Analysis for the Stage Specific Antigen of the Primordial Germ Cells in the Chick Embryo

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ABSTRACT. A monoclonal antibody 2C9 (IgM κ -light chain) was established by fusing the myeloma cells (X63-Ag8-653) with the spleen cells immunized with sexually indifferent gonads from 6-day chick embryos. The 1- to 17-day chick embryos were examined by immunohistochemistry (ABC technique). As a result, the 2C9 antigen first appeared in the cytoplasm of some primordial germ cells (PGCs) of the germinal crescent at 1 day of incubation. The reactivity was also detected in the hypoblastic cells. This antigen may be produced at this stage. After the migrating stage, 2C9-reactive PGCs were increased in number. From this stage to the sexually differentiating stage (7 days of incubation), the 2C9 antibody was reactive all over the cytoplasm of PGCs in both sexes. In the female gonads, the reactivity disappeared at 8 days of incubation, but not in the male. The reactivity of male PGCs was gradually decreased and disappeared until 14 days of incubation. Since the stages of disappearance of this antigen in both sexes seem to depend on the differentiation of the oogonia and spermatogonia, this antigen may disappear in accordance with germ cell differentiation. Cross-reactions were observed in hepatocytes, gastrointestinal endoderm and some mesonephric tubules. By SDS-PAGE and immunoblotting methods, all the extracts from these tissues revealed two bands; 109 kilodalton (kDa) and 64 kDa, suggesting that the 2C9 antibody detects the same molecule in each kind of cells. The 2C9 antibody may be a useful cell-marker and/or probe for analysis of the germ cell differentiation in chick PGCs.—**KEY WORDS:** chick embryo, immunohistochemistry, monoclonal antibody, primordial germ cell.

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Avian primordial germ cells (PGCs) first appear in the epiblast [2, 17, 21, 27] and are translocated to the anterior and anteriolateral primary hypoblast designated as the germinal crescent [22]. PGCs then migrate into the developing embryo via bloodflow [4, 19] by ameoboid movement [13] to settle in the developing gonad (genital ridge). The PGCs have a tendency to be surrounded with somatic cells during the gonadal development. After sex differentiation, the PGCs differentiate into spermatogonia and oogonia in the testis and the ovary, respectively.

The germ cell differentiation is complicated, and a number of investigations on the factors related to the germ cell development have been carried out in the chick [1, 15, 23, 26], mouse [6, 10, 16] and rat [3]. It seems likely that extracellular matrices [5, 17, 27] and chemotactic factors [12] have a relation to the cell adhesion and migration of the PGCs. Although a number of molecules related to the germ cell development have been found out, their roles for the differentiation have not yet been defined.

In this study, we tried to establish a monoclonal antibody for analysis of the unknown molecule related to the differentiation of chick PGCs and to analyze the expressions of the antigen by immunohistochemistry and immunoblotting.

MATERIALS AND METHODS

Animals: Fertilized eggs of White Leghorn, from Kanagawa Prefectural Livestock Experiment Station, were incubated to use for immunogen, immunohistochemistry and immunoblotting. Embryonic stages were classified after Hamburger and Hamilton [8]. BALB/c strain

mice, maintained as a closed colony in the Department of Veterinary Anatomy, the University of Tokyo, were used for immunization and collecting spleen cells.

Production of mAb bound to PGCs: The monoclonal antibody was established by a basic procedure [11]. BALB/c mice were immunized with the indifferent gonad emulsion from 6-day chick embryos (stage 29). Myeloma cells (X63-Ag8-653) were fused with splenocytes from an immunized mouse in the presence of polyethyleneglycol 3500. The fused cells were incubated in HAT selective medium [11] containing 10% fetal calf serum (FCS) in 5% CO₂ under a moist atmosphere at 37°C. They were screened out by indirect enzyme-immunohistochemistry in the chick gonads to obtain hybridomas secreting the antibody bound to PGCs. One of the hybridomas secreting the PGC positive antibody was named 2C9 and was cloned by limiting dilution. The mouse was given an intraperitoneal injection of with the 2C9 hybridoma clone (1×10^8 cells). About 2 weeks later, the mouse ascitic fluid was prepared.

Immunohistochemistry: In addition to whole embryos at 1 to 10 days of incubation, the gonad, liver, and gastrointestinal tract from 11- to 17-day embryos were fixed with Bouin's fluid and were dehydrated in a graded series of ethanol. They were embedded in paraffin and sectioned at 5 μ m. The sections were used for immunohistochemistry using avidin-biotin-peroxidase complex (ABC) technique. The specimens were deparaffinized and treated with 0.3% H₂O₂ in methanol to remove endogenous peroxidase. They were washed in 0.01 M phosphate-buffered saline (PBS) and were blocked with 1% bovine serum albumin (BSA). After briefly washing with PBS,

they were incubated with diluted ascitic fluid containing 2C9 in PBS containing 0.1% BSA (1:15000) for 2 hr. After a rinse with PBS, they were incubated with secondary antibody (biotin-conjugated goat anti-mouse IgG + IgM diluted 1:50, (Tago Inc., U.S.A.)) for 1 hr. After washing again with PBS, they were treated with ABC (Vector, U.S.A.) for 30 min. Peroxidase was then developed with 0.005% 3, 3'-diaminobenzidine (DAB) in PBS containing 0.001% H_2O_2 for 20 min and washed with distilled water. They were observed by light microscopy. The incubations were performed at room temperature at all times.

