Production and Characterization of an Anti-Chicken Transferrin

Osamu Shimizu¹, Keitaro Isokawa², Kota Kanematsu², Jun Ohtsu²,
Toshiaki Iwata², Hiroyuki Matsui², Hiroshi Sato¹ and Yoshihisa Toda²

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Abstract

Several lines of recent evidence have suggested that transferrin plays a significant role in tissue interaction or morphogenesis at early stages of embryo development. In the present study, an anti-chicken transferrin antibody was produced and its basic characteristics were clarified as a basis for use in further studies. An antiserum termed Toraji 3 was raised against chicken transferrin and purified into IgG and ligand-affinity-purified fractions. These three preparations of the antibody gave an intense immunohistochemical signal in visceral yolk sac and developing liver, both of which are known to be major producers of transferrin in early development. In immunoblot analysis, these three preparations detected 70-kDa transferrin, whereas the ligand-affinity-purified preparation showed higher specificity. It was also demonstrated by enzyme-linked immunosorbent assay and immunoblotting that Toraji 3 antibody bound preferentially to chicken transferrin and showed a negligible binding to human transferrin.

Introduction

Transferrin is one of well-characterized β 1-globulins present in serum. While the role of transferrin as a nutrient or a simple iron transporter has been widely accepted^[1,2], several recent studies have suggested its significance in embryogenesis. Copp et al.^[3] reported that transferrin was involved in the completion of neurulation during early embryogenesis, and Isokawa et al.^[4] identified transferrin as one of the molecules involved in cardiac endothelial-mesenchymal transformation. Moreover, it has been reported that a yet unidentified inductor molecule, target tissue and transferrin, all three need to be present simultaneously in the induction process for kidney morphogenesis^[5,6].

These biological effects of transferrin can not be explained by a simple iron transport or nutritional role. Even if the mechanism by which transferrin exerts its activity is merely iron transport, its involvement in various embryological events is of significance. To reveal the level at which transferrin works in these processes, the present study was undertaken in order to produce a specific probe, anti-chicken transferrin, because the chicken embryo is one of the most widely used experimental models in developmental studies. The resulting antiserum and its purified fractions were shown to bind preferentially to 70-kDa chicken transferrin and to have a negligible binding to a human cognate protein, human transferrin. These findings provide a basis for further use of the antibody in further studies.

Materials and Methods

Immunization protocol

One milligram of chicken transferrin (iron complex form, type II, Sigma) in 1 ml of sterile saline was

清水 治¹, 磯川 桂太郎², 兼松 宏太², 大津 順², 岩田 敏明², 松井 弘行², 佐藤 廣¹, 戸田 善久²

1 Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry

2 Department of Anatomy, Nihon University School of Dentistry

To whom all correspondence should be addressed: Dr. Keitaro Isokawa, Department of Anatomy, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101, JAPAN

emulsified with an equal volume of Freund's complete adjuvant (Gibco BRL) and injected intradermally into multiple sites on the back of a New Zealand White rabbit (3 kg in weight) under intramuscular anesthesia (1 ml/kg, Ketalar 50, Sankyo). The animal was maintained in the Laboratory Animal Center, Nihon University School of Dentistry. Twenty-four and 45 days later, the animal was boosted intradermally with the same amount of immunogen in Freund's incomplete adjuvant (Gibco BRL). Pre-immune serum used in this study was collected from the same rabbit prior to immunization. Antisera were collected immediately before each booster injection and 14 days after the second booster. Titration of the antisera was performed with immunoblot analysis, immunohistochemistry and ELISA, and the latter antiserum (designated Toraji 3 antiserum) was selected for use in this study as described in Results.

