Nuclear Localization of Bovine Immunoglobulin G (bIgG) in the Liver of Rats Injected with Hydrocortisone-bIgG Conjugate Intravenously

By
Toshikazu NISHIMURA and Takashi NAKANO

Department of Anatomy, Aichi Medical University School of Medicine, Yazako, Nagakute-cho, Aichi-gun, Aichi-ken 480-1195, Japan

Received for Publication, May 15, 2001

Keywords: Hydrocortisone-bovine IgG conjugate, Rat, Liver, Target cell nuclei, Immunohistochemistry

Summary: Bovine serum albumin conjugated with steroid hormone injected intravenously into rats can enter the hormone-target cell nuclei with its antigenicity kept intact (Nishimura and Nakano, 2000). Antinuclear antibodies (ANA) such as immunoglobulin G (IgG) are found in some autoimmune diseases. To confirm immunocytochemically whether immunoglobulin G (IgG) conjugated with steroid hormone enters in the hormone-target cell nuclei, bovine IgG (bIgG) was conjugated with hydrocortisone 21-hemisuccinate. The hydrocortisone-bIgG conjugate was injected into the vascular system of adrenalectomized rats. Then the rats were killed 2 or 3 h after injection to detect bovine IgG in the hormone-target cell nuclei of the liver by fluorescein-isothiocyanate labeled-anti bovine IgG antibody under confocal laser scanning microscopy. In the liver of both rats injected with hydrocortisone-bIgG, the fluorescence was observed in not only the cytoplasm but also the nuclei of liver cells. In control rat injected with bIgG, few nuclei showed fluorescence. These results demonstrate that bovine IgG conjugated with steroid hormone can enter the hormone-target cell nuclei of rat with its antigenicity kept intact, and suggest that antinuclear antibody in some autoimmune diseases may be transported from blood plasma into the nucleus.

Antinuclear antibodies (ANA) such as immunoglobulin G (IgG) are found in some autoimmune diseases such as autoimmune hepatitis (Alvarez et al., 1999; Nishioka and Morshed, 1999). IgG injected into the cytoplasm does not enter the nucleus (Lanford et al., 1986). Modified IgG combined with a short amino acid sequence (nuclear localization signal) can enter the nucleus from cytoplasm through the nuclear pore complex (NPC) (Lanford et al., 1986; Nigg, 1997). However, the macromolecules, e.g., IgG, are not likely to pass freely through the cell membrane by passive transport. Accordingly, it is unclear how ANA enter the nucleus.

Glucocorticoid receptor is detected in the nuclei of isolated hepatocytes, hepatic stellate cells and Kupffer cells of rat (Raddatz et al., 1996). In neutrophil, the immunostaining of the glucocorticoid receptor is also visible in the cytoplasm, not in the segmented nucleus (Miller et al., 1998), as antineutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases (Niles, 1996; Roozendaal et al., 2000). Thus, the distribution of antibodies in autoimmune liver diseases seems to correlate with that of glucocorticoid receptors.

