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Rapid clearance of circulating protein by early chicken embryo blood cells

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SUMMARY

It has been speculated that free amino acids digested from proteins in bird eggs are transported to the circulation for the nourishment of the embryo. In the present study, we found that early chicken embryo protein in the serum might be utilized efficiently as a nutrient. Proteins injected into the blood of embryonic day 3 (E3) embryos were partially degraded and rapidly cleared. The rapid clearance of the injected proteins might be the result of efficient pinocytosis by blood cells, which then efficiently digested the intracellular proteins. An evaluation of the fluorescence intensity of injected fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) indicated that about half was taken up by the blood cells 80 min after injection. About 4 h after injection, most of the FITC-BSA was digested and the products were released into the serum, which implies that circulating blood cells may serve as a digestive system in early chick embryos. However, the endocytic activity of blood cells decreased after E5, and BSA may reside in the circulation with a longer half-life after E5. These results imply that blood cells would serve as a digestive system only in early embryos. In summary, the mechanism revealed here gives the early embryo the ability to make use of protein as a nutrient without prior digestion outside the embryo.

Key words: chicken embryo, amino acids, protein, blood cells, pinocytosis, digestion.

INTRODUCTION

Nutrition for the developing bird embryo originates entirely from the fertilized egg, which comprises the shell, albumen and yolk, and supplies carbohydrate, fatty acids, minerals, trace elements, vitamins, energy sources and amino acids. It has been established that several amino acids are essential and cannot be synthesized in the embryo (Freeman and Vince, 1974c). There are no free amino acids in eggs; amino acids are present only in the proteins located mainly in the albumen and yolk, which are separated by the vitelline membrane. With the development of the embryo, these proteins are gradually used as a supply of amino acids. At as early as 3 days of incubation, free amino acids can be identified in the yolk of fertilized chick eggs, and these amino acids increase up to day 9 (Williams et al., 1954). It has been proposed that proteolytic enzymes are involved in the hydrolysis of the proteins in the yolk (Emanuelsson, 1951; Emanuelsson, 1955), and proteolytic activity has been detected in the yolk sac during incubation (Ito, 1957). An active amino acid transport system exists in the yolk sac membrane by day 6 of incubation (Holdsworth, 1967). However, there has been little progress in characterizing this amino acid transport system, with only aspartic proteinase cathepsin D having been identified in the yolk (Wouters et al., 1985) and no amino acid transporters having been identified in yolk sac cells.

In a study on quail, Yoshizaki and colleagues (Yoshizaki et al., 2004) observed a two-step process for the digestion of yolk granules. First, the weight of yolk granules in the yolk decreased as some granules were segregated into endodermal cells produced by unequal cleavage at the periphery of the yolk sac. In the second step, yolk proteins were solubilized by the high concentration of salts that resulted from the disruption of the yolk sphere membranes, making the yolk proteins susceptible to digestion by cathepsin D, which is present in yolk. The digestion products were then endocytosed by endodermal cells. At an early stage, the vitelline membrane is digested by hatching enzyme, and the proteins in albumen are endocytosed by yolk sac ectodermal cells (Yoshizaki et al., 2002; Yoshizaki et al., 2000). At a later stage, ovalbumin is transported through the albumen sac into the extra-embryonic cavity and then into the amniotic cavity through the amnion. After oral uptake, ovalbumin is present in the intestine of the hen, with little digestion; from there, it reaches the yolk (Yoshizaki et al., 2002). In the avian embryo, the yolk sac is critical for the absorption of nutrients from the yolk and albumen; it is thought to play a role similar to that of the intestine in adult animals. The yolk sac forms from the area opaca and consists initially of a thin epithelial ectoderm, underlain by a layer of tall endodermal cells that contain many intracellular yolk droplets (Bellairs and Osmond, 1998). It is generally agreed that nutrients are first absorbed into yolk sac cells, especially the endodermal cells, and are then transported into the circulation. However, the mechanism responsible for this process, especially in the early stages, is not fully known. It has been proposed that yolk proteins in the endodermal cells are digested by a proteinase and the products are transported into the circulation. Cathepsins B and D were identified in endodermal cells (Gerhartz et al., 1997), and their activities have been demonstrated in the endodermal cells of quails, even during early embryonic stages (Gerhartz et al., 1999).

Further research is needed to establish the following: whether the digested products are transported in the form of amino acids, oligopeptides or polypeptides; how nutrients are transported from the yolk to yolk sac cells and then to the circulation; and how other processes contribute to nutrient utilization by the embryo. In the present study, we demonstrated that proteins in the blood of early chick embryos were taken up efficiently by circulating blood cells, where they were rapidly digested. This implies a possible mechanism for the use of serum proteins or polypeptides as an amino acid supply in the early chicken embryo.

