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Short communication

Detection of CD2 expression in chicken hematogenic embryo yolk sac lymphoid cells prior to thymus genesis

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Abstract

Lymphoid mononuclear cells from chick embryos at stage 16 were collected prior to fetal liver and thymus genesis to study the differentiation and function of the hematogenic yolk sac and to detect whether CD2 occurs on the surface of lymphoid mononuclear cells. The phenotype and functional activity of the cell surface protein E receptor and the ultrastructure of embryonic E^+ cells were compared with those of mature T cells. Our results indicate 99.36% homology between the E receptors of embryonic lymphocytes and mature T cells. Other similarities, including molecular distribution, motivation, the ability to form an erythrocyte rosette, the structure of the receptor–ligand complex, and the conformation of the signal channel, were detected between embryonic lymphocytes and mature CD2-expressing T cells. These results indicate that CD2 is already expressed prior to fetal liver and thymus genesis and that its expression is not dependent on the thymic microenvironment.

Keywords: CD2; Chicken embryo; Hematogenic yolk sac

1. Introduction

Because of their important biological role and given their potential applications, hemopoietic stem cells have attracted significant attention from biological researchers in recent years; this, has led to the acceptance of the theory of three-stage fetal hematogenesis and the belief that the yolk sac is the first site of embryonic hematogenesis in mammals and birds. Yolk sac hematogenesis appears to produce mostly erythrocyte series cells. Pro-T lymphocytes from fetal liver and bone marrow migrate to the thymus where, under the influence of multiple factors in the thymic microenvironment, they develop into original, precursor, double-negative, double-positive, single-positive, and (finally) immature T lymphocytes [1]. CD2, an important T lymphocyte marker, appears at an early stage of precursor T development [2]. Scientists have successfully studied many features of the differentiation and development of thymic T lymphocytes; however, few studies have focused on lymphoid cells before they enter the thymus.

We previously found that E^+ cells (hereafter referred to as E cells) are able to form erythrocyte rosettes with sheep erythrocytes and the existing lymphoid mononuclear cells in chick embryo blood, indicating that lymphoid cells carry an E receptor for a specific T lymphocyte marker [3,4]. Thus, we designed a study to further define this molecule and to determine whether the E receptor is actually the T cell surface antigen CD2. To accomplish this, we compared embryonic and adult chick mature T lymphoid cell CD2. Our results provide new information on the differentiation, function, and application of hemocytes from the hematogenic

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yolk sac, as well as new clues to the relationship between embryonic hematogenesis and embryonic immune function. They may also lead to the development of a strategy for minimizing immunological rejection during stem cell transplantation therapy.

2. Materials and methods

2.1. Preparation of AET-sheep erythrocyte suspension

Sheep peripheral blood cells (SRBCs) were harvested from Chinese Merino sheep (bred in our lab). Freshly prepared 0.143 M (pH 9.0) s-(2-aminoethyl)-2-isothiuronium bromide hydrobromide (AET, Sigma) was added to the SRBCs at a ratio of 1:4, and the cells were incubated in a water bath at 37 °C for 15 min with shaking every 5 min. The cells were subsequently washed five times with cold stroke physiological saline solution and centrifuged for 5 min at 500*g*. The AET-SRBCs were then resuspended in RPMI-1640 culture medium containing 10% calf serum to yield 10% AET-SRBC suspension. The cells were stored at 4 °C until use.

2.2. Preparation of mononuclear cells

Sixty white Leghorn eggs (purchased from the IAS-CAAS) were opened at 55–56 h of incubation according to routine methods, and embryos at stage 16 were selected as described previously [5]. Blood was then collected from the embryos in tubes containing heparin. Wing vein blood was also collected in heparin-containing tubes from 4-month-old white Leghorn chickens. The lymphocytes in the samples were then isolated. After washing three times with Hank's solution without Ca^{2+}/Mg^{2+}, each sample was divided in two, and one-half was mixed with an equal volume of 1% SRBC for use in erythrocyte rosette testing. The test was conducted according to routine methods using 0.8% glutaral for fixation. The other half of each sample was washed three times with Hank's solution without Ca^{2+}/Mg^{2+} and adjusted to a concentration of 1×10^6 cells/ml.

