Vol. 180, No. 1, 1991
October 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 64-68

LOCALIZATION OF AN ENDOGENOUS ADP-RIBOSE ACCEPTOR, p33, IN POLYMORPHONUCLEAR CELL GRANULES IN CHICKEN LIVER INTERLOBULAR CONNECTIVE TISSUE[1]

Harumi Osago[*], Koichi Mishima[2,@], Mikako Tsuchiya[*], Yoshinori Tanigawa[*], Tetsuhiro Umeno[#], and Makoto Shimoyama[*]

Departments of [*]Biochemistry, [@]Oral and Maxillofacial Surgery, and [#]Internal Medicine, Shimane Medical University, Izumo 693, Japan

Received August 5, 1991

Summary: We investigated immunohistochemically the localization of p33, an endogenous substrate protein for an arginine-specific ADP-ribosyltransferase in chicken liver. Polymorphonuclear-pseudoeosinophilic granulocytes (heterophils) in interlobular connective tissues of the liver were exclusively and strongly stained with the antibody against p33. Strong reactivity was associated with granules in cytoplasm of the heterophils. When the chicken liver nuclear fraction was washed, the transferase activity was released into the 600 x g supernatant fraction while a nuclear enzyme poly(ADP-ribose) synthetase was retained in the pellet fraction. These results indicate that p33 and probably also ADP-ribosyltransferase, found in the liver nuclear fraction [Tanigawa et al. (1984) J. Biol. Chem. 259, 2022-2029, Mishima et al. (1988) Eur. J. Biochem. 179, 267-273], originate from interlobular heterophils of the chicken liver.

Arginine-specific ADP-ribosyltransferase transfers the ADP-ribose moiety of NAD to acceptors such as small molecular guanidino compounds and arginine residues of various peptides and proteins (1). We reported the purification and properties of the ADP-ribosyltransferase and its endogenous substrate protein, p33, from chicken liver nuclei prepared by a standard method in the presence of Ca^{2+} (2,3).

[1]This work, carried out in the Department of Biochemistry, was supported in part by Scientific Research Grants-in-Aid 01770177 and 01580192 and Cancer Research Grant 01010077 from the Ministry of Education, Science and Culture, Japan.

[2]Present address: Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(ß-aminoethylether)-N,N,N',N'-tetraacetic acid.

0006-291X/91 $1.50

Recently, we found that chicken peripheral polymorphonuclear cell (heterophil) granules contained high levels of both ADP-ribosyltransferase activity and p33 (4). Peptide maps of p33 from heterophils and liver were identical. Heterophil ADP-ribosyltransferase showed similar properties to that of liver so far examined, including molecular weight on SDS-PAGE, effects of SH-reagents and salt on the enzyme activity. Since the liver is one of the extramedullary hematopoietic organs in the avian species (5), it is possible that both the enzyme and p33 found in chicken liver are due to the presence of heterophils in the tissue.

To localize p33 histologically, we prepared a monoclonal antibody against the chicken liver p33 and stained the liver section immunohistochemically using this antibody.

We describe here observations that p33 exists not in hepatocyte nuclei but rather in the granules of interlobular heterophils in chicken liver. The possibility that not only p33 but also ADP-ribosyltransferase found in chicken liver nuclear fraction are derived from interlobular heteropils is discussed.

MATERIALS AND METHODS

Materials. Chickens (White Leghorn) were obtained from Hara Farms, Shimane, Japan. [adenylate-^{32}P] NAD (29.6 TBq/mmol) was obtained from New England Nuclear. Other materials were obtained from Seiwa Industry Co. Ltd. (Shimane, Japan) and used without further purification.

Enzyme and protein assays. For the standard assay of ADP-ribosyltransferase activity, the reaction mixture, containing 400 μg of casein, 50 mM Tris-HCl buffer (pH 9.0), 0.1 mM [adenylate-^{32}P] NAD (1 kBq/tube), 5 mM dithiothreitol, 0.1 mM benzamide, a potent inhibitor of poly(ADP-ribose) synthetase (6) and 20 μl of the enzyme preparation in a total volume of 0.2 ml, was incubated at 25°C for 30 min. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid and radioactivity in the acid-insoluble fraction was measured as described previously (2). For the standard assay of poly(ADP-ribose) synthetase activity, the reaction mixture, containing 4 μg of whole histones, 2 μg of DNA, 50 mM Tris-HCl buffer (pH 8.0), 10 μM [adenylate-^{32}P]NAD (0.5 kBq/tube), 10 mM $MgCl_2$, 1 mM EDTA , 0.1 mM novobiocin, a potent inhibitor of arginine-specific ADP-ribosyltransferase (7), and 20 μl of the liver fractions in a total volume of 0.2 ml, was incubated at 25°C for 10 min. Other conditions were as described above. Protein concentration was measured using Bio-Rad protein assay kits.

