THE PATTERN OF MICROTUBULES DURING SPERMATOGENESIS:
A COMPARATIVE IMMUNOHISTOCHEMICAL STUDY
ON THE DISTRIBUTION OF TUBULIN*

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During spermatogenesis, a great number of microtubules temporarily appear around the nucleus of the spermatid at the time the nucleus undergoes transfiguration. Therefore, the transfiguration of the nucleus is considered to be closely related to the distribution of the microtubules.

In this paper, the distribution pattern of microtubules in the spermatid of the rodent, which has an asymmetrical fishhook-shaped nucleus, was compared with that of microtubules in the spermatid of the dog, which has the symmetrical ovoid nucleus.

The results indicate that the microtubules in rodents were localized only on the concave side of the nucleus, while those in the dog spermatid were distributed evenly on the posterior half of the nucleus.

Microtubules in cytoplasm are considered to play an important role in the morphogenesis of cell and tissue. During spermatogenesis, the nucleus of the spermatid changes its form from round to narrow piliform, which is specific to sperm. Synchronized with this transfiguration of the nucleus, a great number of microtubules temporarily appear around the nucleus (3, 4). These observations suggest that the appearance of the microtubules is closely related to the transfiguration of the nucleus.

Meanwhile, the shape of the sperm nucleus varies from species to species. For example, in rodents, such as the mouse and rat, the sperm has a nucleus with an asymmetrical configuration, which looks like a fish-hook, while the dog sperm has a symmetrical ovoid nucleus. If the microtubules relate to the transfiguration of the nucleus in spermatogenesis, there must be some differences between the distribution pattern of microtubules in the sperm of rodents and dogs.

On the other hand, microtubules are thought to be composed of molecules of tubulin, and, in fact, the reconstructed microtubules with the molecules of tubulin *in vitro* have the same properties as those of the microtubules which are observed *in vivo* (5, 8). On that ground, the localization of microtubules is expected to be recognizable by staining tubulin protein by labeled anti-tubulin antibody.

In this paper, the distribution of microtubules in the spermatid of the rodent

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was compared with that of the dog, during spermatogenesis, by staining microtubules with fluorescein-labeled anti-tubulin antibody.

MATERIALS AND METHODS

Preparation of the antigen:

The tubulin was purified by the method as described by Shelanski *et al.* (5). The pig brain was homogenized in the reassembly buffer of Weisenberg (8) (0.1 M MES, 1 mM EDTA, 1 mM GTP, 0.5 mM MgCl_2 , pH 6.4), and the homogenate was centrifuged at $100,000 \times g$ for 1 hr. The equal volume of reassembly buffer containing 8 M glycerol was added to the supernatant, and the mixture was incubated at 37°C for 30 min. The reassembled tubulin was collected by centrifugation at $100,000 \times g$ for 1 hr and was disassembled in the cold reassembly buffer at 0°C for 1 hr. These procedures were repeated three times. The reassembled tubulin finally obtained by the above process was treated with a mixture of 1% sodium dodecyl sulfate (SDS) and 1% β -mercaptoethanol for 3 min at 100°C . The dissolved tubulin was applied to a Sephadex G-100 column, preequilibrated with phosphate buffered saline (PBS) containing 0.1% SDS. The elution was performed with the same solution. The fractions containing proteins were analyzed by the SDS acrylamide gel electrophoresis by the method described by Weber and Osborn (6). The molecular weight of the protein was estimated from the mobility in the SDS-gel. Egg albumin and bovine serum albumin were used for the standard proteins for the determination of molecular weight.

Preparation of anti-tubulin serum:

The second peak obtained by Sephadex G-100 column chromatography contained the tubulin monomer (see results). The protein (about 1 mg) of this fraction was mixed with an equal volume of Freund's complete adjuvant and was injected into the rabbits subcutaneously. The rabbits received the booster injection 40 days after the first injection. The antiserum was harvested from the ear vein 7 days after the booster injection and stored at -20°C until use. It was then diluted 1 : 10 with PBS and was used in the immunohistochemical staining as an anti-tubulin antibody.