2.3. Detection of in vitro erythrocyte rosette formation by light and electron microscopy

Fixed adult and embryonic chick samples were stained with Giemsa–Wright's stain and subjected to light microscopy to determine the number of erythrocyte rosettes. Using this number, the ratio of mononuclear cell to erythrocyte rosette reaction was calculated.

To prepare the samples for transmission electron microscopy, adult and embryonic chick samples were prefixed in 2.5% glutaral for 15 min, washed three times with sucrose-phosphate buffer, then post-fixed for 30 min in 1% osmic acid and serially dehydrated. Following Epon 812 entrapment and the preparation of ultrathin sections, the samples were observed using a JEM-100C transmission electron microscope.

The morphology of the erythrocyte rosettes was determined by scanning electron microscopy (Hitachi S-450 scanning electron microscope) using smears of the adult and embryonic chick samples that were prepared by JB-5 type ion sputtering after acetone volatilization.

2.4. Extraction and analysis of the surface proteins on the E-positive cell membrane

Human T lymphocytes, chick mature T lymphocytes, and chick embryonic E cells were extracted by centrifugal separation after the formation of erythrocyte rosettes [6] and separated according to the following method. Approximately 10^6 cells in a 1 ml volume were added to a 1.5 ml aseptic centrifuge tube, reconstituted with 150 μl of Hank's solution without Ca^{2+}/Mg^{2+}, and incubated in a 45 °C water bath for 1 h with shaking every 15 min. The cells were then collected by centrifugation at 4 °C for 10 min at 2000*g*. The supernatants were transferred to Microcon centrifuge tubes (MW = 10 kDa, Millipore Co.) and spun at 4 °C for 10 min at 10,000*g*. A 20 μl aliquot of each concentrated solution was subjected to SDS–PAGE (10% alkalinity separation paste), transferred onto nitrocellulose filters (Millipore Co.), stained with Ponceau red, and decolorized after marking the position of the molecular weight standard and soaking for 20 min in RPMI-1640 culture medium containing 10% calf serum. The membrane was then placed in a centrifuge tube containing 1% AET-SRBC suspension prepared in RPMI-1640 culture medium containing 10% calf serum and incubated for 20 min at 37 °C. The tube was centrifuged for 5 min at 500*g*, then at 4 °C for 2 h after rinsing with 4 °C NaCl, to detect erythrocyte aggregation.

2.5. Cloning of the chick CD2 mRNA fragment

The following primers were designed according to the sequence of the chick T lymphocyte surface antigen *CD2* (GenBank Accession No. LOC768858):

P1: 5′-AGCCCGTCCCTCAGCCAATC-3′ and

P2: 5′-AGCTTCCGCATCGCCATCTC-3′.

Cellular RNA was extracted using Trizol (Invitrogen Co.) according to the manufacturer's instructions, and cDNA was synthesized from 1.5 μg of the RNA using the EasyScript First-Strand cDNA Synthesis Kit (TransGen Co.). The cDNA was used for PCR according to standard procedures. The reaction conditions were: 30 cycles of denaturation for 30 s at 72 °C, annealing for 30 s at 56 °C, and extension for 1 min at 72 °C; followed by a final incubation for 10 min at 72 °C. The products were separated by agarose gel electrophoresis, purified from the agarose gel and cloned into a T carrier plasmid (TransGen Co.) for subcloning. Insertion of the fragment was confirmed by sequencing.

3. Results

Using blood collected from stage 16 chick embryos, we isolated a considerable number of lymphoid mononuclear cells, which were smaller than irregularly shaped erythrocytes (data not shown). A portion of lymphoid mononuclear cells were able to form erythrocyte rosettes when mixed with SRBCs (Table 1).

Light microscopy indicated E-positive reactions among mononuclear cells isolated from the peripheral blood of stage 16 chick embryos at a frequency of approximately 30%. The morphology of the cells was similar to that of mature chick T lymphoid cells, except that the chick embryonic cells exhibited slightly stronger staining (Fig. 1a). The diameter of the E cells was between 4 and 5 μm, and the cells were regular in shape, with blue staining of the cytoplasm and nucleus using Giemsa–Wright's stain; moreover, the nuclei were relatively large and round or ovoid.

