Changes in subcellular and zonal distribution of glucokinase in rat liver during postnatal development

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Abstract   Subcellular and zonal distribution of glucokinase in rat liver during postnatal development was examined immunohistochemically. Before day 11 after birth, only some hepatocytes were immunostained, and a positive immunostaining was found in the cytoplasm but not in the nucleus. No zonal distribution of glucokinase was observed in livers of such pups. From day 15, at which time a dietary change from milk to laboratory chow begins to take place, glucokinase immunoreactivity increased; this increase was associated with increases in glucokinase activity and in glucokinase protein, and also the immunostaining was observed mainly in the nuclei. At day 21, the glucokinase immunoreactivity was found almost exclusively in the perivenous zone. At day 30, an intense immunostaining was seen both in the perivenous zone and in the periportal zone, being slightly predominant in the former. The present results indicate that dramatic changes in the distribution of glucokinase in developing rat liver may be related to dietary change.

Key words: Glucokinase; Liver; Postnatal development;
Subcellular distribution; Zonal distribution; Rat;
Immunohistochemistry

1. Introduction

The primary function of the liver is to maintain the blood glucose levels through glycolysis, glycogen metabolism, and gluconeogenesis [1]. Glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is one of the hexokinase isozymes and is often called hexokinase IV or high-K_m hexokinase [2]. This enzyme has been identified only in glucose-sensing cells such as hepatocytes, pancreatic islet cells, and some neuroendocrine cells [3]. Glucokinase is considered to play a major regulatory role in hepatic glucose metabolism [2,4–6]. The activity of glucokinase fluctuates with the nutritional and hormonal status [2,4–8]: the enzyme is decreased by fasting and diabetes and increased by refeeding of fasted rats or by insulin treatment of diabetic rats.

It is widely accepted that the activities of gluconeogenic enzymes, such as glucose-6-phosphatase and fructose bisphosphatase, are higher in the periportal hepatocytes than elsewhere in the liver of adult rats, whereas the activities of glycolytic enzymes, such as glucokinase and pyruvate kinase L, are

higher in the perivenous hepatocytes [9]. Therefore, the periportal zone is considered to be gluconeogenic; and the perivenous zone, glycolytic.

Earlier we examined the subcellular distribution of glucokinase in rat liver and found that glucokinase is present in the nucleus as well as in the cytoplasm, in contradiction to the widespread belief of its exclusive localization in the cytoplasm [10]. In addition, we studied the changes in tissue and subcellular distribution of the enzyme during fasting–refeeding [11] and suggested that glucokinase might be translocated between the nucleus and the cytoplasm in hepatocytes. We also reported clear evidence that nuclear glucokinase is translocated to the cytoplasm during perfusion of the liver with high glucose [12]. We proposed the idea that translocation of glucokinase might be involved in the regulation of glucose metabolism in hepatocytes [12].

There have been several reports concerning the appearance of glucokinase in rat liver during ontogenic development [2,7,8,13,14]; however, the changes in subcellular and tissue distribution of glucokinase in extrauterine life has not yet been clarified. Recently, Kirchner et al. [15] reported that immunoreactivity for glucokinase is observed mainly in the periportal zone of neonatal rat liver whereas the enzyme is located mainly in the perivenous zone in adult rat liver. In this study, we re-examined the changes in subcellular and zonal distribution of glucokinase during the suckling–weaning period and found that our data are quite different from their results.

2. Materials and methods

2.1. Antibody
In the immunohistochemical study, we employed rabbit antisera raised against rat liver glucokinase as described previously [11,12]. Monospecific antibodies against rat liver glucokinase were purified by adsorption and elution from rat liver glucokinase immobilized onto poly(vinylidene difluoride) membranes [11]. Non-immune IgG and pre-immune IgG were purified by batch adsorption of the respective non-immune and pre-immune sera onto protein A-Sepharose.

2.2. Animals
Male Wistar rats weighing 180–220 g were used as adult animals. They had free access to water and food (MM-3; Funabashi Farm, Japan). All animals were killed by decapitation prior to removal of their livers. Neonatal rats were born naturally and kept with their mothers until they were sacrified.

