Occurrence of Degradation Products of Chicken Transferrin in Murine Circulating Blood

Ichiro KIMURA*, Koji SAITO** and Eijiro OZAWA
Division of Cell Biology, National Institute of Neuroscience, National Center for Neurology and Psychiatry, Kodaira, Tokyo 187, Japan
Accepted July 27, 1989

Abstract—To determine the true survival of an exogenous substance in the circulation, it is requisite to assess that of the intact molecules. We studied the survival of chicken transferrin (Tf) in murine circulating blood after i.v. injection. With the aid of polyacrylamide-gel isoelectric focusing, direct immunofixation and densitometry, degradation products of chicken Tfs were found, and the disappearance rate of intact molecules was rather higher than that obtained by the single radial immunodiffusion method. These results strongly suggest that the degradation products must be taken into consideration in studies on the clearance of proteinous substances.

The clearance of proteins, especially of glycoproteins, from circulating blood has been extensively studied (1-3). However, most previous reports have not given a full account of the molecular intactness of the circulating materials. They were performed using radioactively labeled preparations, and remaining radioactivity was measured as an index of the amount of surviving molecules. It is likely that these previous data do not always reflect the true survival of "intact" molecules, since radioactive catabolic products, if present, might cause an overestimation of the survival time. To determine the true survival of a given substance, it is essential to quantify the number of surviving intact molecules. In this study, we intravenously injected chicken transferrin (Tf) into mice and examined the molecular properties of circulating materials immunoreactive to chicken Tf antibody. We report here that degradation products of chicken Tfs were found in the murine circulating blood. Intact molecules disappeared at a rather higher rate than the immunoreactive materials.

Materials and Methods

Transferrins: We purified four types of chicken Tf subspecies which differ in their carbohydrate chain structures: two N-acetylneuraminic acid (NeuAc)-bearing serum Tf (Tf2), one NeuAc-bearing serum Tf (Tf1), NeuAc-free Tf (Tf0) derived from neuraminidase-treated Tf2, and ovoTf (oTf). Tf0-2 and oTf were purified as described elsewhere (4). Finally, all the Tf subspecies to be used were dialyzed against 0.9% NaCl.

In vivo studies: After Tf samples were injected into the tail vein of BALB/cAJcl mice (males, 5 to 6 week-old), blood samples (30-40 μl) were collected from the tail by cutting off the tip at various time intervals after injection, placed overnight at 4°C and then centrifuged for 5 min in an Eppendorf centrifuge (type 5412) to obtain serum samples.

Analysis of chicken Tf subspecies: Slab polyacrylamide gel isoelectric focusing (PAGIF), direct immunofixation (DIF), and selective visualization of bands of chicken Tf subspecies and their derivatives were done in combination as described elsewhere (5). Murine sera were analyzed on PAGIF gels after appropriate dilution with 0.9% NaCl.
Sera were mixed prior to PAGIF with 1/100 volume of 10 mM FeCl₃–0.1 M sodium citrate–0.1 M sodium bicarbonate (pH 8.6) in order to completely iron-saturate the chicken Tf's therein and allowed to stand for 30 min at room temperature (5).

**Determination of chicken Tf by single radial immunodiffusion (SRID) method:** Concentrations of all the materials immunoreactive to anti-chicken Tf antibodies were determined by the SRID method as described previously (5), using rabbit anti-chicken Tf2 serum which did not show any immunoreactivity with control murine serum and had the same immunoreactivity to all the chicken Tf subspecies tested, both holotypes and apotypes (4–7).

**Determination of intact Tf molecules:** The amount of chicken intact Tfs in murine serum was determined by means of PAGIF, DIF, staining and densitometry as described elsewhere (5).