SDS-PAGE and Immunoblotting: The gonad, liver, gastrointestinal tract, mesonephros and head from the 6-day chick embryo, were extracted with PBS containing 0.1% triton $\times$ 100. The extracts were centrifuged at $20,000 \times g$ for 30 min. The supernatants and biotinylated molecular weight standard (Bio-Rad Laboratories, U.S.A.) were denatured in the sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 10% glycerol) for 5 min in a boiling water bath. They were separated by SDS-polyacrylamide gel (7.5%) electrophoresis (SDS-PAGE) [14]. The proteins were transferred electrophoretically [24] to polyvinylidene difluoride (PVDF) transfer membrane (Pall Co., Ltd., U.S.A.).

The blotted PVDF membrane was first blocked in PBS containing 0.3% Tween 20 (PBS/T), 15% FCS, and 5% BSA for 2 hr. The membrane was rinsed briefly with PBS/T, followed by incubation with diluted mouse ascitic fluid containing 2C9 (1:100,000) in PBS/T containing 0.1% BSA (BSA-PBS/T) for 4 hr. After washing with PBS/T for 1 hr, the membrane was incubated with the biotin-conjugated secondary antibody (diluted 1:5000) in BSA-PBS/T for 4 hr. After washing again with PBS/T, the membrane was treated with ABC for 1 hr and visualized with DAB in PBS containing H_2O_2 .

RESULTS

Monoclonal antibody 2C9: The mAb 2C9 (IgM, κ -light chain) bound to the PGCs in the 6-day chick embryo (stage 29, Fig. 1A, B). This reaction was observed all over the cytoplasm. The reactivity was also present in hepatocytes, gastrointestinal endoderm and some mesonephric tubules in the same manner with this mAb.

Immunohistochemistry: Temporal and spatial reactivities to mAb 2C9 were examined in the developing chick embryos from 1 to 17 days of incubation (stage 7 to 43). In these stages PGCs can be distinguished from somatic cells by their large size and somewhat peculiar nuclei. In the germinal crescent stage (1 day of incubation), the reactivities were detected in the cytoplasm of PGCs, although some PGCs revealed no reactivity. The reactions were also observed intensely in the hypoblast, but weakly in the epiblast (Fig. 2). From migrating stage (stage 14) to settling stage into the genital ridge (stage 18), similar reactions were observed in the cytoplasm of migrating PGCs in the blood vessels (Fig. 3) and among the mesenchyme, while in the genital ridge (stage 24 to 29), the PGCs were bound to mAb 2C9 in a similar manner (Fig. 1).

After the sex differentiation in the 7-day embryo (stage 31), similarly positive reactions with mAb 2C9 were detected in both sexes (Fig. 4A, B). In the female, the reactions disappeared at 8 days of incubation (stage 34, Fig. 5). At this stage, the cortex of the female gonads had the high activity of proliferation and the PGCs clustered within the cortex. In the male, the PGCs were reacted with the mAb 2C9 after sex differentiation. The mAb 2C9 positive cells decreased after 10 days of incubation (stage 36), and finally disappeared until 14 days of incubation (stage 40, Fig. 6). The specific reactivity with the mAb 2C9 was detected in hepatocytes, gastrointestinal endoderm and mesonephric tubules during all stages ex-

Fig. 1. Immunostaining of chick gonads with mAb 2C9 at 6 days of incubation. Specific reactions are detected in PGCs (A. $\times$ 200). It is noted that these reactions are scattered in the cytoplasm of PGCs (B. $\times$ 400).

Fig. 2. PGCs in the germinal crescent region (1 day of incubation) are stained with mAb 2C9 (arrows $\times 400$), but there are some PGCs with no reactivity (arrowheads). The antigens are polarly present in the cytoplasm of PGCs and hypoblastic cells.

Fig. 3. A migrating PGC in blood vessels at 2 days of incubation. The 2C9 antigens are scattered all over its cytoplasm (arrow $\times 400$). Yolk sac endoderm also expresses the antigens, but blood cells do not.

Fig. 4. After sex determination of chick gonads, the mAb-positive PGCs persist in both male (A.) and female (B.) at 7 days of incubation.

amined (Table 1). However, there were no reactivities in the metanephros.

SDS-PAGE and Immunoblotting: A molecular weight analysis of the molecule carrying 2C9 epitope was carried out in the each of 6-day embryonic body parts, heads, gonads, livers, gastrointestinal tracts and mesonephros by SDS-PAGE and was blotted to PVDF membrane, resulting in two bands; a strongly stained 109 kDa band and a weakly stained 64 kDa band. Except for the head, all of the tissues containing mAb 2C9 positive cells showed two bands. There were no detectable bands in the head extract lane (Fig. 7).