Radiolabeled steroid hormones like [3H]-hydrocortisone, may enter the target cell nuclei in vivo (Beato et al., 1969; Rüstow et al., 1975). In the classic, or genomic model of steroid hormones, free lipophilic nonproteinbound hormones cross the cell membrane under passive transport to bind to the receptor in the cytoplasm, and the hormone-receptor complex is translocated into the target cell nucleus to exert genomic effects (Bamberger et al., 1996; Guiochon-Mantel et al., 1996; Hун et al., 1996; Jensen and DeSombre, 1972; Madan and DeFranco, 1993; Mangelsdorf et al., 1995; Mendel, 1992). In contrast, recently, steroids coupled with high-molecular weight substances including steroid hormone-bovine serum albumin conjugates (steroid-BSAs) are used to study the membrane-initiated...
or nongenomic activities of steroid hormones, because steroids coupled with high-molecular weight substances cannot pass through the cell membrane and do not enter the cytosol. (Gametchu et al., 1999; Revelli et al., 1998; Rosner et al., 1999; Rossato et al., 1999; Watson and Gametchu, 1999; Watson et al., 1999; Wehling, 1997). Furthermore, glucocorticoid receptor immunoreactive sites are associated with the plasma membrane and coated or regular vesicles in hippocampal and hypothalamic neurons of rat (Liposits and Bohn, 1993). Estrogen receptor α tends to be found on the cell membrane of rat pituitary tumor cells (Norfleet et al. 1999). Steroid hormones circulate in blood plasma in three physical states: free, albumin-bound, and steroid hormone-binding proteins such as corticosteroid-binding globulin (CBG) (Kuhn, 1988; Pescovitz et al., 1990; Rosner et al., 1988). CBG is detected within the hormone-target cell nuclei (Kuhn, 1988; Rosner et al., 1988; Selcer and Leavitt, 1988). Pietras and Szego have suggested that endocytotic vesicles serve as vehicles for the nuclear transfer of steroid hormones (Pietras and Szego, 1984; Szego, 1984). Colloidal gold labeled-steroid hormone-BSA conjugates (steroid-BSA-gold) bind to the target cell membrane (Spindler et al., 1991; Moats and Ramirez, 2000; Nishimura and Nakano, 1997) and are taken up by receptor-mediated endocytosis (Moats and Ramirez, 2000; Nishimura and Nakano, 1997). Steroid-BSA-gold bind to nuclear receptors in the sectioned cells (Beppu, 1989; Okuda et al., 1989). Using electron microscope we earlier reported that steroid-BSA-gold entered the hormone-target cell nuclei of the normal rats killed 2 h after the intravenous injection (Nishimura and Ichihara, 1997; Nishimura and Nakano, 1999), and then confirmed that antigenicity of BSA is retained in the nuclei of the hormone-target cells from an immunocytochemical experiment with steroid-BSAs without colloidal gold (Nishimura and Nakano, 2000). Though BSA is likely to be degraded rapidly in the nucleus (Yamaizumi et al., 1978), IgG introduced directly into the nucleus is retained (Lanford et al., 1986). In the present study, we injected bovine IgG conjugated with hydrocortisone (hydrocortisone-bIgG) into rat tail vein, then examined the localization of bIgG in the liver of adrenalectomized rat killed 2 or 3 h after the injection.

**Materials and Methods**

**Materials**

Hydrocortisone 21-hemisuccinate and 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride were purchased from Sigma (St. Louis, MO, USA), and bovine immunoglobulin G from Biogenesis (Poole, England). Fluorescein-isothiocyanate (FITC)-labeled rabbit anti-bovine IgG antibody (Anti-bIgG antibody-FITC) was purchased from Inter-Cell Technologies, Inc. (Hopewell, NJ, USA).

**Conjugation of Bovine IgG with Hydrocortisone 21-Hemisuccinate**

Bovine IgG (bIgG) was conjugated with hydrocortisone 21-hemisuccinate by a slight modification of the method described in the conjugation of α-ecdysone 22-hemisuccinate to thyroglobulin by Chang and O’Connor (1979). Briefly, 16-mg hydrocortisone 21-hemisuccinate dissolved in 600 μl of pyridine-water (1:1) was mixed with 103-mg 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride at 4°C. Then, 20-mg bIgG dissolved in 500 μl H2O was added to the mixture. The reaction mixture was allowed to stand overnight at room temperature, dialyzed with 300 ml H2O for 30 min. and repeated six times, and then with 30% aqueous pyridine (300 ml) for over 2 h, repeated ten times, and lyophilized. The reaction product, hydrocortisone-βIgG, was stored at 4°C until use.

**Pretreatment of Antibody**

Anti-bIgG antibody-FITC of 0.5 ml was stirred with 20 mg of normal rat liver-powder for 1 h at freezing temperature, and centrifuged described previously (Nishimura and Nakano, 2000). The supernatant was used as the antibody.

**Administration of Hydrocortisone-bIgG or bIgG to Rats**

Three adrenalectomized rats (Wistar strain, 10-week-old males) were injected with 1 ml of reaction mixture (5 mg/ml) or bovine IgG (5 mg/ml) dissolved in saline containing 5% glycerol. After 2 or 3 h, the rats were perfused, fixed, frozen and sliced as described previously (Nishimura and Nakano, 2000).

**Control Experiment**

The control rats, which received no injections, were fixed by perfusion as above. The liver was removed, cut into pieces, fixed, and sectioned as above.

**Immunofluorescence Staining**

The tissue sections were fixed with methanol for 20 min, dried, and washed with Dulbecco’s phosphate-buffered saline (PBS−) for 5 min. Sections were reacted with normal rabbit serum for
30 min, then with the antibody for 2 h at room temperature, washed with PBS\(^-\), stained with 0.01% Evans blue as described previously (Nishimura and Nakano, 2000), and observed by differential interference contrast and immunofluorescence using a confocal laser scanning microscope (LSM-GB200: Olympus, Japan).