**Electrophoretic analysis of immunoprecipitates:** In order to certify the degradation products of chicken Tfs in murine sera, the materials immunoprecipitable with anti-chicken Tf immunoglobulin (Ig) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoprecipitation was carried out by adding sufficient Ig to completely precipitate the reactive materials. After standing overnight at 4°C and pelleting in an Eppendorf centrifuge for 5 min, precipitated materials were washed three times in 0.9% NaCl and then analyzed by SDS-PAGE according to Laemmli (8). Gels were stained with Coomassie brilliant blue R-250. Partially purified Ig from preimmune and anti-sera was obtained by ammonium sulfate precipitation and DEAE-cellulose column chromatography according to Livingston (9).

**Protein determination:** Protein was determined by the method of Lowry et al. (10) using bovine serum albumin as the standard.

**Results**

**Properties of purified Tf subspecies:** We purified four kinds of chicken Tf subspecies: Tf2 and Tf1 from adult serum, o Tf from unfertilized egg white, and Tf0 from neuraminidase-treated Tf2. The results of carbohydrate content analyses confirmed previous data (11) and certified that there was a selective removal of NeuAc residues from Tf2 by neuraminidase treatment (data not shown).

Tf2, Tf1 and Tf0 were not distinguishable from their mobility in SDS-PAGE, but o Tf traveled slightly faster than the other subspecies (data not shown, see Ref. 12). On a PAGIF gel, Tf0 and o Tf were not distinguishable. The pl value of the holotypes was 5.70 for Tf2, 5.85 for Tf1, and 6.07 for o Tf and Tf0 (4, 5). The pl value of the apotype was 6.15 for Tf2 and 6.30 for Tf1. In our isoelectric focusing system, apotypes of o Tf and monoferric Tfs were not focused as a distinct band. Double immunodiffusion tests on chicken Tf subspecies showed that they all had identical immunological reactivity to rabbit antisera against Tf2 and o Tf (data not shown).

**Molecular properties of chicken Tfs after injection:** Since each chicken Tf subspecies possesses a distinct pl value depending on the number of terminal NeuAc residues and iron-binding, molecular changes such as degradation or desialylation, if they occur, are detectable on a PAGIF gel. In addition, the partial degradation products or derivatives of Tfs may also be detectable. The analytical method employed, which combines PAGIF and DIF, is useful to selectively detect the chicken Tfs and their derivatives immunoreactive to antibodies.

Results concerning o Tf, Tf1 and Tf2, both their apotypes and holotypes, presented in Fig. 1, demonstrate that neither desialylation nor sialylation occurs, but there is iron-removal from holo Tfs and iron-binding to apo Tfs. Additionally, the degradation products of chicken Tfs or their derivatives were demonstrated. The presence of materials that are immunoreactive to anti-Tf antibody and have rather acidic pl's are obvious in the figure. These materials were not detectable just after injection, but became more notable with the passage of time. Occurrence of degradation products with molecular weights lower than that of Tf was also demonstrated by SDS-PAGE analyses of the immunoprecipitates (Fig. 2).

**Clearance of intact Tfs:** The above data show that the materials which are reactive to
Chicken holo and apoTfs (5 mg/ml of 0.9% NaCl) were intravenously injected at 0.5 mg/13 g of body weight. Murine sera collected at various time intervals after injection were analyzed on a PAGIF gel after appropriate dilution with 0.9% NaCl. Chicken Tfs and their derivatives which were immunoreactive to anti-Tf antibody were selectively visualized by DIF and staining. The pl value of the holotype was 5.70 for Tf2, 5.85 for Tf1 and 6.07 for oTf. The pl value of the apotype was 6.15 for Tf2 and 6.30 for Tf1. Under our experimental conditions, apo-oTf could not be focused as a distinct band. The arrow indicates the degradation products or modified molecules of chicken Tf subspecies. aHours after injection. bPresence (+) and absence (-) respectively indicate whether or not murine sera were treated with iron before PAGIF analysis.