MATERIALS AND METHODS

Reagents

The following were purchased from Sigma-Aldrich (St Louis, MO, USA): bovine serum albumin (BSA; A7030), human serum albumin (HSA; A1653), horseradish peroxidase (HRP; P6782), fluorescence isothiocyanate (FITC)-BSA conjugate (A9771), nocodazole (M1404) and 2,4,6-trinitrobenzenesulfonic acid (TNBS; P2297). The preparation of trinitrophenyl (TNP)-BSA and TNP-HSA was adapted from the method of Bondada and Robertson (Bondada and Robertson, 2003). Briefly, 80 mg of BSA or HSA dissolved in 2.5 ml of 0.1 mol l^{-1} $NaHCO_3$ were added slowly with stirring to a 317 μl solution that contained 95 μl of 5% TNBS. The mixture was covered with aluminium foil and incubated overnight at 4°C with gentle stirring. The reaction was then dialyzed against 2 l of saline, with five changes; the absorbance was measured in a spectrophotometer at wavelengths of 280 and 340 nm, and the ratio of TNP to albumin was calculated. Antiserum against TNP was produced in mice immunized with TNP–HSA, and antiserum against BSA was produced in chickens immunized with BSA. Rabbit anti-BSA IgG (A1113) was purchased from Invitrogen (Carlsbad, CA, USA).

Fertilized eggs

Fertilized eggs of Jingbai 938, a White Leghorn (*Gallus domesticus* Brisson) strain purchased from the Experimental Station of the China Agricultural University, were incubated at 37.8°C in 70% relative humidity.

Protein quantification

HRP was microinjected into E3 embryo blood, and at specified times points a volume of blood was removed and diluted in cold PBS containing 0.1% gelatin. Some of the diluted blood was further diluted in PBS containing 0.1% gelatin and 0.05% Triton X-100, and some was further diluted in PBS containing 0.1% gelatin. The HRP activity was measured with reference to a standard curve, using 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 as substrates. The amount of HRP contained in the blood cells was determined as the amount in the blood (cells+serum) minus that in the serum.

The concentration of BSA was measured by a direct competitive enzyme-linked immunosorbent assay (ELISA) or an antibody sandwich ELISA (if not indicated otherwise in the figure legend, antibody sandwich ELISA was used). For the direct competitive ELISA, the wells of an assay plate were coated with BSA and incubated with rabbit anti-BSA, which had been incubated previously with diluted serum samples or serially diluted BSA; the wells were washed, incubated with diluted HRP-conjugated anti-rabbit IgG, and washed again. Substrate (TMB and H_2O_2) was added to the wells, and the reaction was terminated with 2 mol l^{-1} H_2SO_4. For the antibody sandwich ELISA, a plate coated with chicken anti-BSA was incubated with diluted serum samples or serially diluted BSA; the wells were washed, incubated with diluted rabbit anti-BSA, washed again, incubated with diluted HPR-conjugated anti-rabbit IgG, and then washed again. Substrate (TMB and H_2O_2) was added, and the reaction was terminated with 2 mol l^{-1} H_2SO_4. The absorbance of the reactions in the plates was read on a microtiter plate reader, and the data were analyzed using SPSS 13.0, with a curve estimation program. The BSA concentrations in the samples were determined with respect to a standard curve constructed from serially diluted BSA reacted in the same plate.

The blood removed from the embryos that had been injected with FITC-BSA was divided into three parts. The first part was used to calculate the cell density, and the second part was diluted in PBS containing 0.05% Triton X-100. The serum and washed cells of the third part were separately diluted in PBS containing 0.05% Triton X-100. The fluorescence of the samples was measured using a microplate reader (GENios; Tecan, Maennedorf, Switzerland), and the concentration of FITC was determined with respect to a standard curve constructed from serially diluted FITC-BSA reacted in the same plate. For the injections and the removal of blood from the embryos, the aorta was used at E3 and E3.5; the yolk sac vein at E4, E5 and E5.5; or the allantoic vein at E8, E11.5 and E12. When used, nocodazole (0.1–0.2 μl), dissolved at 5 mg ml^{-1} in DMSO, was injected 20 min before FITC-BSA or HRP.

Western blot analysis

TNP-BSA (10 μg) was microinjected into the embryo blood at E3, and at specified time points blood was removed and diluted in cold saline (0.9% NaCl). For each sample, the serum was diluted in SDS-PAGE loading buffer, and each lane containing 0.005 μl serum was electrophoresed. After being washed in cold PBS, the cells in 1 μl blood were diluted in SDS-PAGE loading buffer, incubated in 95–100°C water for 5–10 min and centrifuged at 14,000 r.p.m. for 10 min, for loading in one lane. After SDS-PAGE, the proteins were transferred from the gel to a nitrocellulose membrane (0.22 μm). The membrane was blocked with Tris-buffered saline containing Tween-20 (TBS-T) and 2% non-fat dry milk, followed by incubation with mouse antiserum against TNP, diluted in TBS-T containing 1% non-fat dry milk. After the membrane was washed, it was incubated with HRP-conjugated anti-mouse IgG. Finally, the membrane was washed, incubated with chemiluminescent HRP substrate (Millipore, P36599; Billerica, MA, USA), and exposed to X-ray film. The densities of the bands were analyzed and compared using Quantity One 4.6.2 software (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry and microscopy

Embryos were injected with FITC-BSA. Blood samples were taken as described above, and the blood cells were washed three times in cold PBS. The blood cells were analyzed by flow cytometry (FACSCalibur analyzer; BD Biosciences, Mountain View, CA, USA) and were observed under an inverted fluorescence phase-contrast microscope.