2.3. Immunohistochemistry
The liver was excised, sliced at a 1.5–2-mm thickness, and immersed overnight in saturated picric acid, 4% paraformaldehyde (Zamboni's solution) [16] at 4°C. After the fixation, the liver pieces were dehydrated, embedded in paraffin, and sectioned at a thickness of 2–3 μm. The sections were placed on slides precoated with 0.1% poly-L-lysine

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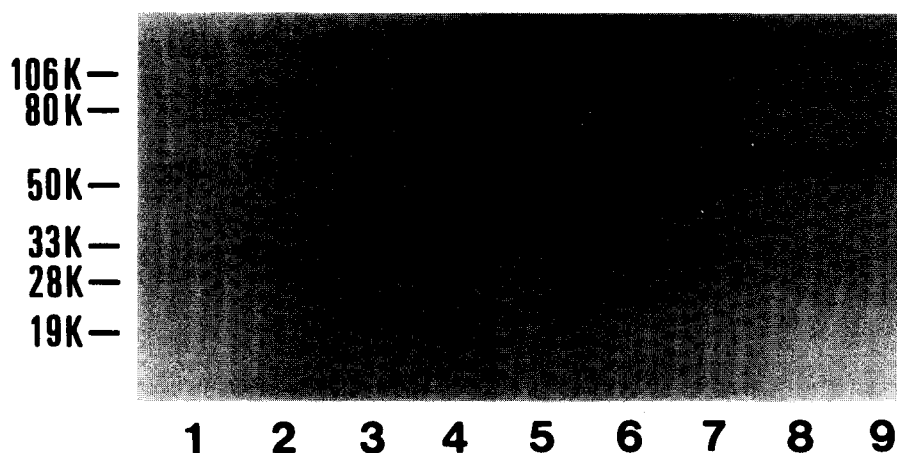Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, Japan.

Fig. 1. Changes in glucokinase protein level in rat liver after birth. The immunoblot analysis was carried out on liver extracts obtained from 3-day (lane 1), 6-day (lane 2), 11-day (lane 3), 15-day (lane 4), 21-day (lane 5), 26-day (lane 6), 30-day (lane 7), and 40-day (lane 8)-old rats, and from adult rats (lane 9).

solution (Sigma, USA), deparaffinized with Histo-Clear (National Diagnostics, USA), and treated with a solution (0.1 mg/ml in 50 mM Tris-HCl buffer, pH 8.0) of pronase E (Sigma, USA) for 10 min at room temperature. Next, the sections were placed in phosphate-buffered saline (PBS) containing 0.3% (w/v) H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity. After having been washed with PBS, the sections were incubated for 1 h at 37°C with a solution containing 2% non-fat milk and 5% normal goat serum. Then, they were incubated consecutively with affinity-purified anti-glucokinase IgG (diluted 1:20 in 5% normal goat serum) overnight at 4°C, with biotin-labeled goat serum against rabbit IgG (Seikagaku, Japan) for 1 h at room temperature, and with streptavidin–peroxidase (Seikagaku, Japan) for 1 h at room temperature. Each incubation was followed by 3–4 washings with PBS. Finally, the sections were soaked in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.05% 3,3'-diaminobenzidine·4 HCl and 0.01% (w/v) H_2O_2 for 15 min at room temperature, washed with distilled water, dehydrated, mounted on Entellan New (Merck, Germany), and examined by light microscopy. As negative controls, semi-thin sections were incubated with anti-glucokinase rabbit IgG adsorbed by an excess of purified rat liver glucokinase, with non-immune rabbit IgG, or with pre-immune rabbit IgG, followed by consecutive incubation with biotin-labeled goat serum against rabbit IgG and streptavidin–peroxidase. Some sections were directly incubated with the second antibody without any preceding incubation with anti-glucokinase IgG.

2.4. Immunoblotting of glucokinase

The liver cytosol was separated by SDS-PAGE and transferred to a

nitrocellulose membrane. Blocking, incubation with antibody, and washing for immunoblotting were performed as described previously [10]. Affinity-purified anti-glucokinase IgG was diluted 1:200 with the buffer used for immunoblotting.

2.5. Assays of hexokinase and glucokinase activities and protein

Both enzyme activities were measured according to the method of Hara et al. [17]. Protein was assayed by the biuret method with bovine serum albumin as a standard.

3. Results

3.1. Specificity of anti-glucokinase antibody

We have previously reported the specificity of affinity purified anti-glucokinase antibody that was obtained from the same rabbit as used in this study [11]. Thus, a single immunoreactive band migrating identically to glucokinase and having a relative molecular mass of 52,000 was seen with the antibody after electrophoresis and immunoblotting of extracts of rat liver (Fig. 1).

3.2. Specificity of immunostaining

We have carefully verified the specificity of immunostaining as described previously [10–12]. In this study, we found that the specificity of immunostaining was improved when a solution containing 2% non-fat milk and 5% normal goat serum was used instead of 5% normal goat serum to block non-specific staining.