**Results**

In the liver of rat sacrificed 2 h after the injection of hydrocortisone-boIgG, fluorescence implying the presence of boIgG was observed on the sinusoidal wall. In many cells showing high interference contrast under differential interference contrast microscope, the fluorescence was localized not only in the cytoplasm but also the nuclei of several cells (Fig. 1a). In some cells showing low interference contrast at the perisinusoidal site, the fluorescence was also observed in the nucleus (Fig. 1b). In what seemed to be Kupffer cells in the sinusoid, fluorescence was observed to be intense in the cytoplasm and faint in the nucleus. However, the fluorescence in the nucleus seemed to be diffuse from the cytoplasm (Fig. 1c).

In the liver of control rat sacrificed 2 h after injection of boIgG, the fluorescence was found in the cytoplasm of cells showing high interference contrast. In almost all cell nuclei, no fluorescence was localized (Fig. 1d). The fluorescence in the cells appearing to be Kupffer cells was similar to that of experimental rat (data not shown).

In the liver of rat sacrificed 3 h after injection of hydrocortisone-boIgG, the fluorescence was also observed in the vesicles and the nuclei of many cells showing high interference contrast (Fig. 2a, b), and in the nucleus of the cells showing low interference contrast in perisinusoidal site (Fig. 2c). The immunostaining in the nuclei of the cell showing high interference contrast displayed two patterns; in one, the fluorescence was diffuse in the nucleus (Fig. 2a), while the other had a new moon-like shape along the nucleoplasmic side of the nuclear envelope (Fig. 2b). Some vesicles were located close to the nucleus (Fig. 2a). The fluorescence pattern in what seemed to be Kupffer cells was similar to that of rat killed 2 h after injection (data not shown). The antibody did not react with the liver section from normal rats that had not been injected (Fig. 2d). In the cells showing high interference in experimental rats, fluorescence was also seen one part of the nuclear periphery (Fig. 3a).

In the cell with segmented nuclei, typical of neutrophils, the fluorescence was seen in the cytoplasm, but not in the nucleus (Fig. 3b). In rats injected with hydrocortisone-boIgG or boIgG, the values were 3.31 ± 1.53 in the rat killed 2 h after injection, and 8.51 ± 0.38 in 3 h, against 0.26 ± 0.21 in the IgG injected-rat (Table 1).

**Discussion**

The present study showed that hydrocortisone-boIgG injected intravenously into rat came to be located in the liver cell nuclei and the cytoplasm of neutrophil, as ANA and ANCA in autoimmune liver diseases (Alvarez et al., 1999; Nishioka and Morshed, 1999; Niles, 1996; Roozendaal et al., 2000). The result suggests the possibility that the distribution of antibodies in autoimmune liver diseases correlates with that of glucocorticoid receptors. Our investigation also revealed a higher percentage of nuclear immunostaining of liver cells of rat killed 3 h after the injection than at 2 h post-injection; a result is supported by the report that IgG is retained in the nucleus (Lanford et al., 1986).

Serum albumin injected intravenously persists in the bloodstream of different animals for prolonged periods of time (Long et al., 1963; Owen et al., 1975). Few BSA injected intravenously into mice were taken up by the nonparenchymal liver cells.

<table>
<thead>
<tr>
<th>Injected Molecule</th>
<th>Time after Injection (Hour)</th>
<th>Total Number of Nuclei*</th>
<th>Number of Nuclei Showing Fluorescence (% of Total ± SD)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone-boIgG</td>
<td>2</td>
<td>1783</td>
<td>59 (3.31 ± 1.53)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1880</td>
<td>160 (8.51 ± 0.38)</td>
</tr>
<tr>
<td>Bovine IgG</td>
<td>2</td>
<td>1936</td>
<td>5 (0.26 ± 0.21)</td>
</tr>
</tbody>
</table>

* Total of nuclei counted in single image under LSM-GB200.
** Values are means ± standard deviation.
In autoimmune liver diseases, ANA are basically directed to components within the cellular nucleus such as DNA, RNA, or proteins (Nishioka and Morshed, 1999). The fluorescent staining of ANA exhibits various patterns; homogenous, nuclear dots, rim, etc. (Nishioka and Morshed, 1999; Strassburg and Manns, 1999). This study showed several immunostaining patterns of bIgG in the nuclei, but it is unclear whether or not bIgG binds to the components within the nucleus.

The result in control rat is supported by the report that isolated hepatocytes take up monomeric IgG of different animals by receptor-mediated endocytosis (Sancho et al., 1984). Serum proteins can be taken up by fluid-phase or receptor-mediated endocytosis by hepatocytes, and then recycled back to the basolateral membrane, degraded in lysosomes, or directed to bile (Crawford, 1996). However, ANA are detected in autoimmune liver diseases (Alvarez et al., 1999; Nishioka and Nakano, 1999).

