, although the increase was not as steep as that before 100 $\mu\text{g}/\text{ml}$. At 200 $\mu\text{g}/\text{ml}$ the Con A binding/Day-5 blastocyst was significantly ($P < 0.001$) higher than that of the Day-2 to Day-4 embryos. Among the latter embryos, due to the great variation exhibited, only the values for Day-4 blastocysts were significantly ($P < 0.05$) different from those for the Day-2 (2-cell) embryos. In all these studies the non-specific binding always increased linearly with the Con A concentration used.

At the 8-cell stage recovered on Day 3, some embryos had not undergone compaction. However, the compacted and the uncompact embryos showed no difference in their Con A binding capacity and the data were therefore pooled.

Text-fig. 2. Scatchard plot of Con A binding by different stages of mouse embryo: 2C = 2-cell (○—○); 4C = 4-cell (●—●); 8C = 8–10-cell (◇—◇); 4B = Day-4 blastocyst (◆····◆); 5B = Day-5 blastocyst (△---△).

The total number of Con A molecules bound/embryo and the apparent association constant at various stages of development were estimated graphically from the Scatchard plot (Text-fig. 2) and are shown in Table 2. There was a rapid increase in number of Con A molecules bound and a decrease in association constant for the Day-5 (late blastocyst) embryos.

Table 2. The number of Con A molecules bound/embryo and their association constant at various stages of development as estimated from the Scatchard plot (Text-fig. 2)

Stage of embryos	No. of Con A molecules bound/embryo	Apparent association constant (M^{-1})
2-cell	12×10^7	3.0×10^6
4-cell	13×10^7	2.9×10^6
8–10-cell	17×10^7	2.1×10^6
Early blastocyst	19×10^7	1.9×10^6
Late blastocyst	150×10^7	0.83×10^6

Discussion

The Scatchard plot analysis is more refined than the method of saturation binding employed in the earlier study with mouse blastocysts (Wu & Chang, 1978). However, both studies have difficulties inherent in the handling and preparation of the embryos and the procedures used. This could affect significantly the accuracy of the values obtained. For example, the digestion of zonae pellucidae by the pronase treatment could conceivably result inadvertently in the pronase attack on the protein molecules of the plasma membrane. Any injuries to the membrane would then alter the membrane compositions, thereby affecting the Con A binding. To minimize such a possibility, the pronase treatment was carried out with great caution. All pronase-treated embryos were washed twice and incubated for 3 h at 37°C so that possible injuries to the membranes could be eliminated. Since pronase-treated embryos after 1, 2, 3 or 4 h in culture appeared to have the same Con A-binding capacity as those treated with acidic Tyrode's solution or dissected with micro-needles, it is very likely that the membrane injuries caused by the pronase treatment were either minimal or could be readily repaired during culture for 1 h. Our observations showed that prolonged pronase treatment invariably resulted in the separation, enlargement and disintegration of the blastomeres, with the early cleavage stages of embryos being more susceptible than the morula and blastocyst stages. Magnuson & Stackpole (1978) indicated that, after removal of the zona by pronase, agglutination of 8- to 16-cell mouse embryos by Con A increased progressively during a 6-h incubation but that no such changes occurred in late blastocysts. It is not clear whether this is due to pronase-induced changes or is simply an artefact caused by the culture conditions. The latter consideration was the main reason for not carrying out the experiment with one-cell embryos in this study.

Another factor of importance is the duration of washing of embryos following incubation in the Con A solution. As shown in the 'Results', the amount of Con A bound to the embryo depended on the concentration of Con A used. Since the washing solution (PBSA) contained no Con A, the washing process favoured the dissociation of the bound Con A from the binding sites on the cell membrane over the association (or binding). This would tend to cause under-estimation of the Con A binding to the embryo.

During the preimplantation stage of development the number of Con A molecules bound/embryo tended to increase with each division, probably related to the increase in cell surface area. However, the 7-fold increase between the Day-4 and Day-5 blastocysts, as shown earlier (Wu & Chang, 1978) and confirmed here, could be due to an increase in the density of the

binding sites and/or an increase in the cell surface caused by the cell division and the giant cell transformation of the abembryonic trophoblast cells. Carollo & Weitlauf (1979) suggested that the increase in Con A binding in implanting mouse blastocysts during delayed implantation (Wu & Chang, 1978) could be related to changes in binding site density and changes in surface area. We are currently investigating the binding site density at the ultrastructural level.

The number of Con A binding sites in the unfertilized to 8-cell mouse embryos have been estimated by Pienkowski (1974) to be about 2.0×10^8 , using ^{125}I -labelled Con A at $50 \mu\text{g/ml}$. This value is much higher than our estimates, probably due to the fact that Pienkowski exposed the embryos to the Con A solution at 37°C whereas we did it as 4°C . At 37°C the Con A not only can bind to the cell surface but may also be taken into the cell, resulting in overestimation of the Con A binding sites on the embryo.

Although the number of Con A binding sites/embryo appeared to be increasing with development, the mouse embryos became less agglutinable and were not agglutinable even when Con A concentration reached as high as $5000 \mu\text{g/ml}$ by Day 4 (Pienkowski, 1974; Rowinski *et al.*, 1976). Evidently, agglutinability cannot be correlated with the number of binding sites alone, and other factors such as the density and the lateral mobility of the binding sites are probably more important and more relevant (Nicolson & Poste, 1976a, b; Konwinski, Vorbrodt, Solter & Koprowski, 1977).

The association constants between Con A and mouse embryos, as estimated in the present study, are lower than that of Con A and SV40-transformed hamster cells, $1 \times 10^7 \text{ M}^{-1}$ (Vlodavsky & Sachs, 1975), comparable to that of Con A and ovalbumin glycopeptide, $2.5 \times 10^6 \text{ M}^{-1}$ (Young & Leon, 1974), but much higher than that of Con A and α -methyl-D-mannoside, $1.5 \times 10^5 \text{ M}^{-1}$ (So & Goldstein, 1968). The change in the association constant at different stages of mouse development suggests that the Con A binding sites, probably glycoproteins, on the embryo surface may vary, during development, in a relative ratio of different binding groups (or sugars) and/or in the groups surrounding the binding sites. This is also accompanied by an increase in the total number of Con A binding sites on the implanting mouse blastocyst. The finding is in line with that of Pinsker & Mintz (1973) who showed by chromatographic analysis that cell-surface glycoproteins change with development in mouse embryos.

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