Antibody purification

An IgG fraction (Toraji 3-IgG) was purified from Toraji 3 antiserum with a protein G affinity column (Ampure PA, Amersham) according to the manufacturer's protocol. For further purification of Toraji 3-IgG, chicken transferrin, the same preparation as that used for the immunogen, was gel-purified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, a portion containing transferrin was cut out from multiple unfixed 10% gels by referring to stained adjacent lanes, minced and incubated overnight at 37°C in 2 volumes of 50 mM ammonium bicarbonate (pH 7.8) containing 0.1% SDS. Approximately 400 μ g of chicken transferrin thus prepared was allowed to stand overnight at room temperature to adsorb onto a pre-activated disk (U12 disk unit; Nalgene) in 0.5 M carbonate buffer, pH 9.3, and stabilized with 0.1% sodium borohydride in phosphate-buffered saline (PBS). This disk was then used to purify the Toraji 3-IgG into a ligand-affinity-purified (LAP) fraction. The buffer system employed for Toraji 3-LAP purification was described previously^[4].

Immunohistochemistry

Fertilized eggs (Oh-hata Sheibah, Shizuoka) were incubated and staged according to Hamberger and Hamilton^[7]. Stage 14 and 31 unfixed chicken embryos were cryoprotected with a graded series of sucrose (10%, 15% and 20%) in PBS, embedded in Tissue-Tek OCT compound (Miles) and frozen in liquid nitrogen-cooled 2-methyl butane. Cryosections, 8 μ m thick, were subjected to indirect fluorescence immunohistochemistry. After blocking treatment with 1% bovine serum albumin (BSA, Sigma) in PBS for 1 h, the sections were incubated in a 1:200 dilution of Toraji 3 antiserum or approximately 40 μ g/ml Toraji 3-IgG or Toraji 3-LAP in 1% BSA-PBS for 1 h. Binding of the primary antibody was visualized by incubating sections with a 1:100 dilution of fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit IgG; Cappel) in 1% BSA-PBS for 1 h. The sections mounted in 90% glycerol in PBS containing 0.2 M n-propylgallate^[8] were examined with a Nikon Optiphot epifluorescence microscope. For controls, the primary antibody was omitted or replaced with pre-immune rabbit serum at a relevant concentration of IgG.

Electrophoresis and immunoblot analysis

Purified chicken and human transferrins were purchased from Sigma and Collaborative Research Products, respectively. Unfractionated total proteins of stage 21 chicken embryos and stage 31 embryonic liver were prepared by sonication with five 5-s bursts (Ultrasonic Disruptor, UD-200; Tomy) in PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 1 μ M pepstatin A, 1 μ M leupeptin; Sigma).

Protein concentration was determined with a bicinchoninic acid assay^[9] using a kit with a BSA standard (MicroBCA; Pierce). SDS-PAGE was performed according to Laemmli^[10], except that the sample buffer consisted of 63 mM Tris (pH 6.8), 2% SDS, 6 M urea and 60 mM dithiothreitol (Boehringer Mannheim).

Electrophoretically separated proteins were transblotted onto PVDF membrane (Immobilon-P; Millipore) for 2-4 h in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (Sigma), pH 10.4, containing 10% methanol. The membrane was treated for 1 h at 37°C with 5% BSA in 20 mM Tris, pH 9.0, containing 150 mM NaCl, and then incubated sequentially for 1 h each at room temperature in Toraji 3 antibody

and alkaline phosphatase (AP)-conjugated secondary antibody (1:5000 dilution, goat anti-rabbit IgG; Cappel) diluted with 1% BSA in 20 mM Tris - 500 mM NaCl, pH 7.4, containing 0.05% Tween 20 (TBSS-T). The dilution of the pre-immune serum and antiserum was 1:200 and that of the IgG and LAP fractions of Toraji 3 was 20 μ g/ml. Color development was carried out in a bromo chloro indolyl phosphate/nitroblue tetrazolium system^[11]. Total proteins in the gel were stained with Coomassie brilliant blue (Fluka)^[12].