Fractionation of chicken liver. Fractionation was performed with 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 8.0), 2 mM 2-mercaptoethanol, 3 mM $CaCl_2$, 1 mM EDTA and 0.5 mM EGTA. All the procedures were carried out at 0-4°C. Isolated liver (5 g) was homogenized in 20 ml of the medium, with 4 strokes in a Teflon homogenizer at 10,000 rpm. The homogenate was filtered through three layers of gauze, followed by 10 min centrifugation at 600 x g. The pellet was suspended in 20 ml of the same medium (crude nuclear fraction) and centrifuged again under the same conditions. The final pellet, suspended with 20 ml of the medium and the supernatant were used as 600 x g pellet and supernatant, respectively.

Purification of chicken liver p33, hybridoma production and purification of monoclonal antibody to p33. p33 was purified from chicken liver as described previously (3). Male 10-20 g BALB/c mice were immunized by giving an intramuscular injection of 10 μg of the p33 emulsified in complete Freund's

adjuvant. After 3 weeks, booster injections of incomplete Freund's adjuvant containing 10 μg of the antigen were given at identical sites. Antisera were titered one week later by ELISA using Hybri-Clonal™ EIA Mouse G+M Screening Kit (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD) according to the supplier's manual. Mice with high titers (A_{490} values > 2x background with antisera dilutions ≥1:50,000) were injected intraperitoneally with 10 μg of the antigen, and 4 days later, the spleens were obtained. Cell fusions were performed by standard procedures (8). Hybridoma colonies were screened for the production of the specific antibody against p33 by ELISA. The hybridoma line producing a high titer of anti-p33 antibody was grown for >100 generations without detectable changes in antibody production. A monoclonal antibody to p33 was purified from the hybridoma supernatant using a protein A-Sepharose CL-4B column and was named L-HYM-2.

Immunohistochemistry. For immunohistochemical localization of p33, chicken liver was fixed with 10% formalin, embedded in paraffin, cut at 5 μ, and stained using an immunoperoxidase procedure (9, 10) with the monoclonal antibody to chicken liver p33, L-HYM-2. In some cases, counterstaining with hematoxylin-eosin was carried out. The preparations placed under a light microscope were photographed.

RESULTS AND DISCUSSION

Immunohistochemical staining with the monoclonal antibody to the chicken liver p33, L-HYM-2, was performed on formalin-fixed sections of chicken liver (Fig. 1). Strong reactivity was associated with granules of particular cells

