Immunohistochemical Localization of Inhibin in Porcine and Bovine Ovaries

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Summary. The immunohistochemical localization of inhibin in porcine and bovine ovaries was studied, using monoclonal antibodies to bovine follicular fluid 32K inhibin (bFF 32K inhibin) and a polyclonal antiserum to porcine follicular fluid 32K inhibin (pFF 32K inhibin). In order to obtain a precise immunohistochemical staining, various fixations were tested with an immunoblotting procedure. Acetone fixation followed by celloidin embedding proved to be most appropriate for the immunohistochemical study of inhibin. A consistent pattern emerged in all sections prepared from porcine and bovine ovaries. Granulosa cells were specifically stained by the antibodies, and especially the cells constituting the inner layer were more intensely stained than the cells close to the theca.

The existence of ovarian inhibin has been demonstrated since 1976 (De Jong and Sharpe, 1976), but it had not been successfully purified until 1985, when we isolated and characterized ovarian inhibin from porcine and bovine follicular fluids (MIYAMOTO et al., 1985; FUKUDA et al., 1986). In earlier reports, we demonstrated that two polypeptide subunits of 20K and 13K daltons are linked by disulfide bridges to form an inhibin molecule with an apparent molecular weight of 32K daltons. In regard to the localization of inhibin, Erickson and Hsueh (1978) reported that inhibin is produced by cultured rat granulosa cells. Immunohistochemical demonstration of inhibin-containing cells, however, had been hampered by a lack of applicable antibodies for immunohistochemistry of inhibin, due to the unavailability of pure inhibin preparations as the antigen. Recently, we have prepared monoclonal antibodies to bovine follicular fluid 32K inhibin (MIYAMOTO et al., 1986) as well as polyclonal antibodies to porcine follicular fluid 32K inhibin (HASEGAWA et al., 1986). Using these antibodies we have here conducted an immunohistochemical study of inhibin localization in bovine and porcine follicles.

MATERIALS AND METHODS

Preparation of tissue samples

Bovine or porcine ovaries were obtained from a local slaughterhouse. These ovaries were collected immediately after slaughter and were dipped in acetone for several hours.
After the primary acetone fixation, the ovaries were cut into halves and further immersed in fresh acetone, which was thrice renewed at one hour intervals. The tissue samples were then kept standing in acetone overnight. The well dehydrated specimens were then embedded in celloidin in the following way: The specimens were transferred to a methanol solution containing 2% celloidin (Hōkoku Chemicals Co. Ltd., Japan), in which they were incubated for 24 hr; they were then transferred to a 4% celloidin-methanol solution and further incubated for 2 days. Finally the celloidin solution was changed to an 8% solution and the tissue specimens were incubated for another 4 days. The 8% celloidin methanol solution was then gradually concentrated over 3-7 days by spontaneous evaporation of methanol to about a 15% celloidin concentration. The tissue samples were then immersed in a large excess of 70% ethanol solution. After incubation at 4°C for a few days, the tissue blocks, in which celloidin was uniformly solidified, were sectioned at 30 μm. Thereafter, sections were placed in 70% ethanol and kept for another several days at 4°C. The removal of celloidin from the sections was carefully performed in the following way: celloidin sections were carefully rinsed with ethanol three times and then immersed in an ether-ethanol solution (ether: ethanol = 1:1 v/v) for 10 min. Most of the solidified celloidin was removed by the ether-ethanol treatment. The sections were then dipped in a 96% methanol solution for 10 min and transferred to an 80% ethanol solution and incubated for 10 min. The sections were then placed onto glass slides pre-coated with a 1% gelatin solution. The glass slides were then completely dried after standing at room temperature for a few hours. They were thoroughly washed with water for 30 min and then air dried. The glass slides with sections thus prepared were used for further experiments.