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Falcon 3915; Becton Dickinson) were coated overnight at 4°C with 0.1 μ g/well chicken and human transferrin in 50 mM carbonate buffer, pH 9.6. After treatment with 1% BSA in the coating buffer for 4 h at 4°C, each plate was incubated for 2 h at room temperature with Toraji 3 antibody and then with 1:5000-diluted AP-conjugated goat anti-rabbit IgG in 1% BSA-TBSS-T. Color development was carried out in 1 mg/ml p-nitrophenyl phosphate (Sigma) in 10 mM diethanolamine buffer, pH 9.6, and absorbance at 405 nm was measured with a microplate reader (Titertek Multiskan PLUS, Flow Laboratories).

Results

Titration of antisera

Toraji 1 (antiserum collected 24 days after immunization), Toraji 2 (antiserum collected 21 days after the first booster injection) and Toraji 3 (antiserum collected 14 days after the second booster injection) were titrated qualitatively by immunoblotting and immunohistochemistry, and quantitatively by ELISA. In the qualitative assays, Toraji 3 gave a more intense immunoreaction than Toraji 1 or Toraji 2. In ELISA, the plates were coated with 0.1 μ g/well chicken transferrin, and Toraji antisera were applied in serial 1:2 dilutions starting at 1:500 dilution.

Based upon our own convention, titers were expressed as the dilution that gave half the value of the saturated reading (1.831 at OD405 under the conditions used). The resulting titers were 1:2000 for Toraji 1, 1:8000 for Toraji 2 and 1:16000 for Toraji 3. As the titer of Toraji 3 was sufficient for further immunolog-

Fig. 1 Immunoblot analysis with Toraji 3 antiserum, Toraji 3-IgG and Toraji 3-LAP. Approximately 50 μ g of unfractionated total proteins from stage 21 embryos (A) and stage 31 liver (B) were separated by 10% SDS-PAGE performed under reducing conditions. To show total proteins, gels were stained with Coomassie (lane 1). Proteins transferred to a membrane were stained with pre-immune serum (lane 2), Toraji 3 antiserum (lane 3), Toraji 3-IgG (lane 4) and Toraji 3-LAP (lane 5). Asterisks indicate chicken transferrin migrating at about 70 kDa.

Fig. 2 Immunofluorescence staining with Toraji 3-IgG. Unfixed frozen sections of stage 31 developing liver (A-C) and stage 14 visceral yolk sac (D-F) were stained with approximately 40 $\mu\text{g/ml}$ Toraji 3-IgG (A, B, D and E) and control pre-immune rabbit serum (C and F) at a relevant concentration of IgG. Bars indicate 100 μm .

ical applications, this antiserum was purified into IgG and LAP fractions.

Application of Toraji 3 for immunoblot analysis and immunohistochemistry

Toraji 3 antiserum, Toraji 3-IgG and Toraji 3-LAP were used for immunoblot analysis and immunohistochemistry. In immunoblots (Fig. 1), all three preparations of Toraji 3 antibody detected a specific band of chicken transferrin migrating at about 70 kDa. Detection of non-specific bands (one higher than 100 kDa, some ranging between 32-48 kDa and one at about 24 kDa) was reduced markedly in the Toraji 3-LAP immunoblot in comparison with the Toraji 3 antiserum or Toraji 3-IgG.

While this observation supports the validity of the antibody purification procedure, such a difference was not demonstrated in immunohistochemistry, i.e. Toraji 3 antiserum and Toraji 3-IgG did not necessarily produce non-specific staining in tissues. No difference was demonstrated between the staining patterns obtained with Toraji 3 antiserum and those with its IgG or LAP fractions (data not shown). This indicated

that Toraji 3 antiserum was sufficiently specific for use at the immunohistochemistry level. Tissue staining with Toraji 3-IgG is shown in Figure 2. This antibody showed intense staining in developing liver and visceral yolk sac epithelium, both of which are known to be major producers of transferrin in the chicken embryo.

The staining in these tissues was perinuclear and/or appeared in apical granules, suggesting the presence of transferrin in the secretory pathway. Pre-immune serum did not show any fluorescence.