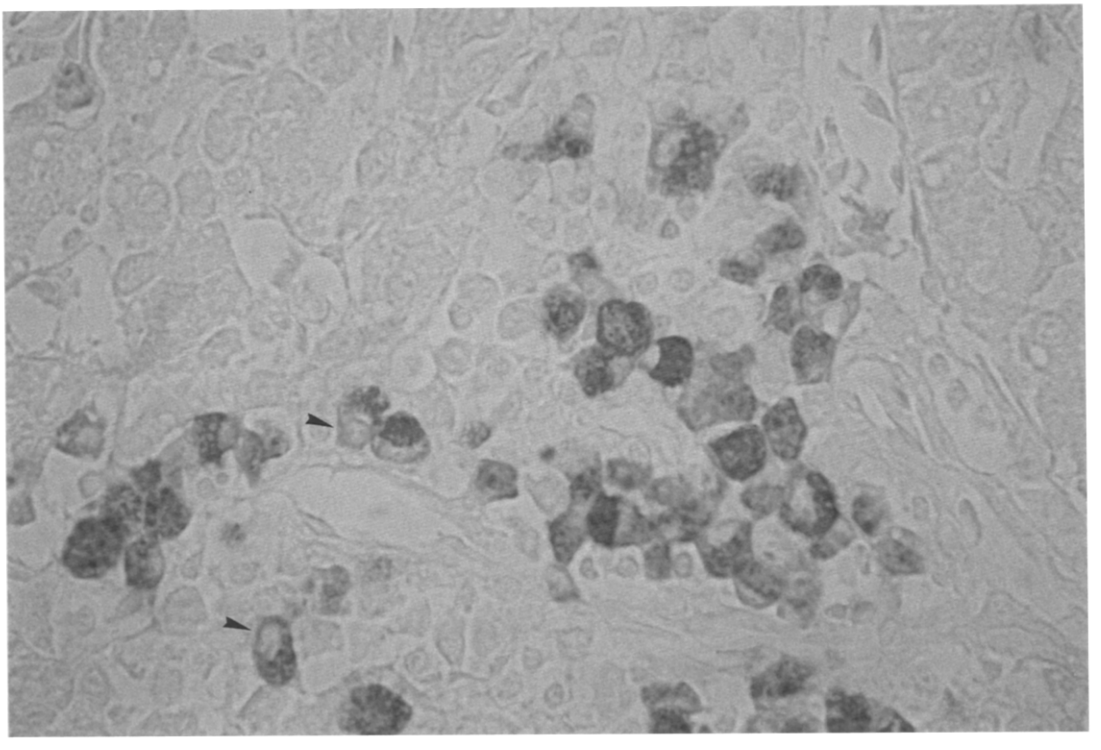

Fig. 1. Immunoperoxidase staining of formalin-fixed chicken liver sections with L-HYM-2 monoclonal antibody. Granules of polymorphonuclear cells present in interlobular connective tissue but not the cell nuclei (arrowheads) reacted strongly.

located in interlobular connective tissue and the reaction was negative for the cell nuclei (Fig. 1, arrowheads). The liver parenchymal cells were not stained. Control sections incubated with L-HYM-2 absorbed on the p33 under the same conditions were uniformly negative (data not shown). By counterstaining with hematoxylin-eosin and by hematoxylin-eosin staining, we confirmed that immunohistochemically reactive cells present in the interlobular connective tissue were polymorphonuclear-pseudoeosinophilic granulocytes. In the avian species, the cells are usually designated "heterophils", the counterpart of mammalian neutrophils (11). This result is consistent with the finding that in chicken peripheral heterophils p33 is localized in the granular fraction separated by Percoll density gradient centrifugation (4). We reported that in the presence of Ca^{2+}, more than 90% of the granular enzymes are precipitated in the nuclear fraction and that the peptide map of heterophil p33 digested with trypsin is identical to that of liver p33 (4). Thus, the chicken liver nuclear fraction prepared by the standard method (presence of Ca^{2+}) was probably contaminated with granules of the interlobular heterophils.

With Ca^{2+}-free medium, more than 70% of marker enzyme activities of the heterophil granules, including ADP-ribosyltransferase, were released into the 600 x g supernatant whereas in the presence of Ca^{2+}, more than 90% of these activities were found in the 600 x g pellet (4). We examined the ADP-ribosyltransferase activities in 600 x g supernatant and pellet fractions prepared with or without Ca^{2+}, respectively. In Ca^{2+}-free medium, the ADP-ribosyltransferase and a nuclear enzyme, poly(ADP-ribose) synthetase also were released into the supernatant fraction (data not shown). Next, we prepared crude nuclear fraction with medium containing Ca^{2+}, washed the fraction as described in Materials and Methods and assayed poly(ADP-ribose) synthetase and ADP-ribosyltransferase activities in the pellet and supernatant fractions, respectively. Table I shows the release of two enzymes from the crude nuclear fraction. After washing the nuclear fraction, 48.7% of the ADP-ribosyltransferase was released into the 600 x g supernatant, while only 8.6% of poly(ADP-ribose) synthetase was found in this fraction (Table I). These results suggest that the chicken liver ADP-ribosyltransferase is essentially not present in hepatocyte nuclei and further, that the ADP-ribosyltransferase and p33 found in chicken liver nuclear fraction are due to co-precipitation of the interlobular polymorphonuclear cell granules with liver cell nuclei.

We cannot exclude the possibility that in addition to the heterophil granular enzyme, the chicken liver contains an ADP-ribosyltransferase originating from the hepatocyte nuclei because more than half of the ADP-ribosyltransferase activity in the crude nuclear fraction was retained in

Table I. Release of ADP-ribosyltransferase and poly(ADP-ribose) synthetase into the 600 x g supernatant with wash of chicken liver crude nuclear fraction

Fraction	Activity			
	Transferase[a]		Synthetase[b]	
	nmol/g wet liver/h			
Crude nuclear fraction	205	(100.0)[c]	60.4	(100.0)
600 x g Supernatant	100	(48.7)	5.2	(8.6)
600 x g Pellet	114	(55.6)	48.0	(79.5)
Recovery	214	(104.3)	53.2	(88.0)

[a]ADP-ribosyltransferase

[b]poly(ADP-ribose) synthetase

[c]Percent distribution of enzyme activities is shown in parentheses. Enzyme activities in the crude nuclear fraction are set at 100%.

the 600 x g pellet fraction even after washing. To clearly identify the origin of chicken liver ADP-ribosyltransferase(s), immunohistochemical staining with an antibody specific to the ADP-ribosyltransferase, as described for p33 in this paper, has to be done.

Acknowledgments: We thank Professor M. Oki and Dr. T. Tsumori for immunohistochemical analysis.

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