We have considered that the existence of ANA suggests vesicular trafficking from the extracellular milieu into the cell nuclei, and some mechanism that disturbs the digestion by lysosomal enzymes. Simian virus 40 (SV40) is taken up by endocytosis and enters the nucleus with intact virion 2 h after infection (Cole, 1996; Hummeler et al., 1970). We earlier indicated the possibility that the membrane of SV40-containing vesicle fuses with a single bilayer diaphragm in the nuclear envelope to transport virus particles within the nucleoplasm, and reported that cell membrane markers such as concanavalin A, taken up together with SV40, entered the nucleus (Nishiura et al., 1991). Androgen-binding protein or sex steroid-binding protein coupled with testosterone was observed in the target cell nuclei (Gerard et al., 1991; Gerard, 1995; Gerard et al., 1994). We suggested that gold-labeled testosterone-BSA conjugate (testosterone-BSA-gold) injected into rat was endocytoxed by the hormone-target cells and entered the nucleoplasm through the nuclear double membranes by vesicular trafficking, and not by NPC, as SV40 did (Nishimura and Nakano, 1997). In the liver of mouse injected with human IgG intravenously, the IgG is stored in the form of droplets in the cytoplasm of hepatocytes up to 2 h after injection, and after this time no immunofluorescent material implying human IgG is demonstrated in the hepatocytes (Mitrenga et al., 1975). The present study showed in experimental rats that some vesicle containing bIgG was localized close to the nucleus 3 h after the injection. The result suggests that hydrocortisone-bIgG would be transported to the nucleus by the vesicle, as SV40 and testosterone-BSA-gold did (Nishimura and Nakano, 1997; Nishimura et al., 1991), and that there is some mechanism that disturbs the digestion by lysosomal enzymes, and is supported by the reports that both polypeptide growth factors such as growth hormone and their receptors are transported to the nucleus via the endosomal pathway (Keresztes and Boonstra, 1999; Lobie et al., 1994).

In conclusion, the present study demonstrates that hydrocortisone-bIgG enters the liver cell nuclei with the antigenicity of bIgG intact. This finding confirms that steroid hormones act as carriers of foreign proteins from the extracellular milieu into the target cell nuclei, and indicates the possibility that the distributions of ANA and ANCA in autoimmune liver diseases correlate with that of glucocorticoid receptors.
Acknowledgements

We thank Mr. M. Miyake and Y. Nakagomi for technical advice regarding photographs.

References


41) Owen NC, Immelman A and Grib D. The elimination of albumin, polyvinylpyrrolidone and dextran from the cir-

Fig. 1. Liver of rat sacrificed 2 h after injection of hydrocortisone-bIgG (a–c) or bIgG (d). Immunostaining is observed in nuclei (arrows) and cytoplasm of cells showing high interference contrast (a), and nucleus (arrow) of cell showing low interference contrast (b). (c) The very strong fluorescence of the cytoplasm obscures the boundary between nucleus and cytoplasm of cell appearing to be Kupffer cells. (d) Immunostaining of bIgG is found in cytoplasm of cells showing high interference contrast. No fluorescence is localized in the cell nuclei. Bars = 10 μm.
Nuclear Localization of Hydrocortisone-Bovine IgG in Rat Liver


49) Rossato M, Nogara A, Merico M, Ferlin A and Foresta C.

Fig. 2. Liver of rat sacrificed 3 h after injection of hydrocortisone-IgG (a–c), and normal rat liver (d). (a) (b) Immunostaining of cells showing high interference contrast. (a) Fluorescence (large arrow) is diffuse in the nucleus. A vesicle (small arrow) is localized close to the nucleus. (b) Fluorescence (arrow) is found in the new moon type along the nucleoplasmic side of the nuclear envelope. Immunostaining (arrow) is found in the nucleus of cell showing low interference contrast (c). No immunostaining is found (d). Bars = 10 μm.


54) Strassburg CP and Manns MP. Antinuclear antibody (ANA) patterns in hepatic and extrahepatic autoimmune disease. J Hepatol 1999; 31:751.


Fig. 3. Liver of rat sacrificed 3 h after injection of hydrocortisone-bIgG. (a) In the cell showing high interference contrast, immunostaining is seen one part of the nuclear periphery (arrow). (b) In the cell with the segmented nuclei (arrow), immunostaining is seen in the cytoplasm, but not in the nucleus (b). Bars = 10 μm.