3.3. Changes in glucokinase and hexokinase activities and in glucokinase protein level during postnatal development

Glucokinase activity was not detectable in the liver of rats up to the age of 11 days (Fig. 2). The enzyme began to increase from the 15th postnatal day, in agreement with the findings of several investigators [2,13,14], and reached adult values at day
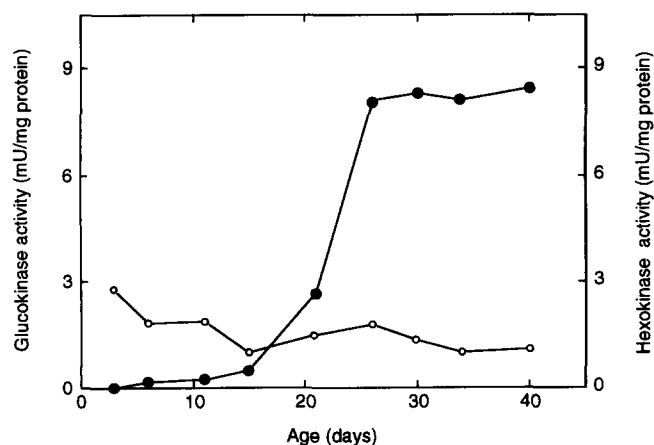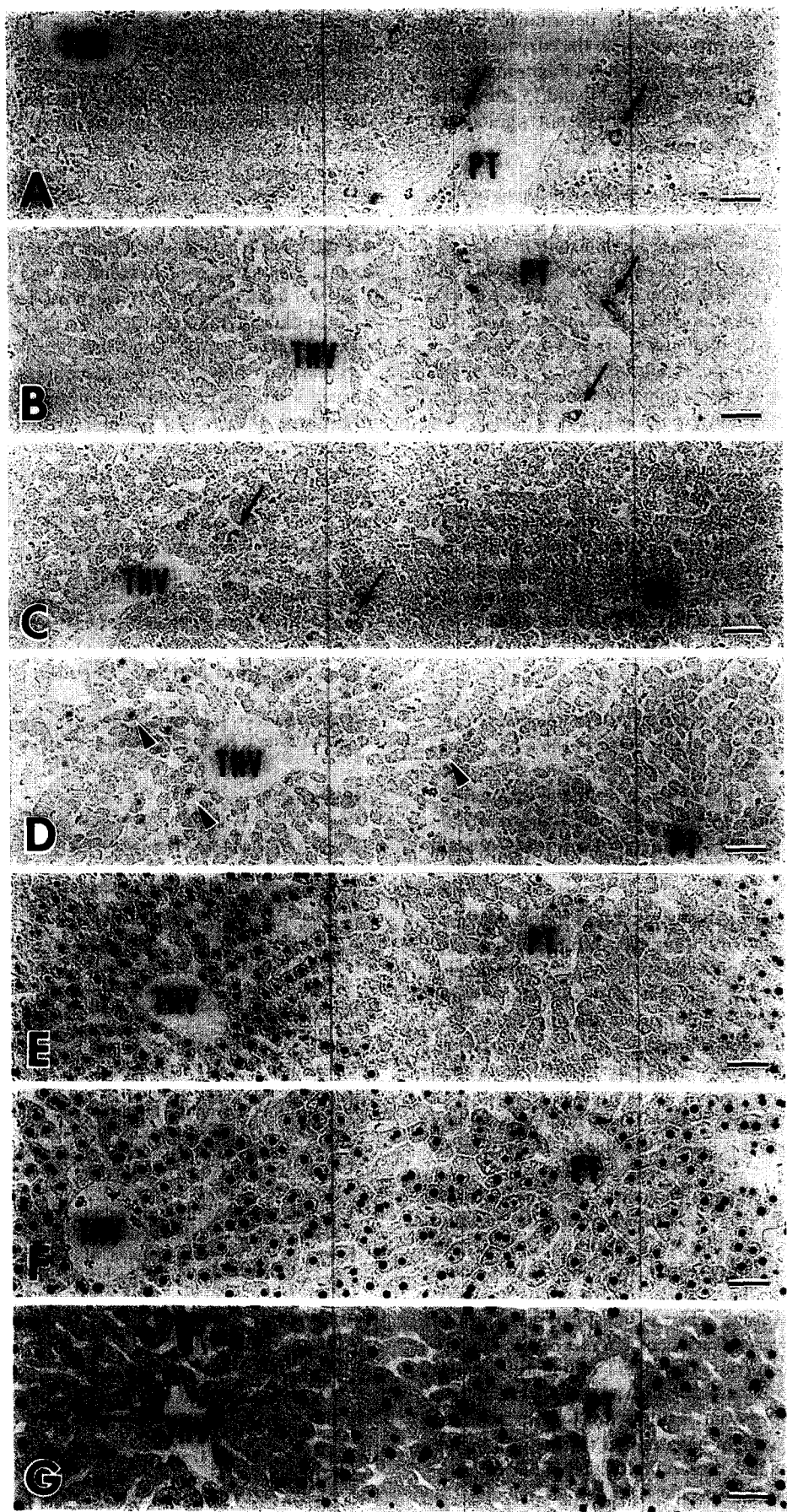

Fig. 2. Changes in glucokinase and hexokinase activities in rat liver during postnatal development. Values are means of three individual experiments. ●, glucokinase activity; ○, hexokinase activity.