RESULTS

Proteins are rapidly cleared and partially degraded in the serum of E3 embryos

The incubation period of chicken eggs is 21 days. The E3 embryo is at an early stage, when definitive erythropoiesis has not yet begun and 1 day after the circulatory system has begun to develop (Bellairs and Osmond, 1998). When BSA or HRP was injected into E3 (Hamburger and Hamilton stage 18–19) embryo blood it was cleared quickly from the circulation (Fig. 1A). At 10 min after injection, the serum concentration of BSA detected by direct competitive ELISA was higher than that measured by antibody sandwich ELISA, and it was significantly higher 50 min later ($P<0.0001$, *t*-test), although the levels were the same immediately after injection (Fig. 1B). Peptides with one or more epitopes can be detected by direct competitive ELISA, and those with two or more epitopes can be detected by antibody sandwich ELISA; the same standard protein was used in the two kinds of ELISA. Therefore, we speculated that the proteins injected into the embryo blood were partially degraded in the blood. To confirm this hypothesis, TNP-BSA was injected, and the BSA peptides coupled with TNP in the serum were evaluated by western blot analysis using an antibody

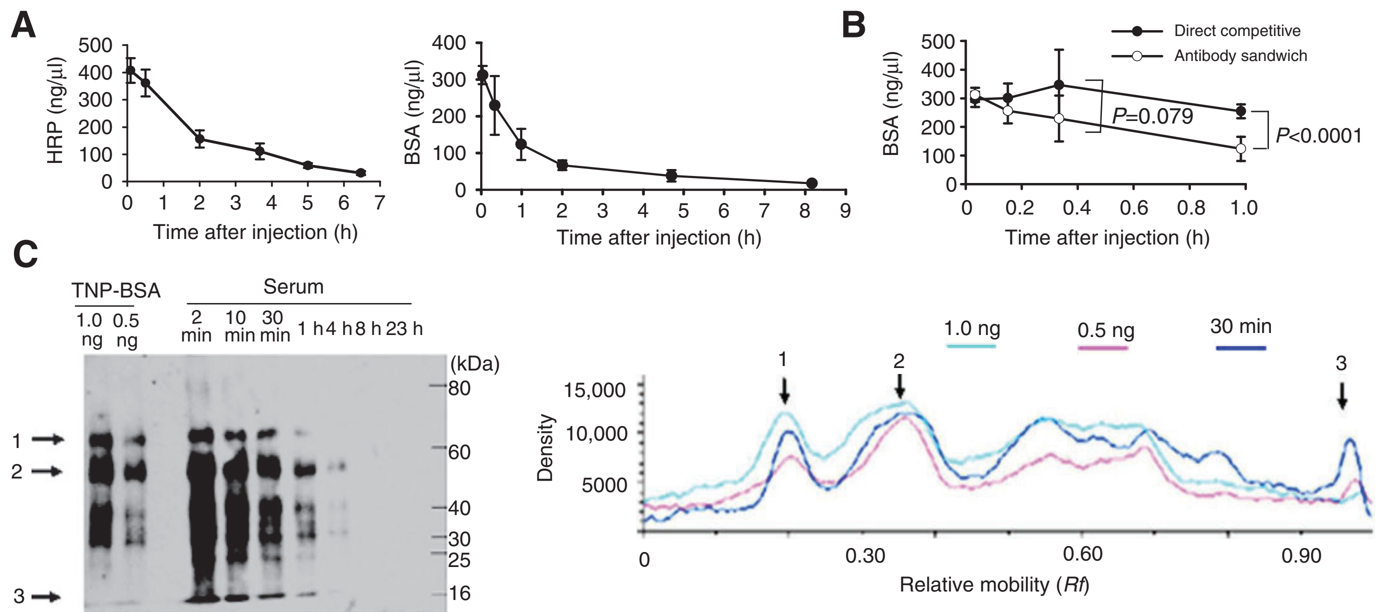

Fig. 1. Partial degradation and clearance of protein from the serum of E3 embryos. (A) HRP or BSA (10 µg) was injected into embryo blood, and the concentration in serum was determined at different times. (B) After the injection of 10 µg of BSA, the partial degradation of BSA in the serum was evaluated by direct competitive and antibody sandwich ELISAs. (C) TNP-coupled peptides in serum collected at different times after injection were detected by western blotting with anti-TNP. The two left lanes in the left figure were loaded with 1 ng and 0.5 ng of TNP-BSA, respectively, as standard markers. The densities in the lanes 1.0 ng, 0.5 ng and 30 min were analyzed and compared, as shown on the right; the positions indicated by 1, 2 and 3 on the left correspond respectively to those on the right.

to TNP (Fig. 1C). The band patterns of 1 ng and 0.5 ng of the TNP-BSA standard marker were nearly the same, except that the lane with 1 ng had more protein in each band. The TNP-BSA serum sample contained mostly large peptides, giving a band pattern similar to that of the TNP-BSA standard; however, there were more smaller TNP-BSA peptides in the serum sample (Fig. 1C, lane marked '30min'), indicating that the injected protein had been partially degraded in the embryo blood, although not necessarily in a biologically significant manner.