**Immunohistochemistry**

**Antibodies.** Two monoclonal antibodies to bovine follicular fluid 32K inhibin, named 256H and 44H, were used for the present study. Both antibodies recognized intact bFF 32K inhibin as well as its larger precursors. A recognition site of 256H is localized in the 20K subunit of bFF 32K inhibin, whereas 44H has a major recognition site on the shorter 13K polypeptide (MIYAMOTO et al., 1986). The antibody 256H is quite specific for bovine inhibin, whereas 44H also recognizes porcine or rat inhibin as well.

A polyclonal antiserum to porcine follicular fluid 32K inhibin (pFF 32K inhibin) has been raised in a rabbit. Characteristics of the antiserum have been described in detail in an earlier report (HASEGAWA et al., 1986). Briefly, the antiserum could bind not only porcine inhibin but also bovine, rat and human inhibins, as determined by immunoneutralization tests and RIA.

**Immunostaining.** Immunostaining was performed by an indirect method (STERNBERGER et al., 1970) or a PAP method (NAKANE and PIERCE, 1967). Briefly, in the indirect method, the sections on the slides, from which celloidin had been removed, were incubated in 1% normal horse serum for 2 hr. After washing, the sections were incubated with the monoclonal antibodies (1:100 or 1:200 dilution) or normal mouse serum (1%) overnight. After washing, the sections were reacted with a complex of anti-mouse IgG and horseradish peroxidase (Bio Rad) for 4 hr. The sections were finally stained with DAB. In the PAP method, the polyclonal antiserum of 1:2000 dilution was used as the primary antibody.

**Absorption of tissue staining**
In the absorption test, 0.6 ml of purified porcine follicular fluid inhibin (80 μg/ml) was
mixed with 0.2 ml of the polyclonal antiserum to pFF 32K inhibin (1:500 dilution) or with 0.2 ml of the monoclonal antibody 44H (1:500 dilution) and incubated for 1 hr at 37°C. After the incubation, the mixture was centrifuged at 3000 rpm for 10 min, and the supernatant then subjected to the PAP procedure.

**Immunoblotting**

In order to verify whether or not fixatives alter the immunogenecity of inhibin molecules, we carried out following test using an immunoblotting procedure. Serially diluted bovine follicular fluid inhibin (20 µg/ml for x 1) was spotted onto nitrocellulose films. The nitrocellulose films were blocked first with 3% BSA for 1 hr and then with 10% horse serum for another 1 hr. After washing with Tris-HCl buffer saline, pH 7.5 (3 rinses, 10 min each), the films were incubated with different fixatives, i.e., 10% formaldehyde, Bouin solution and Bouin-Hollande solution (Roméis, 1968), or PBS for 24 hr. Thereafter, the films were washed thoroughly with Tris-HCl buffer saline, pH 7.5 (TBS), containing 0.05% Tween 20 (3 rinses, 10 min each) and then with TBS. The films were then subjected to immunoblotting. Immunoblotting was done by the Towbin et al. method (1979). Incubation with primary antibody, 44H, was carried out at a dilution of 1:500. Affinity-purified goat anti-mouse IgG-conjugate to HRP (Bio Rad) was used at a dilution of 1:500.

**RESULTS**

In order to select an appropriate fixative, an immunoblotting procedure was used. As shown in Figure 1, inhibin spots on nitrocellulose films which were incubated with Bouin solution, Bouin-Hollande solution or 10% formaldehyde solution were faintly stained, whereas the control inhibin spots were clearly visualized by the immunoblotting. This fact indicates that these fixatives are not suitable for inhibin immunohistochemistry. Actually, we were unable to obtain sufficient immunostaining for inhibin when using sections from ovaries fixed with these fixatives (data not shown). In this study we have chosen acetone fixation, for the reason that inhibin can be precipitated by treatment with acetone without any loss of its biological activity (Baker et al., 1982). We also have used celloidin embedding, because nitrocellulose tightly binds inhibin without loss of its immunogenecity, as shown in the control in Figure 1 and our earlier report (Miyamoto et al., 1986).