Scanning electron microscopy indicated several significant differences in morphology between the stage 16 chick embryo E cells (Fig. 1b) and mature T lymphoid cells (Fig. 1c). Both were round or oval in shape; however, the volume of the E cells was larger than that of the mature chick T lymphoid cells ($\Phi = 3$–4 μm), and small introcessions were distributed across the E cell surface, whereas the surface of the mature chick T lymphoid cells bore digitiform villi like Polliack et al. [7] had reported.

Transmission electron microscopy also indicated significant differences between the chick embryonic cells (Fig. 2a) and mature chicken T lymphoid cells (Fig. 2e). For example, the cell body in the embryonic cells was larger than that in the mature chicken T lymphoid cells, and numerous small introcessions were distributed across the cell surface. In addition, compared to the T lymphoid cells, the embryonic cells had a larger nuclear-cytoplasmic ratio, a broader perinuclear cisterna, a clear nuclear membrane, a large and obvious nucleolus, little heterochromatin, large cytoplasmic mitochondria, numerous lysosomes, and few ribosomes. In comparison, the T cells bore many surface microvilli and their nuclear chromatin was highly condensed; also, there was an abundance of free ribosomes in the cytoplasm, but few mitochondria.

In both cell types, however, the mode of connection with the SRBCs and the mode of entry into the cells via the E receptor were the same. In both cases, a flocculent cap-shaped connection was observed on the surface (Fig. 2b, f), through which the compound was able to cross into the cytoplasm and enter directly into the perinuclear cisterna along with microtubules (Fig. 2c, d, g).

Table 1
Number of erythrocyte rosette reactions involving mononuclear cells from the peripheral blood of chick embryos

No	Number of cell count	Chick embryo E cell count	Positive rate (%)
1	200	64	32
2	200	58	29
3	200	63	31.5
4	200	65	32.5
5	200	62	31
Average	200	62.4	31.2

The observed erythrocyte rosette reactions indicate that mature human T lymphoid, mature chick T, and chick embryo E cells contain a surface protein capable of interacting with sheep erythrocytes to form erythrocyte rosettes and that the molecular weight of the human-derived protein is greater than that of the chick-derived protein. Each of these proteins was approximately 50 kD in size (Fig. 3a). Using RT-PCR, we amplified three cDNA fragments from chick embryo and fowl peripheral blood E-positive cells, the lengths of which were 300, 400, and 500 bp respectively (Fig. 3b). Based on the lengths of the fragments, we identified fragment 2 as the gene of interest. Therefore, fragment 2 was purified, cloned into pGEM-T, and sequenced. The data indicated 99.36% homology between the fowl and chick embryo sequences and 99.6% homology between the sequence of the amplified fragment and the sequence of chick *CD2* in the NCBI database (Fig. 4). BLAST analysis showed that the fragment consisted of three exons without introns.

4. Discussion

We analyzed the expression of chicken CD2 on hematogenic chicken embryo yolk sac lymphoid cells and cloned a *CD2* cDNA fragment from fowl mature T cells and yolk sac hematogenic chick embryo blood erythrocyte rosette-positive cells. We showed that circulatory blood cells from stage 16 chick embryos express CD2 on their surface, similar to mature chick T lymphoid cells.

Since the discovery of a T lymphoid cell surface antigen using a monoclonal antibody, there have been several reports of novel CD molecules in chicken lymphoid cells [8]; however, until now, few reports on chicken CD2 have been published. Upon completion of the chicken genome project, the sequence of chicken *CD2* was deduced using a computer. We previously showed the presence of numerous lymphoid mononuclear cells in stage 12 (48 h) chicken embryos and demonstrated that a portion of them are able to form erythrocyte rosettes upon exposure to sheep erythrocytes; moreover, they are able to induce a T lymphoid cell-specific ANAE-positive reaction, and the proportion of positive cells increases significantly during chick embryo development [3,4]. These data indicate an abundance of lymphoid cells in the blood during yolk sac hematogenesis, which is independent of the presence of local factors from the embryonic liver and thymus, except for gigantocytes. Moreover, the morphology of these cells was similar to that of mature lymphoid cells, and the level of homology between the CD2 expressed on the E^+ and mature chick T lymphoid cells was 99.36%.