Protein clearance is attributable mainly to the capacity of blood cells to take up protein

Although proteins may be partially degraded in the serum, this process may not be efficient enough to rapidly clear proteins from the circulation. FITC-BSA was used to trace the fate of injected protein. Most circulating blood cells were able to take up the injected FITC-BSA, as was the case for TNP-BSA. Blood cells began to take up the BSA almost immediately after it was injected (Fig. 2A,B). A large fraction of the polypeptides coupled with TNP was clearly detected prior to 75 min after injection, whereas little or none was detected at 4.5 h after injection (Fig. 2A). Furthermore, more FITC (perhaps coupled to several forms of the protein) was detected in the cells at 6 h after injection than at 40 min after injection, suggesting that most of the protein had been degraded and trapped in the cell by 6 h after injection (Fig. 2B). At 8 h after injection there was little FITC detected in the cells (Fig. 2B). Correspondingly little BSA remained in the serum in the above experiment (Fig. 1A).

The capacity of the blood cells to clear injected protein was evaluated (Fig. 3). The quantity of FITC-BSA and its products of degradation were measured as the quantity of FITC coupled to BSA, peptides or amino acids. At 80 min after injection, nearly half of the injected protein or its products was trapped in the cells. At about 4 h after injection, most of the protein had been degraded. The increase of FITC in the cells of 1 µl blood from 20 to 80 min after injection was 244 units (the definition of 1 unit is given in the legend of Fig. 3) and the weighted mean in the serum during this period was 411 units, indicating that within 1 h the blood cells took up nearly 60% (244/411) of the peptides in the serum, at a constant concentration (Fig. 3A). Although the fluorescence in the cells was strongest at 2 h after injection (Fig. 3A), in the experiment using HRP the quantity of active HRP was lower at this time point compared with that at 30 min or 1 h after injection (Fig. 3B). This confirmed that the absorbed peptides were degraded in the cells and argues that the capacity of the blood cells was sufficient to rapidly clear the injected protein from the circulation.

Microtubules are not involved in rapid clearance of circulating proteins

In our preliminary studies we injected FITC-BSA alone or with unlabeled BSA. We observed no effect of BSA on the uptake of FITC-BSA by the embryo blood cells (data not shown). Furthermore, the similar rates of clearing for HRP and BSA (Fig. 1A) showed that rapid clearance was not restricted to a specific protein. These findings indicated that protein uptake did not occur by receptor-mediated endocytosis. FITC-BSA or HRP was injected along with nocodazole, a cytoskeleton-destabilizing drug (Lee et al., 1980), to evaluate the contribution of macropinocytosis to protein clearance. Nocodazole did not suppress FITC-BSA uptake by embryo blood cells (Fig. 4A,B) or obviously affect HRP clearance from the serum (Fig. 4C). These results indicated that microtubules were not involved in the clearance of circulating protein. However, we could not yet reject the contribution of macropinocytosis to the clearance.

Rate of protein clearance from the circulation varies at different stages

The ability of blood cells to take up FITC-BSA decreased with embryo development, with little uptake observed at E6 (Hamburger and Hamilton stage 29) and almost none at E7 (Hamburger and