**Immunohistochemical staining with monoclonal antibodies**

Ovarian sections which were fixed and embedded by the methods above mentioned were stained with monoclonal antibodies, 256H and 44H. As shown in Figures 2A and B, both antibodies 44H and 256H could stain granulosa cells of the antral follicles in bovine ovaries. On the other hand, only 44H could stain granulosa cells of the antral follicles in a porcine ovary (Fig. 2C, D), but 256H provided little, if any, specific staining for inhibin (Fig. 2E) when used with a porcine ovary. These results can be explained by the differences in specificity of these antibodies, i.e., 44H recognizes not only bovine inhibin but also porcine, human and rat inhibin. As shown in Figures 2A, B, C and D, the inner granulosa cell layer was strongly stained in all cases. This suggests that inhibin exists at a higher concentration in the inner granulosa cells than in the cells close to the theca.

Granulosa cells in the primary or secondary follicles were not stained in this study.
Immunohistochemical staining with a polyclonal antibody

Figure 3 shows the results of immunostaining of bovine follicles with the polyclonal antiserum to pFF 32K inhibin. Though the antiserum was raised against pFF 32K inhibin, it also recognizes bovine inhibin, as previously reported (HASEGAWA et al., 1986). Granulosa cells in bovine antral follicles were stained with the antiserum as shown in Figures 3A and B. Figure 3B shows a positive staining of bovine follicular fluid that was firmly preserved with acetone and celloidin treatment. Absorption of the antiserum with purified pFF 32K inhibin prohibits the specific staining as shown in Figure 3C.

Figure 4 also shows immunostaining features of porcine follicles with the above described antiserum. Granulosa cells in porcine antral follicles were also stained with the antiserum as shown in Figure 4A. Specific staining of the granulosa cells also disappeared after absorption of the antiserum (Fig. 4B). Granulosa cells constituting the inner layer were more intensely stained than the cells close to the theca.

Granulosa cells in the primary or secondary follicles were not stained in this study.

DISCUSSION

We first tried several different methods of fixation, such as 5% acrolein, Bouin, Bouin-Hollande and 10% formaldehyde. When using ovaries fixed with these fixatives, granulosa cells were indeed faintly stained, but the features were insufficient and relatively inconsistent (data not shown). Actually the immunoblotting test revealed that the immunogenecity of inhibin molecules suffered some damage caused by these fixatives containing aldehyde compounds (Fig. 1). Therefore, immunoblotting may be valuable in selecting appropriate fixatives for studying immunohistochemical localization of proteins of interest.
We next tried a frozen section method without chemical fixation. With this, however, only faint specific staining was obtained with a high background reaction in these sections (data not shown), probably because inhibin molecules in these sections leaked out during the fixation procedure.

Based upon these findings, together with the fact that a nitrocellulose film tightly binds inhibin molecules that can be easily immunostained as shown in Figure 1, a combination of acetone fixation and methanol celloidin embedding was used in the present series of experiments. Throughout the fixation and embedding procedures, inhibin molecules must remain in an insolubilized form without suffering molecular damage, because inhibin can be precipitated by these organic solvents without loss of its biological activity (Baker et al., 1982). As shown in all figures, relatively clear immuno-
staining features were obtained by acetone fixation and celloidin embedding. This method may be useful for the immunohistochemistry of soluble proteins of which antigenecities are susceptible to commonly used fixatives.

As we previously reported, porcine or bovine follicular fluid 32K inhibin do consist of two polypeptide subunits of 30K and 13K daltons (MIYAMOTO et al., 1985; FUKUDA et al., 1986). Studies with cDNA revealed that the 13K subunits of bovine, porcine and human inhibin are identical to each other in amino acid sequence, while the 20K sub-

Fig. 3. Immunostaining of inhibin by the PAP method using a polyclonal antibody (1:2000 dilution). A and B. Sections are from bovine ovaries. The follicular fluid is also stained (B). A: ×150, B: ×30. C. A section of the bovine ovary immunostained with the antibody after incubation with pFF 32K inhibin (Absorption test). ×190. GC granulosa cell, FF follicular fluid.