Binding of Toraji 3-IgG to human transferrin

It is important to assess the binding of Toraji 3 antibody to human transferrin, because the addition of human transferrin to culture medium is inevitable in order to establish serum-free conditions.

As shown in Fig. 3, Toraji 3-IgG stained chicken transferrin intensely, and the equivalent amount of human transferrin was stained only faintly under identical conditions. This observation was verified by ELISA. An assay plate was coated with 0.1 μ g of chicken or human transferrin per well, and Toraji 3-IgG was applied in serial 1:2 dilutions across the plate starting at 8.8 μ g/ml. In this assay, chicken transferrin was easily detected by 28 ng/ml Toraji 3-IgG and gave significant level of signal in ELISA. In contrast, human transferrin was hardly detected. The signal, or absorbance at OD405, of human transferrin detected with 4.4 μ g/ml Toraji 3-IgG was about 260 times lower than that of chicken transferrin.

Fig. 3 Binding of Toraji 3-IgG to chicken and human transferrin on transblotted membrane. Purified chicken (lane 1) and human (lane 2) transferrin was used for 10% SDS-PAGE under reducing conditions. Half a microgram of each protein in gel was stained with Coomassie (A). A one-tenth amount (0.05 μ g) of each protein in the adjacent lanes was transblotted to PVDF membrane and stained with 20 μ g/ml Toraji 3-IgG (B). Note that human transferrin is stained much less intensely with this antibody than approximately the same amount of chicken transferrin.

Discussion

In the present study, we obtained a sufficient level of antibody response in a rabbit that was immunized using a relatively short immunization protocol. The resulting Toraji 3 antiserum was purified into IgG and then LAP fractions. All three of these preparations of Toraji 3 antibody gave satisfactory results in immunohistochemistry, while Toraji 3-LAP was more suitable for immunoblot analysis of unfractionated proteins. Toraji 3 antibody detected a specific band migrating at 70 kDa in 10% SDS-PAGE under reducing conditions. Published molecular weights for transferrin tend to be somewhat higher, ranging from 76-81 kDa^[13]. Under the conditions we used, however, chicken transferrin did migrate at 70 kDa and a similar variation in transferrin mobility has been reported previously^[14].

The significance of our Toraji 3 antibody is two-fold. First, the antibody is polyclonal. Even if a monoclonal antibody gives acceptable results in immunohistochemistry and/or immunoblot analysis, this does not necessarily mean that the antibody can inhibit the bioactivity of the antigen. By contrast, a polyclonal antibody is a heterogeneous population of immunoglobulins and is thus very likely to affect the function of the antigen by binding to its critical epitope(s). In this context, Toraji 3 would be expected to act as a block-

ing antibody, perturbing transferrin utilization by cells.

Second, Toraji 3 bound preferentially to chicken transferrin and had only minimal binding capacity for human transferrin. This property of Toraji 3 will be extremely useful when serum-free conditions are necessary in future experiments, because serum-free culture is usually maintained in a chemically defined medium supplemented with human transferrin and/or insulin. In fact, we have successfully immunoprecipitated synthetically radiolabeled chicken transferrin using Toraji 3-IgG in the presence of a large excess of human transferrin (unpublished data).

One of the most likely explanations for this minimal interspecies cross-reactivity may be the difference in primary structure between avian and mammalian transferrin. Indeed, the amino acid sequence homology between chicken transferrin^[15] and its human cognate protein^[16] is 50.4% according to the Genetyx-Homology program (version 2.0.0.; Software Development Co., Ltd). However, an antibody directed against rabbit transferrin (its amino acid sequence^[17] also shows a low homology, 50.6%, with chicken transferrin) binds to the chicken protein as well as human transferrin to a similar extent upon ELISA and immunoblotting (unpublished data). It appears that the immunogenicity of individual proteins and the kinetics of antigen-antibody binding are rather complex^[18,19].

The availability of Toraji 3 antibody, whose characteristics have been clarified in the present study, provides an opportunity to perform *in vitro* functional studies to reveal the level at which transferrin works in various cellular events during early chicken embryogenesis.