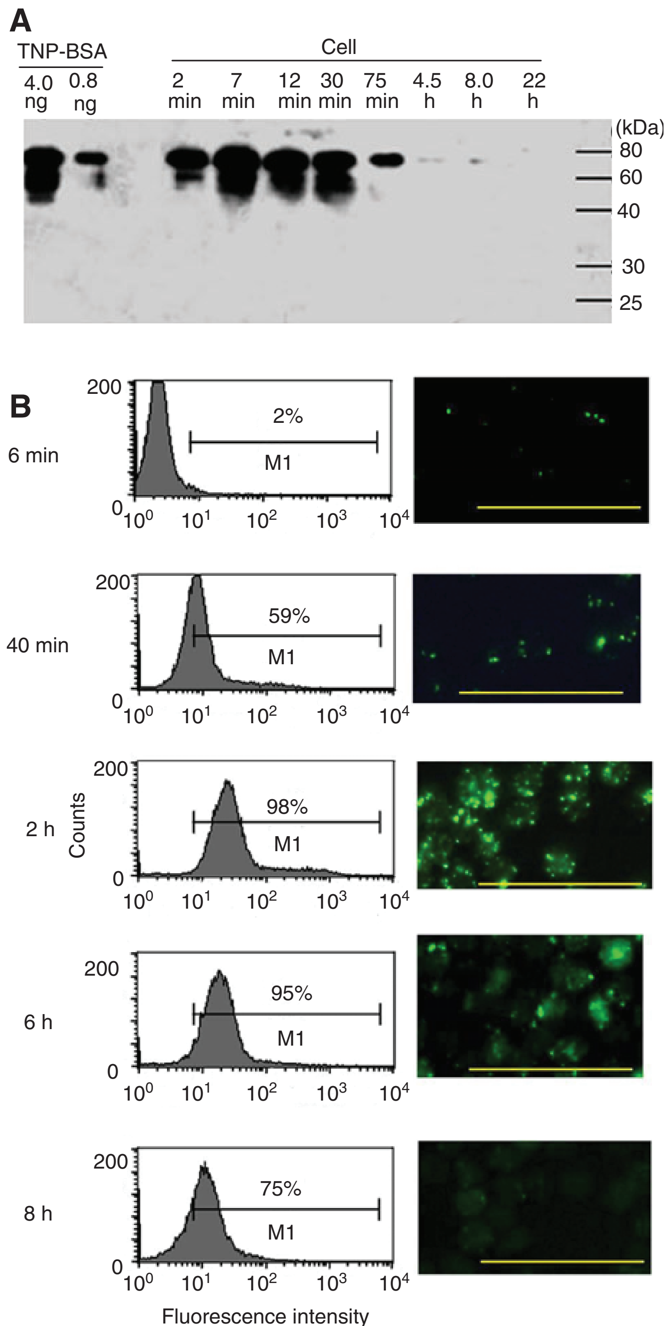

Fig. 2. Protein uptake by E3 embryo blood cells. (A) At the specified times after the injection of TNP-BSA, the TNP-labeled proteins/polypeptides in the blood cells were detected by western blotting with anti-TNP antibody. (B) At the specified times after the injection of 10 μg of FITC-BSA, blood cells were analyzed by flow cytometry and observed under an inverted fluorescence phase-contrast microscope. Bar: 50 μm.

Hamilton stage 31; Fig. 5A). Correspondingly, the clearance rate of BSA from the serum became slower with embryo development (Fig. 5D, lower panel). There seemed to be no great difference in BSA clearance among embryos after E8, although BSA clearance was slightly faster at E8 than at E12 (Fig. 5D, lower panel). Blood cell morphology differed at different stages of embryo development, and membrane ruffling was active before E6 but inactive after E7 (Fig. 5B). The uptake of FITC-BSA per cell was similar at E3 and E4 (Hamburger and Hamilton stage 24) but was much lower at E5.5 (Hamburger and Hamilton stage 28; Fig. 5C, right panel). Although the concentration of blood cells increased with embryo development (Fig. 5C, left panel), the uptake capacity of the blood cells seemed to rapidly decrease between E5 to E6 (Fig. 5A,C). These results imply that the rapid clearance of injected proteins is attributable mainly to the capacity of blood cells to take up protein in the early embryo stages.

The measurement of serum BSA by direct competitive ELISA and antibody sandwich ELISA showed that partial degradation also occurred at late developmental stages (Fig. 5D, middle panel). After the injection of FITC-BSA, the fluorescence intensity in the circulation rapidly decreased and then increased at all stages (Fig. 5D, upper panel). Further study is needed to ascertain whether this was related to partial degradation.

DISCUSSION

The use of egg nutrients by the developing embryo has been studied extensively (Moran, 2007). Free amino acids and intact proteins can both be transported to the embryo, but the embryo is thought to use primarily intact proteins such as α-liventin, β-liventin, γ-liventin (Nace, 1953) and ovalbumin (Sugimoto et al., 1999). The main products absorbed were originally proposed to be amino acids and not intact proteins (Freeman and Vince, 1974c), but there is still no direct evidence that amino acids are transported from the yolk or albumen to the embryo. In addition, yolk sac cells on villi use receptor-mediated endocytosis to absorb intact very-low-density lipoproteins rather than to transport digestion products (Lambson, 1970; Noble et al., 1988). Furthermore, intact ovalbumin exists widely in many organs in the embryo (Sugimoto et al., 1999), which indicates that proteins taken up by yolk sac cells can be transported intact through these cells to the circulation.

The total incubation period of the chicken embryo is 21 days, and the circulatory system begins to develop after E2 (Hamburger and Hamilton stage 12). Most of the organs appear between E3 and E5 (Freeman and Vince, 1974a); for example, the stomach, pancreatic anlagen and lung primordial cells develop at E3, the adrenal gland, gonadal anlagen and metanephros at E4, and the spleen, bursa of Fabricius and thymus anlagen at E5. The liver primordia appear at the end of E2, grow rapidly after E4, and gluconeogenesis in liver begins at E7. By E4, the chicken embryo has almost completed all the important early stages of development. Embryos at all the stages are thought to feed mainly on the nutrients in the yolk sac endodermal cells, and proteolytic activity is similar among the cells at different stages (Gerhartz et al., 1999). It has been shown previously that when chick embryo explants of 11–13 somites (at about 2 days of incubation) are cultured *in vitro* in the presence of ovalbumin, the ovalbumin is taken up by the area opaca and then transported to the embryo, in the form of digested products (Hassell and Klein, 1971). Embryos of mammals (Beckman et al., 1997) and birds (Klein, 1968) that were cultured *in vitro* preferred intact proteins over nutrients from the environment. Before the area opaca becomes vascularized, the embryo is nourished by intracellular yolk droplets, which are most conspicuous in the large yolky endodermal cells of the area opaca, although smaller droplets are present in the ectodermal, mesodermal and endodermal cells of the embryo itself. The intracellular yolk droplets in the chick embryo are consumed by about 48 h of incubation, at which time the embryonic circulation has become established and the intracellular yolk of the area opaca has become available (Bellairs and Osmond, 1998). After the extra-embryonic circulation is established, the term yolk sac is usually substituted for area opaca; the nutrition contained