CD2 is known as E receptor, also as T11, or lymphocyte function-associated antigen 2 (LFA-2) [9]. CD2 is distrib-

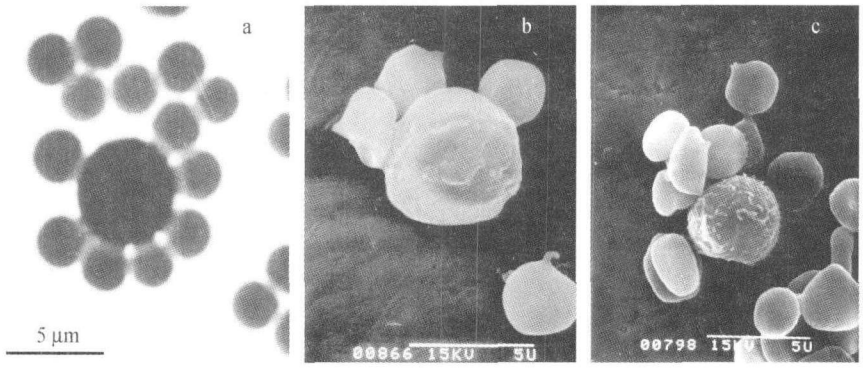

Fig. 1. Observation of erythrocyte rosette-positive cells by light and scanning electron microscopy. (a) A chick embryo E cell observed by light microscopy; (b, a) chick embryo E cell observed by scanning electron microscopy; (c, a) chick mature T lymphocyte observed by scanning electron microscopy.

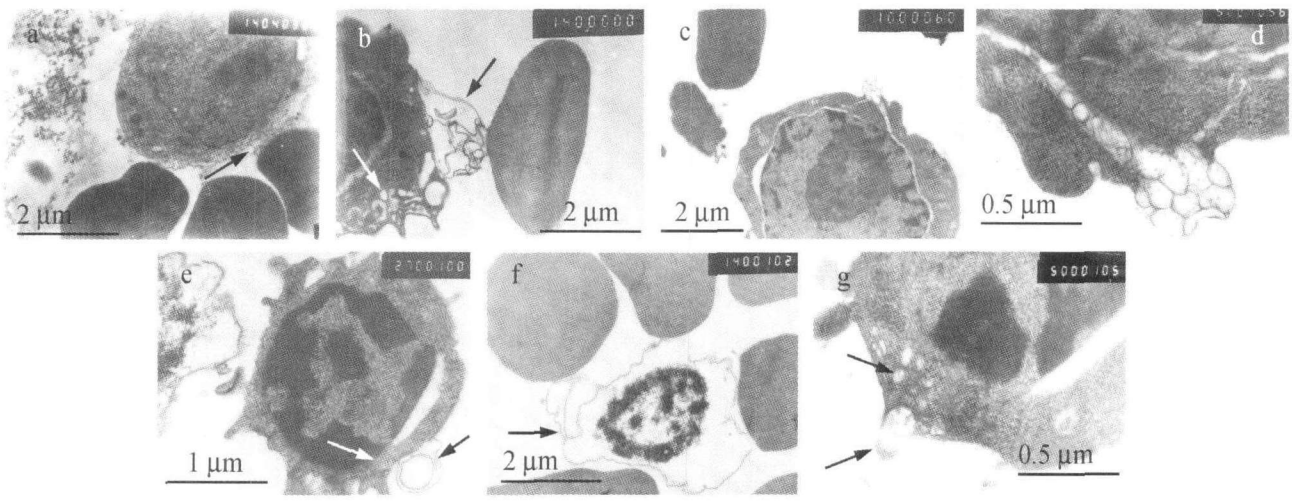

Fig. 2. Ultrastructure of the erythrocyte rosette-binding part observed by transmission electron microscopy. (a) The electro-dense and flocculent connection between a chick embryo E cell and SRBC; (b) a typical cap-shaped connection between a chick embryo E cell and SRBC, and microbule section close together; (c) the structure of E-receptor extended from the chick embryo E cell membrane surface recovery into the perinuclear cisterna; (d) enlarged view of c; (e) E-receptor and microbule of a chick mature T cell; (f) cross-section of the cap-shaped connection between a chick mature T cell and SRBCs; (g) a typical cap-shaped connection between a chick mature T cell and SRBC, and microbule section close together.