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Fig. 3. Changes in immunohistochemical distribution of glucokinase in rat liver during postnatal development. The immunohistochemical analysis was carried out on liver sections obtained from 3-day (A), 6-day (B), 11-day (C), 15-day (D), 21-day (E), and 30-day (F)-old rats, and from adult rats (G). Arrows indicate the immunostaining in the cytoplasm, and arrowheads that in the nuclei. THV, terminal hepatic venule; PT, portal triad. Bars = 50 μm.

26 (Fig. 2). The enzyme protein level determined by immunoblots showed a close correlation with the enzyme activity (Fig. 1). It should be noted that a faint band migrating identically to glucokinase was seen at days 3, 6, 11, and 15 after birth. On the other hand, hexokinase activity did not significantly change during postnatal development.

3.4. Changes in immunohistochemical distribution of glucokinase during postnatal development

A clear and positive glucokinase immunostaining was observed in the nuclei of parenchymal cells in sections from adult rats (Fig. 3G). This is a confirmation of our previous study on hepatic glucokinase distribution [10,11]. At days 3, 6, and 11 after birth, no glucokinase immunoreactivity was observed in most of the hepatocytes (Fig. 3A–C); however, a positive immunoreactivity was detected in some of them. Interestingly, immunoreactivity was seen in the cytoplasm of those cells but not in their nuclei. At day 15, glucokinase immunoreactivity was observed mainly in the nuclei (Fig. 3D). At day 21, a dramatic increase in immunoreactivity was found in the nuclei of hepatocytes, especially in those in the perivenous zone (Fig. 3E). At day 30, a strong immunoreactivity was detected in the nuclei of perivenous and periportal hepatocytes, being slightly more intense in the former cells (Fig. 3F). The intensity of the immunostaining was similar to that seen in adult rats.

4. Discussion

The specificity of the reaction has to be evaluated very carefully in immunohistochemical studies. One of the most critical points in performing immunohistochemistry is to block non-immunological binding of the antibody to tissue components (i.e. non-specific staining). In this study, we found that non-specific staining was blocked almost completely by incubation of sections with a solution containing 2% non-fat milk and 5% goat serum.

The present data clearly demonstrated that (i) the change in glucokinase immunoreactivity in rat liver during postnatal development shows a close correlation with that of hepatic glucokinase protein levels and with that of glucokinase activity, (ii) these changes also parallel the change in hepatic glucokinase mRNA during postnatal development [7], (iii) glucokinase immunostaining is present in the cytoplasm of hepatocytes until day 11 post-partum and mainly in the nuclei from day 15 onwards, and (iv) the increase in glucokinase immunoreactivity during the weaning period is first observable in the perivenous zone and then extends to the periportal zone.

Our results indicated that a preference of glucokinase distribution for the periportal zone was not detected in the neonatal rat liver in contrast to the finding of Kirchner et al. [15]. In their study, high amounts of brown-color deposits of peroxidase reaction products were observed in sections of livers of rats younger than 15 days of age, whereas the glucokinase protein level was very low in livers of such neonatal rats. These discrepancies may be due to method-dependent differences; for instance, endogenous peroxidase activity was not blocked in their study. In addition, they did not treat sections with a protease such as pronase E; this might have caused the use of a higher amount of anti-glucokinase antibodies, which could have resulted in an increase in non-specific staining.

From the age of about 13 days, young rats begin to consume some of the solid food provided for the mother rat [18]. The appearance of glucokinase in hepatocytes coincides with the start of weaning, which is characterized by a nutritional shift from high-fat milk provided by the mother rat to a carbohydrate-rich diet supplied as laboratory food pellets [2,13,14]. Insulin acts as a triggering factor for the developmental induction of hepatic glucokinase [2,5–8]. In this study, we found that the preference of glucokinase localization for the cytoplasm in hepatocytes of rats of 3–11 days of age shifted to a preference for the nucleus in rats older than 15 days of age. In addition, a similar preference of immunostaining for the cytoplasm is observed in sections of 72 h fasted rat liver [11] and of streptozotocin-diabetic rat liver (data not shown). Refeeding of glucose to starved rats [11] and injection of insulin into diabetic rats (data not shown) restored glucokinase activity, glucokinase protein levels, and nuclear localization. Taken together, these results may suggest that insulin regulates glucokinase translocation in hepatocytes as well as hepatic glucokinase induction. Furthermore, it seems likely that impairment of glucokinase translocation induced by insulin deficiency and/or insulin resistance may be associated with hyperglycemia in diabetes.

It has been reported that the development of zonal distribution of gluconeogenic enzymes, such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, are completed around day 12 after birth [9]. On the other hand, our results clearly indicated that the zonal distribution of glucokinase, a glycolytic enzyme, develops during the weaning period.

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