Fig. 4. Immunostaining of inhibin by the PAP method using a polyclonal antibody (1:2000 dilution). A. A section from a porcine ovary. B. A section of porcine ovary immunostained with the antibody after incubation with 32K inhibin (Absorption test). A, B: ×170. GC granulosa cell.
units of bovine and porcine inhibin are slightly different from each other (MASON et al., 1985; FORAGE et al., 1986). As was also previously reported, the monoclonal antibody 44H recognizes the common 13K subunit (MIYAMOTO et al., 1986). Therefore 44H recognizes not only bovine inhibin, but also porcine or human inhibins as well. On the other hand, a recognition site for 256H is located in the 20K subunit of bovine inhibin (MIYAMOTO et al., 1986), and the antibody can not bind 125I-labelled porcine inhibin (data not shown). Therefore, as shown in Figure 2, both bovine and porcine ovaries clearly showed specific immunostaining when using 44H, while only the bovine ovary gave a specific staining feature when using 256H. Studies with cDNA have also revealed that the 20K and the 13K subunits are encoded in different mDNA's (MASON et al., 1985; FORAGE et al., 1986). The fact that two antibodies directed to different subunits gave a consistent staining feature suggests that these subunits coexist in the same cells. Recently it has been reported that the 13K subunit dimer exists in porcine follicular fluid (VALE et al., 1986; LING et al., 1986). At present it is not possible to describe the localization of such a subunit dimer in the ovarian cells without more quantitative data, though the staining feature obtained by using the 13K subunit specific antibody, 44H, was limited to granulosa cells.

The polyclonal antiserum exclusively recognizes the common 13K subunit, and an immunoblotting study revealed that the antiserum could stain the 13K subunit as well as both intact inhibin molecules from porcine and bovine species (data not shown). Therefore, both bovine and porcine follicles were specifically immunostained with the polyclonal antiserum. The specific staining features could be neutralized by pretreatment of the antiserum with an excess amount of purified porcine inhibin, indicating the specificity of the staining results. When using the monoclonal antibodies, the results of such an absorption test were not so apparent (data not shown), probably because of very low affinity constants of these antibodies to inhibin (Ka=10^{-6}-10^{-7}), making it difficult to saturate these antibodies with the antigen.

In the present study, granulosa cells in the primary or secondary follicles were not stained. However this does not necessarily mean that the granulosa cells in these follicles do not contain inhibin, since we observed that granulosa cells from primary or secondary follicles could release immunoreactive inhibin in vitro, though the amount was much lower than from the granulosa cells in the antral follicles (unpublished data).

As shown in all figures, granulosa cells in the inner part of the follicle were intensely stained. Although the inner part of the granulosa cell was always strongly stained, there were two types in the staining pattern. The granulosa cells may be stained with gradually increasing intensity toward the inner part (Fig. 2A), whereas they may also be stained as though the inner part was exclusively stained to form a distinctive cell layer with regard to the distribution of inhibin (Fig. 2B). These results indicate the presence of subpopulations of granulosa cells according to their different positions in the polarity of the tissue. Definitive evidence of heterogeneity appearing with the developmental stages of granulosa cells has been obtained for aromatase (ZOLLER and WEISZ, 1979), LH receptor (BORTOLUSSI et al., 1979), prolactin (DUNAIF et al., 1982) and cyclic nucleotide (DAIL et al., 1980). The staining features obtained by the present study are similar to those of the prolactin receptor (BORTOLUSSI et al., 1979) and cAMP (DUNAIF et al., 1982), of which developments were also restricted primarily to the innermost granulosa cells. At present it is not clear whether the asymmetrical differentiation of granulosa cells in terms of inhibin localization has a physiological significance, though it is evident that granulosa cells are really a heterogeneous system.
In conclusion, our present results demonstrate for the first time the tissue localization of inhibin predominant in the inner part of granulosa cell layer for both porcine and bovine follicles.

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