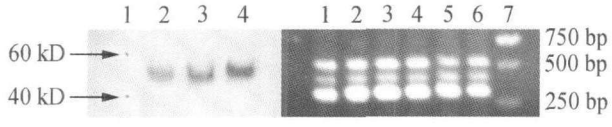

Fig. 3. An erythrocyte rosette reaction and RT-PCR analysis. In a: 1, marker; 2, chick embryo E cell; 3, chick mature T cell; 4, human T cell. In b: 1, 2, and 3, chick mature T cell; 4, 5 and 6, chick embryo E cell; 7, marker.

uted across the surface of thymocytes, mature T lymphoid cells, and most NK cells, where it functions in cellular adhesion and as a signaling molecule with important roles in cell maturation, reorganization, activation, and apoptosis [10]. Lymphoid cell proliferation is a key part of the immune response, and CD2 is associated with the activation and proliferation of lymphoid cells; thus, CD2 is an important topic in immunogenesis and immunoregulation studies. In recent years, important progress has been made regarding the molecular structure, activation pathway, and biological function of CD2. Therefore, the conformation and signal pathway of CD2 have attracted great interest from immunologists wishing to understand the mechanism of action of E receptor on lymphoid cells. E receptor has been shown by immunofluorescence to produce obvious capping of the lymphoid cell surface [11]. The three-dimensional crystal structure of CD2 clearly indicates that the ligand- and monoclonal antibody-binding regions of CD2, as well as the region involved in adhesion, are located in its extracellular domain and that the epitopes involved in each of these functions overlap [10]. During the process of signal transduction, CD2 binds with tubulin in the cytoskeleton, and tubulin plays an important role in the distribution of CD2 on the cell surface; moreover, tubulin guides CD2 to nearby target molecules [12]. However, there are no reports on the conformation of E receptor and the morphological characteristics correlated with its functions in signaling. We analyzed the ultrastructure of erythrocyte rosettes using fowl and stage 16 chick embryo cells and

Fig. 4. Comparison of the sequenced *CD2* gene fragment and the published sequence of adult chicken T cell *CD2*.

found that there were complicated areas of linkage between CD2 and its ligand. In particular, a floccus extended from the E^+ cells to the SRBCs, forming a cap close to the E^+ cell side. Upon disaggregation from the SRBC, the floccus entered the cytoplasm through the microtubule system and eventually entered the nucleus. Several microtubules were observed in the cytoplasm near the E receptor; however, longitudinal sections of the microtubules were not produced. It is clear that tubulin plays a critical role in the cell surface distribution of CD2 and that CD2 can affect the intracellular redistribution of tubulin; thus, the binding of CD2 with tubulin creates an information channel that extends to the nucleus and affects the activation, proliferation, and functional regulation of E^+ cells, as well as a series of immunological reactions. Our erythrocyte rosette results indicate that the CD2 expressed on the surface of chick embryo blood cells prior to fetal liver and thymus genesis is the same as that expressed on chick T lymphoid cells. Our results also provide a morphological basis for further research on the structure and function of CD2 and the mechanism of action of E receptor on lymphoid cells.

Our results demonstrate that hemopoietic stem cells already contain differentiated lymphocytes and erythrocyte series cells and that they express CD2 prior to thymus genesis. Such studies will have great theoretical significance for research on the spatiotemporal relationship between hemopoietic stem cells and their ancestral cells and the effect of the yolk sac microenvironment on hemopoietic stem cells and embryonic immune function. In addition, they will provide a theoretical basis for applied research into pathopoiesis mechanism of autoallergic disease, immunological tolerance, immunological rejection, and stem cell therapy.

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