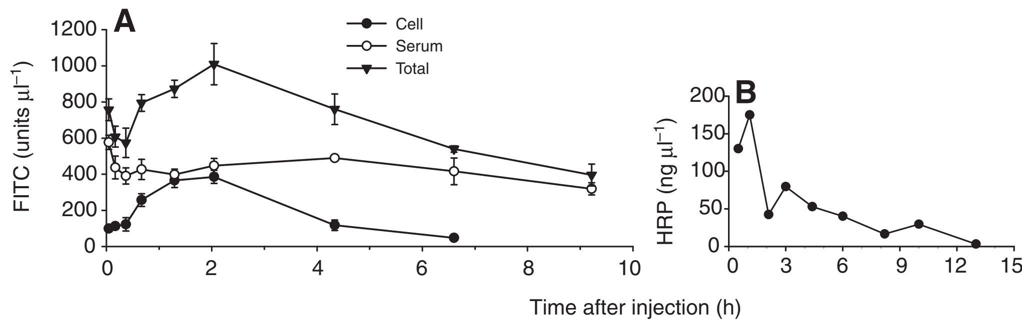

Fig. 3. Distribution of injected peptides in circulating blood cells and serum at specified times after injection in E3 embryos. (A) The concentration of FITC-coupled peptides in blood cells, serum and blood (total: cell+serum) after the injection of 10 μg FITC-BSA. FITC-coupled peptides were quantified based on FITC, where 1 unit of FITC was equal to the amount of FITC contained in 1 ng of FITC-BSA. (B) The concentration of active HRP in the circulating blood cells after the injection of 10 μg of HRP per embryo.

in the yolk sac endodermal cells is obtained mainly by their unequal cleavage from the yolk cell and their uptake of material from the yolk (Mobbs and McMillan, 1981; Yoshizaki et al., 2004). The yolk sac develops quickly, and it encloses the whole yolk at E5. As discussed above, yolk protein is absorbed first by the yolk sac endoderm for digestion and is then transported across these cells to the circulation for the nourishment of the embryo. However, the exact form of the digested products transported across the vascular pole of the endodermal cells is not known, although polypeptides and peptides are likely to be transported and preferred by embryos cultured *in vitro*. In the present study, we provide evidence that the rapid uptake and efficient intracellular digestion of the proteins or peptides in blood cells might enable early embryos to make use of intact protein without extraembryonic digestion. Furthermore, the circulation appears to provide an environment favorable for the partial degradation of proteins, which enhances the efficiency of digestion.

In our initial study, we injected BSA into the E3 embryo circulation and were surprised by the rapid clearance of BSA from the serum. We surmised that some cells in the circulation were responsible for removing the BSA, and a western blot analysis revealed BSA in the blood cells (data not shown). Using injected FITC-BSA, we showed that the blood cells efficiently cleared the injected protein by uptake and intracellular digestion (Figs 2 and 3). The clearance rate for injected BSA and the protein uptake capacity of blood cells gradually decreased with embryo development, especially after E5 (Fig. 5A,C,D), which is further evidence that the blood cells play a major role in clearing injected BSA from the serum in early embryos.

Definitive erythropoiesis begins at E5 in chick embryos. Before E5, the circulation contains mainly primitive blood cells arising from the division of blood island mesodermal cells and subsequent mitotic division (Freeman and Vince, 1974b). Therefore, we propose that the decline in protein uptake by blood cells after E5 is caused by the onset of definitive erythropoiesis.

