EXPRESSION OF CELLULAR RETINOIC ACID BINDING PROTEIN (CRABP) IN ADULT RAT BRAIN

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ABSTRACT

The presence of cellular retinoic acid binding protein (CRABP) was shown in adult rat brain by immunoblot and gel-filtration analysis. Immunohistochemical study demonstrates that neurons are CRABP-positive, but glial cells and blood vessels are not. The presence of CRABP in mature neurons suggests that retinoic acid and CRABP are involved in the differentiation of neuronal cell lineage *in situ*, like as in neuroblastoma cells in culture.

Retinoic acid, an active metabolite of retinol (vitamin A), has the ability to induce differentiation of various cancer cells (1, 3, 13). The biological action of retinoic acid is considered to be mediated by cellular retinoic acid binding protein (CRABP) (12, 15, 17). IMR-32 cells, a human neuroblastoma cell line, that contain a large amount of CRABP, cease to grow, differentiate and extend neurites when exposed to retinoic acid (3).

CRABP is highly expressed in the adult testis, but only a small amount is found in the adult brain (11). Recently, a large amount of CRABP was shown to be transiently expressed in the central nervous system during early development (7-9), raising the possibility that retinoic acid and CRABP are involved in the neuronal differentiation during development *in vivo*. To clarify the role of retinoic acid in neurogenesis, it is of great interest to investigate the lineage of CRABP-positive cells. In the present study, we show that CRABP is localized in neuronal cells, but not in glial cells of rat brain.

MATERIALS AND METHODS

Detection of Retinoic Acid Binding Activity

Brains of Wistar rats (3 months old) were homogenized in 50 mM Tris-HCl (pH 7.5) and centrifuged at 100,000 *g* for 30 min. The supernatant (500 μ g protein) was incubated with 2 nM [³H]retinoic acid (50 Ci/mmol, New England Nuclear, Boston) in the absence and the presence of 2 μ M unlabelled retinoic acid or 2 μ M retinol at 4°C for 8 h and then applied to a Sephadex G-50 column (1×30 cm), which had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl.

Immunoblotting

The expression of CRABP in rat brain was examined by immunoblotting as described previously (9). CRABP was purified from porcine testis according to the procedure described by Ong and Chytil (10). Anti-CRABP antiserum that specifically reacts with CRABP, but not with cellular retinol binding protein (CRBP) or with bovine P2 protein (7, 9), which shows more than 60% homology to the N-terminal region of CRABP (4, 15), was

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used to detect CRABP in rat brain. Purified CRABP (25 ng) and proteins of the rat brain cytosol (50 μ g) were analyzed by SDS-polyacrylamide gel electrophoresis using 15% gels as described by Laemmli (5). After electrophoretic transfer to the nitrocellulose filter (16), the sheet was incubated with anti-CRABP antiserum for 2 h at room temperature, washed with phosphate-buffered saline (PBS, pH 7.4), and the immunoreactivity on the sheet was detected by goat peroxidase-conjugated anti-rabbit immunoglobulin and 4-chloronaphthol. The protein content was estimated by the method of Lowry *et al.* (6).

Immunohistochemistry

Wistar rats (3-month-old) were anesthetized with pentobarbital and perfused with 4% paraformaldehyde in PBS. After perfusion, the brain was coronally dissected and embedded

in paraffin. Six-micron paraffin sections were mounted on gelatin-chromium alum-coated microscopic slides. Sections were deparaffinized and preincubated with PBS containing 2% bovine serum albumin (BSA) and 5% heat-inactivated goat serum, then incubated with 1,000 $\times$ diluted anti-CRABP antiserum for about 72 h at 4°C. After the incubation with the first antibody, the endogenous peroxidase activity was abolished by soaking the sections in 0.2% H_2O_2 in methanol for 20 min. Immunoreactivities on the section were detected by the system of biotinylated-goat anti-rabbit IgG and peroxidase-labelled avidin (Histo-Gen, BioGenex Lab., U.S.A.). Peroxidase activity was detected by diaminobenzidine as substrate. Sections were lightly counterstained with hematoxylin. Specificity of the immunostaining was examined by replacing the primary antibody with pre-immune IgG from the same rabbit.

Fig. 1 Identification of CRABP in rat brain. a: Sephadex G-50 gel filtration column chromatography of the cytosolic proteins of rat brain. [3H]Retinoic acid binding activity in the absence ($\bullet$ — $\bullet$), and in the presence of a 1,000-fold amount of unlabelled retinoic acid ($\circ$ — $\circ$) or retinol ($\square$ — $\square$). An arrowhead indicates the position of void volume, and an arrow indicates the position for CRABP of porcine testis. b: SDS-polyacrylamide gel (15%) electrophoresis. Lane 1, porcine testis CRABP (1 μ g); lane 2, brain cytosolic proteins (25 μ g). Stained with Coomassie brilliant blue. c: Immunoblot analysis using anti-CRABP. Lane 1, porcine testis CRABP (25 ng); lane 2, brain cytosolic protein (25 μ g)

RESULTS

On Sephadex G-50 column chromatography of the brain cytosol, specific [3 H]retinoic acid binding activity was eluted at the molecular weight of porcine testis CRABP. This binding activity was completely inhibited in the presence of a 1,000-fold excess amount of unlabelled retinoic acid, but only partly inhibited in the presence of a 1,000-fold excess amount of unlabelled retinol (Fig. 1a). To identify the retinoic acid binding protein, immunoblot analysis was carried out by using anti-CRABP. A protein with a molecular weight similar to that of porcine testis CRABP was detected in the brain protein (Fig. 1, b and c).

Localization of CRABP-positive cells in adult rat brain was examined by immuno-

staining using the same anti-CRABP. Neurons in cerebral cortex and hippocampus were stained with the anti-CRABP serum, although the intensity of staining was slightly different among various regions (Fig. 2). No immunoreaction was detected with pre-immune serum. The immunoreaction was located in the cytoplasm of neurons, but not in the nucleus. Because immunoreaction was not detected in the neuropil in grey matter, glial cells appeared to be CRABP-negative. Also, no immunoreaction was detected in the blood vessels.

DISCUSSION

In adult tissues, CRABP was abundantly found in the testis, mainly in the Sertoli cells and sper-

Fig. 2 Immunohistochemical staining of CRABP. Coronal sections of the brain cut through the plane of infundibulum. a: Neurons in the cerebral cortex and hippocampus were stained with antiserum against CRABP. Arrow, CRABP-positive neurons in hippocampus; arrowhead, CRABP-positive neurons in cerebral cortex. b: The section was not stained with pre-immune serum. Bar, 100 μ m

matogonia (2). In the brain, a small amount of CRABP was detected (11), but its distribution has been unclear. We found that a large amount of CRABP was transiently expressed in the central nervous system during early development. In the neural tube of early mouse embryo, CRABP is expressed in neuroblasts located in lateral walls of the ventral part of the neural tube. At present, biological significance of retinoic acid and its specific binding protein in neurogenesis is not clear. If we know the types of CRABP-positive cells in the adult central nervous system, it will provide a clue for clarifying possible functions of retinoic acid and CRABP in determining cell lineage during neurogenesis.

In the present study, we show that CRABP with a molecular size similar to that of porcine testis, is present in the mature rat brain and mainly located in neuronal cells, but not in glial cells. These suggest that CRABP-positive cells in the central nervous system of the early embryo are the precursor of some neuronal cells. Retinoic acid may be involved in the differentiation and growth of neuronal cells in the brain, like as in neuroblastoma cells in culture.

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