antibodies are present in murine circulating blood not only as intact molecules but also as degraded or modified materials. This means that the data obtained by solely immunological methods do not reflect the values for "intact" Tf molecules. Therefore, we tried to examine the clearance of "intact" Tfs with the aid of PAGIF, DIF, staining and densitometry. This procedure allowed us to selectively and quantitatively analyze the "intact" chicken Tfs in murine serum. In this experiment, "intact" Tfs refers to those which were focused on the PAGIF gel identically to standard Tfs. As can be seen from Fig. 3, clearance rates of "intact" Tfs were rather higher than those obtained by SRID, which quantitated all the materials reactive to anti-Tf antibodies. Half-lives estimated with exponential clearance phases from 6 to 22 hr after injection were about 6.9 hr for intact Tf1, 9.6 hr for immunoreactive Tf1, 5.7 hr for intact Tf0, and 7.0 hr for immunoreactive Tf0: apparent half-lives of Tf1 and Tf0 were about 1.4 and 1.2 times longer than the true half-lives of Tf1 and Tf0, respectively. The
clearance rates were dependent on the carbohydrate chain structures.

Discussion

The most important finding in the present study was that some degradation products of exogenous chicken TfS were found in murine circulating blood (Figs. 1 and 2). This implies that clearance data that were obtained on the basis of immunoreactivity or remaining radioactivity due to labeled molecules do not represent the true clearance of proteinous molecules. Thus, in studies on the clearance of proteinous substances such as glycoproteins (1–3), for an exact interpretation of the data, it is very important to take into consideration the presence of degradation products.

When an excess amount of chicken adult serum was analyzed on a PAGIF gel, such degradation products were never detectable (data not shown). Furthermore, when murine serum containing chicken TfS was incubated at 37°C for 24 hr, no such materials were detectable. Although the mechanism of the occurrence and the biological significance of degradation products remain to be elucidated, these observations suggest the possibility that heterologous TfS are taken up by, degraded in, and exocytosed as degradation products from cells such as hepatocytes.

Acknowledgment: This work was partly supported by a grant from the National Center of Neurology and Psychiatry (NCNP grant No. 86-01) of the Ministry of Health and Welfare, Japan.

References

1 Ashwell, G. and Morell, A.G.: The role of surface carbohydrate in the hepatic recognition and transport of circulating glycoproteins. Adv. Enzymol. 41, 99–128 (1974)
2 Neufeld, E.F. and Ashwell, G.: Carbohydrate recognition system for receptor-mediated pinocytosis. In Biochemistry of Glycoproteins and Proteoglycans, Edited by Lennarz, W.J., p. 241–266, Plenum Press, New York and London (1980)
3 Ashwell, G. and Harford, J.: Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. 51, 531–554 (1982)
4 Kimura, I., Hasegawa, T. and Ozawa, E.: Indispensability of iron-bound chick transferrin for chick myogenesis in vitro. Dev. Growth Differ. 24, 369–380 (1982)
5 Kimura, I.: Developmental change in microheterogeneity of serum transferrin of chickens. Dev. Growth Differ. 25, 531–535 (1983)
6 Kimura, I. and Ozawa, E.: Further studies on the developmental change in myotrophic activity of chicken serum: Relation between activity and transferrin. Dev. Growth Differ. 25, 523–529 (1983)
7 Li, I., Kimura, I. and Ozawa, E.: A myotrophic protein from chick embryo extract: Its purification, identity to transferrin, and indispensability for avian myogenesis. Dev. Biol. 94, 366–377 (1982)
8 Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–688 (1970)
9 Livingston, D.M.: Immunoaffinity chromatography of plasma proteins. Methods Enzymol. 77, 423–450 (1981)
graphy of proteins. Methods Enzymol. 34, 723–731 (1974)

10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

11 Williams, J.: A comparison of glycopeptides from the ovotransferrin and serum transferrin of the hen. Biochem. J. 108, 57–67 (1968)

12 Kimura, I., Hasegawa, T., Miura, T. and Ozawa, E.: Muscle trophic factor is identical to transferrin. Proc. Japan Acad. 57(B), 200–205 (1981)