DISCUSSION

In the present study, we established the mAb 2C9 which recognizes specific molecules in the PGCs of the chick embryo. The immunohistochemical localization of an antigen against the 2C9 was detected in the PGC cytoplasm of the 6-day embryo, and the reactivity was PGC-specific in the gonads. Since hepatocytes, gastrointestinal endoderm and mesonephric tubules at the same stage were reacted with this mAb, the 2C9 antigen seems to be widely distributed in the tissues of chick embryo.

The localization of 2C9 antigen was examined in the chick embryo from 1 to 17 days of incubation. The reactivity of PGCs with 2C9 mAb at the germinal crescent

Fig. 5. A gonad in a female at 8 days of incubation. The reaction of 2C9 antigens in PGCs has completely disappeared.
 Fig. 6. A gonad in a male at 14 days of incubation. PGCs with 2C9 antigens decrease in number after 10 days of incubation and disappears until 14 days of incubation.

Fig. 7. Immunoblotting analysis of some tissue extracts in the chick. The extracts of gonad (lane 3.), liver (lane 4), mesonephros (lane 5) and gastrointestinal tract (lane 6) show two bands, a main 109 kDa band and a minor 64 kDa band. The head loaded lane does not show any bands (lane 2). Lane 1 is loaded biotinylated SDS-PAGE standards.

Table 1. Expressions of 2C9 antigen in the chick embryo

Stage of embryo ^{a)}	- 7	14 to 18	21 to 29	31 ^{b)}	34	40 -
Differential degree of PGCs	germinal crescent	migrating stage	within the germinal ridge	sexual differentiation	oogonia (♀)	spermatogonia (♂)
Germ Cells	+	+	+	+/+	+/-	-/-
epiblast	±					
hypoblast	+					
blood cells		-	-	-/-	-/-	-/-
hepatocyte			+	+/+	+/+	+/+
gastrointestinal endoderm		+	+	+/+	+/+	+/+
mesonephros			+	+/+	+/+	+/+
neural tube		±	-	-/-	-/-	-/-

a) By Hamburger and Hamilton (1951) [9].

b) After stage 31, sexuality could be determined histologically. ♂/♀.

stage (1 day of incubation) was almost the same with that of 6-day embryonal gonads except that some PGCs did not express this antigen. A similar reaction to mAb 2C9 was detected in hypoblast cells. It is thought that the antigen derived from hypoblast cells may be translocated to the PGCs, or that some signals from hypoblast stimulate the production of an antigen in the PGCs. However, we cannot define this concept in the present study. After this stage, in both male and female gonads, the reaction of PGCs was detected continually from the migrating stage to 7-day embryonic stage. The reaction in PGCs disappeared in the female at 8 days of incubation, whereas in the male it still persisted until 14 days of incubation. PGCs are thought to differentiate into oogonia at 8 days and spermatogonia at 13 days of incubation, respectively [9]. The disappearance of the reaction may have a strong relation with the differentiation of spermatogonia and oogonia. In the other positive somatic cells examined, 2C9 antigen still remained in a small amount at each later stage. The finding indicates that the 2C9 antigen may appear and act mainly during embryogenesis.

Since glycogen granules accumulate in chicken PGCs during early embryogenesis, periodic acid Schiff (PAS) staining has been used as a cell-marker for PGCs. PAS-positive granules in chicken PGCs first appear at the germinal crescent stage and persist until 6 or 7 days of incubation [4, 19, 22]. The reactions of PGCs with mAb 2C9 were similar to those with PAS staining in the period of these appearance. The other 2C9 antigen-detectable tissues also have PAS-positive granules. These findings suggest that the antigen may contain glycoconjugates. If this is the case, it will be related to the differentiation of PGCs.

In the electrophoretical examination, all mAb 2C9 positive tissues showed two bands, a main 109 kDa band and a minor 64 kDa band, suggesting that the proteins reacted with 2C9 mAb have the same molecules as are located in the cytoplasm of hepatocytes, gastrointestinal endoderm and mesonephric tubules.

An analysis of various protein molecules in the chick PGCs has been carried out by many authors. Urven *et al.* [26] reported the immunohistochemical expression of EMA-1 [6] in the chick embryo. The EMA-1 antigen was expressed in the plasma membrane from the initial appearance of PGCs to the settling stage in the genital ridge. The reactivity with EMA-1 disappeared at 7 to 8 days of incubation in the female. In the male, the reactions of PGCs with EMA-1 decreased at 11 days of incubation. It is thought that the EMA-1 recognizes a high molecular weight glycoprotein [7]. Its spatial expressions in other tissues in the chick were almost similar to those of the 2C9. It seems that these two epitopes, 2C9 and EMA-1, may have a close relation with each other in molecular cascades. Furthermore, Yoshinaga *et al.* [28] reported the immunohistochemical expressions of Le^x antigen [16] in the early mouse embryo, and suggested that the Le^x antigen was similar to the EMA-1 and SSEA-1 in the period of appearance [20]. It seems that the

2C9 antigen shows a pattern similar to that of these antigen in the period of appearance.

Since the mAb 2C9 is able to trace PGCs from the germinal crescent stage to settling stage, it may be one of useful cell-markers and/or probes for the analysis of germ cell differentiation in the chick embryo.

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