Macropinocytosis can be induced by growth factors in some tumor cells (Chinkers, 1979; Haigler, 1979; West et al., 1989). Given that blood cell mitotic division is active before E5, we wondered whether macropinocytosis was also induced by growth factors in blood cells and whether it played a major role in the clearance of circulating proteins in early embryos. However, the efficient uptake of protein by blood cells appeared not to be affected by microtubule destabilization with nocodazole (Fig. 4). Considering the situation in some tumor cells, where macropinocytosis is stimulated by certain growth factors but is not inhibited by nocodazole (Haigler, 1979; Sandvig and van Deurs, 1990), the present results do not exclude a role for macropinocytosis in the clearance. In addition, pinocytotic vesicles with a diameter of more than 0.5 μm were frequently found beneath the cell membrane (data not shown), indicating that macropinocytosis might occur in early embryo cells. BSA at different concentrations in the serum of E3 embryos had a similar half-life, and the uptake of FITC-BSA by blood cells was not suppressed by co-injection with BSA (data not shown). This indicates that protein clearance does not occur *via* receptor-mediated endocytosis. Although other endocytic pathways have been

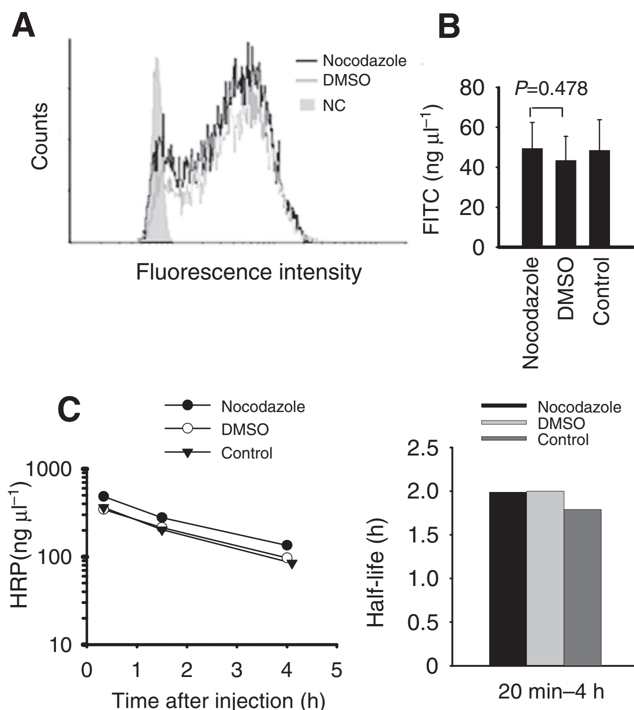

Fig. 4. The effect of nocodazole on the clearance of circulating protein. (A) At 1 h after the injection of 10 μg FITC-BSA along with nocodazole or DMSO, E3.5 (Hamburger and Hamilton stage 22) embryo blood cells were analyzed by flow cytometry. NC (negative control), without injection of FITC-BSA. (B) The levels of FITC-coupled peptides in E3.5 embryo blood cells were compared among the group to show the effect of nocodazole on uptake. Control, FITC-BSA injected alone. (C) The effect of nocodazole on the clearance of HRP from E3 embryo serum. The half-life (right panel) was calculated from the data in the left panel.