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References

- [1] Thorstensen, K. and Romslo, I.: The role of transferrin in the mechanism of cellular iron uptake, *Biochem. J.*, 271, 1-10, 1990
- [2] Barnes, D. and Sato, G.: Serum-free cell culture: a unifying approach, *Cell*, 22, 649-655, 1980
- [3] Copp, A. J., Estibeiro, J. P., Brook, F. A. and Downs, K. M.: Exogenous transferrin is taken up and localized by the neurulation-stage mouse embryo *in vitro*, *Dev. Biol.*, 153, 312-323, 1992
- [4] Isokawa, K., Rezaee, M., Wunsch, A., Markwald, R. R. and Krug, E. L.: Identification of transferrin as one of multiple EDTA-extractable extracellular proteins involved in early chick heart morphogenesis, *J. Cell Biochem.*, 55, 1-2, 1993
- [5] Thesleff, I. and Ekblom, P.: Role of transferrin in branching morphogenesis, growth and differentiation of the embryonic kidney, *J. Embryol. Exp. Morph.*, 82, 147-161, 1984
- [6] Ekblom, P., Thesleff, I., Saxen, L., Miettinen, A. and Timpl, R.: Transferrin as a fetal growth factor. Acquisition of responsiveness related to embryonic induction, *Proc. Natl. Acad. Sci. USA*, 80, 2651-2655, 1983
- [7] Hamburger, V. and Hamilton, H. L.: A series of normal stages in the development of the chick embryo, *J. Morphol.*, 38, 49-92, 1951
- [8] Giloh, H. and Sedat, J. W.: Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein complexes by n-propylgallate, *Science*, 217, 1252-1255, 1982
- [9] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C.: Measurement of protein using bicinchoninic acid, *Anal. Biochem.*, 150, 76-85, 1985
- [10] Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4,

Nature, 227, 680-685, 1970

- [11] Harlow, E. and Lane, D. (eds.): *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988
- [12] Fairbanks, G., Stecks, T. L. and Wallach, D. F. H.: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane, *Biochemistry*, 10, 2606-2617, 1971
- [13] Aisen, P. and Listowsky, I.: Iron transport and storage proteins, *Ann. Rev. Biochem.*, 49, 357-393, 1980
- [14] Cavanaugh, P. G. and Nicolson, G. L.: Lung-derived growth factor that stimulates the growth of lung-metastasizing tumor cells: Identification as transferrin, *J. Cell. Biochem.*, 47, 261-271, 1991
- [15] Jeltsch, J. M. and Chambon, P.: The complete nucleotide sequence of the chicken ovotransferrin, *Eur. J. Biochem.*, 122, 291-295, 1982
- [16] Yang, F., Lum, J. B., McGill, J. R., Moore, C. M., Naylor, S. L., van Bragt, P. H., Baldwin, W. D. and Bowmann, B. H.: Human transferrin: cDNA characterization and chromosomal localization, *Proc. Natl. Acad. Sci. U. S. A.*, 81, 2752-2756, 1984
- [17] Banfield, D. K., Chow, B. K. C., Funk, W. D., Robertson, K. A., Umelas, T. M., Woodworth, R. C. and MacGillivray, R. T. A.: The nucleotide sequence of rabbit liver transferrin cDNA, *Biochim. Biophys. Acta Gene Struct. Expression*, 1089, 262-265, 1991
- [18] Geysen, H. M., Tainer, J. A., Rodda, S. J., Mason, T. J., Alexander, H., Getzoff, E. D. and Lerner, R. A.: Chemistry of antibody binding to a protein, *Science*, 235, 1184-1190, 1987
- [19] Getzoff, E. D., Geysen, H. M., Rodda, S. J., Alexander, H., Tainer, J. A. and Lerner, R. A.: Mechanisms of antibody binding to a protein, *Science*, 235, 1190-1196, 1987