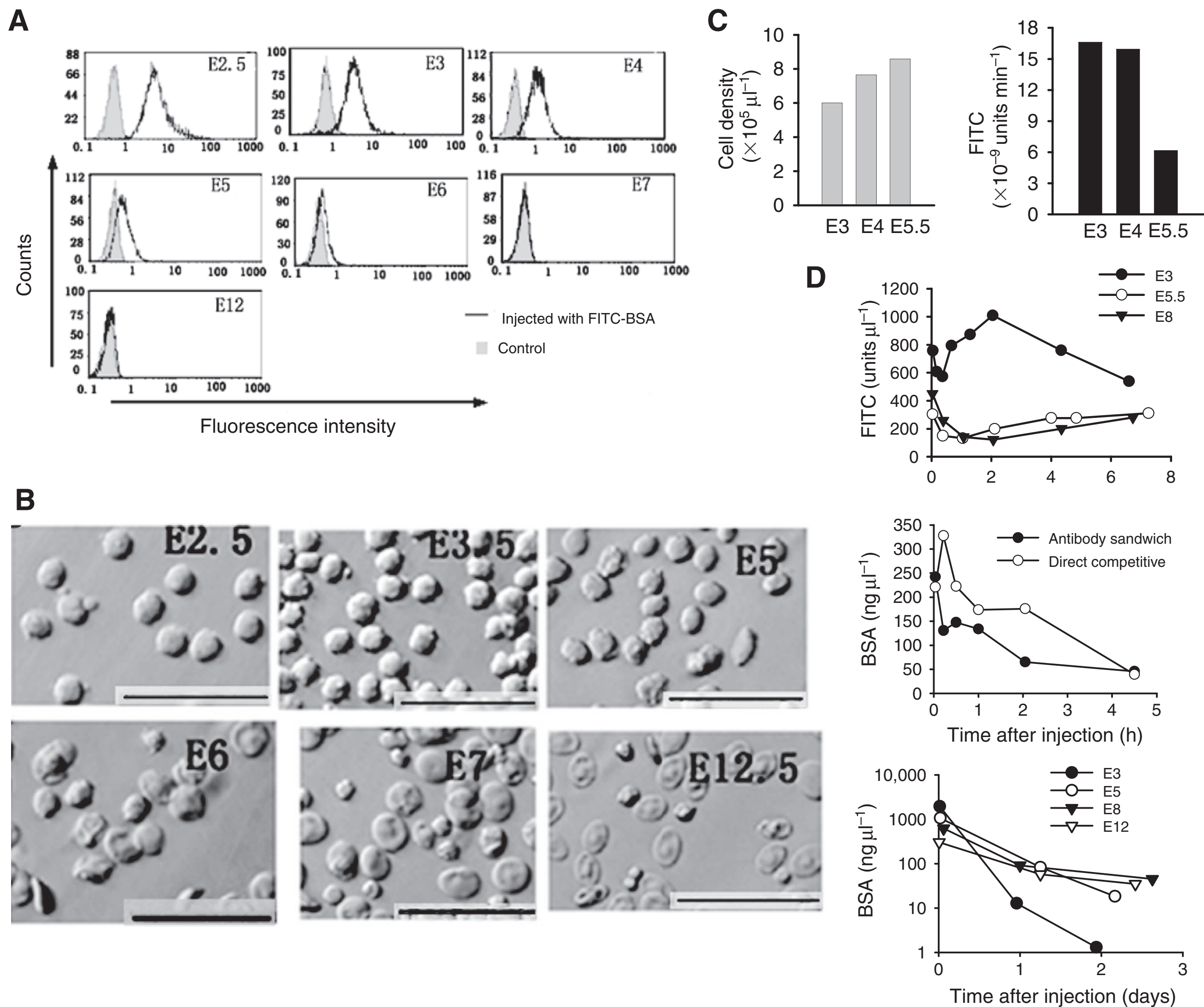

Fig. 5. Protein clearance from the circulation of embryos at different stages. (A) FITC-BSA uptake by blood cells at different embryonic stages was analyzed by flow cytometry. Blood cells from E2.5 (Hamburger and Hamilton stage 16), E3, E4, E5, E6, E7 and E12 embryos were analyzed at 1 h after the injection of 5, 5, 10, 15, 20, 30 and 60 μg of FITC-BSA, respectively. (B) Embryo blood cell morphology at different embryonic stages. Bar: 50 μm. (C) Left panel: blood cell density at different embryonic stages. Right panel: average uptake of FITC-BSA per cell, defined as the average increase in FITC-coupled peptides per minute in cells of 1 μl blood between 20 and 60 min after injection, divided by blood cell density and the weighted mean in the serum. FITC-coupled peptide was quantified based on FITC, where 1 unit of FITC is equal to the amount of FITC contained in 1 ng of FITC-BSA. (D) The clearance of protein from the circulation of embryos at different stages. Upper panel: concentration of FITC remaining in the blood after the injection of 10, 80 and 200 μg of FITC-BSA into E3, E5.5 or E8 embryo blood, respectively. Middle panel: after the injection of 250 μg of BSA into E11.5 embryo blood, the partial degradation of BSA in the serum was evaluated by direct competitive and antibody sandwich ELISA. Lower panel: after the injection of 50, 300, 600 and 600 μg of BSA into E3, E5, E8 and E12 embryos, respectively, BSA was cleared from the serum at different rates at different embryonic stages.

identified and studied extensively (Johannes and Lamaze, 2002), it would be surprising if blood cells were to efficiently take up protein by a mechanism other than macropinocytosis or receptor-mediated endocytosis. However, further direct evidence is still needed. We propose that the efficient uptake of protein by blood cells may be related to their active mitotic division, considering that their uptake capacity decreased after the onset of definitive erythropoiesis.

Injected FITC-BSA was taken up efficiently by early embryo blood cells, followed by rapid intracellular digestion (Fig. 3). Two hours after injection, the fluorescence in the blood cells began to decrease, whereas the fluorescence intensity in the serum slightly increased, which implies that some digested products were not trapped in the blood cells but were rapidly released into the serum as free amino acids. Thus, even though early embryo blood cells require amino acids as nutrients to maintain active mitotic division, they may also supply free amino acids to other embryonic tissues. The rapid digestion of proteins implies the presence of efficient vesicle transport and high proteolytic enzyme activity in these cells.

Scavenger endothelial cells in the chicken liver play a major role in clearing macromolecular waste from the circulation (Seternes et al., 2002; Smedsrød, 2004). Our earlier studies indicated that the scavenger function is not fully active in the embryo until E5 (G.W. and Z.L., unpublished observations), and the present study demonstrated that circulating blood cells efficiently cleared proteins from the serum before E5. Thus, the efficient clearance of proteins by blood cells in the early-stage embryo may be a substitute for the

scavenger function of the liver and may also serve as an innate immune system by removing dangerous agents.

In summary, we showed that circulating blood cells in early embryos have the ability to intracellularly digest proteins or peptides from the serum, which may then be used as nourishment for the embryo. This process might be critical for the early embryo as proteins are not digested extensively in the yolk at early stages.

Further research is required to clarify the processes of transporting protein from the yolk to the circulation and intracellular digestion of protein by early embryo blood cells, and whether free amino acids are transported from the yolk to the early embryo circulation, to understand the ability of early embryo blood cells to digest other nutrients such as carbohydrates and fatty acids, and to elucidate the defense functions of early embryo blood cells.

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