Preparation of FITC-labeled anti-rabbit IgG goat γ -globulin:

The goat γ -globulin against rabbit IgG was conjugated with fluorescein isothiocyanate (FITC) at 4°C under continuous stirring for 6 hr, and the conjugate was purified by the method described by McDevitt *et al.* (2).

Preparation of the cells:

The testes of mouse (ddy strain, about 30 grams in weight), rat (Wistar strain, about 400 grams in weight) and dog (about 10 kilograms in weight) were minced in 1% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.2). The minced testis of each kind of animal was suspended in the same fixative and fixed for another 15 min at room temperature. After the tissue debris was removed by filtration through gauze, the suspension was centrifuged. Then, the precipitate was repeatedly washed with 0.01 M phosphate buffer, pH 7.2, containing 0.9% NaCl (PBS). Finally, the precipitate of the last washing was resuspended in a small volume of

PBS, placed on the albuminized slide glasses and air-dried at 37°C. After immersing in PBS, the slides bearing cell suspension were treated with 0.05% Nonidet P-40 for 15 min, washed several times with PBS and incubated in the 1 : 10 diluted anti-tubulin serum for 30 min. After washing well with PBS, the specimens were stained with FITC-labeled anti-rabbit IgG goat γ -globulin for 30 min. After washing with PBS, the specimens were mounted with glycerin and observed by fluorescent microscopy.

RESULTS

Specificity of anti-tubulin antiserum:

The assembled tubulin, prepared by the method of Shelanski *et al.* (5), was dissolved in the solution containing 1% SDS and 1% β -mercaptoethanol, and was applied on the column of Sephadex G-100 which was preequilibrated with PBS containing 0.1% SDS. The elution pattern gave two peaks as shown in Fig. 1. The analysis of the contents in the second peak, by the SDS acrylamide gel electrophoresis, showed a single band of protein (Fig. 2). The molecular weight of this protein was estimated about 52,000 by comparing the mobility of the protein to that of the bovine serum albumin and of the egg albumin in the SDS gel. Thus, the protein of the second peak is considered to be pure tubulin monomer. The antiserum prepared against tubulin monomer specifically reacted with the spindle fibers and the cytoplasmic fibers of mouse fibroblast (L-cell) (Figs. 3 and 4). Since these fibers are reported to be composed of tubulin (1, 7), the antiserum prepared in this experiment was considered to be specific to tubulin.

Distribution of tubulin in mouse spermatids:

The mouse spermatids firstly reacted with anti-tubulin serum and were then

FIG. 1. Elution pattern of the reassembled tubulin by Sephadex G-100 column eluted with PBS containing 0.1% SDS. There are two peaks corresponding to the fraction number 23 and 28.

FIG. 2. SDS acrylamide gel electrophoretic pattern of the fraction 28 in Fig. 1. Single band is recognized.

FIG. 5. The mouse spermatid which has a round nucleus. The specific fluorescence is recognized in the symmetrical pattern on the posterior half of the nucleus. $\times 1,400$

FIG. 6. In mouse spermatid in early stage, specific fluorescence is seen slightly deviated on one side of the nucleus. $\times 1,400$

FIGS. 7, 8, 9. The mouse spermatid which has a fishhook-shaped nucleus. The specific fluorescence is located on the concave side of the nucleus. $\times 1,400$

FIG. 10. The mature mouse spermatozoa. No specific fluorescence is observed. $\times 1,400$

FIG. 3. The mouse L-cell in metaphase was treated with antitubulin serum, and then reacted with anti-rabbit IgG goat γ -globulin labeled with FITC. The spindle fibers show intense fluorescence. $\times 1400$

FIG. 4. The mouse L-cell in interphase. The cytoplasmic fibers show intense fluorescence. $\times 700$

treated with FITC-labeled anti-rabbit IgG goat γ -globulin. In the early spermatid, which had a round nucleus and was supposed to be in the cap phase, the specific fluorescence was recognized as a bundle of the fibers around the posterior half of the nucleus. The fibers, which demonstrated strong fluorescence, ran parallel to each other in a direction tangential to the nucleus (Fig. 5). In spermatid in an advanced stage, having an elliptic nucleus, the specific fluorescence tended to appear slightly concentrated on one side of the nucleus (Fig. 6). As spermatogenesis proceeded, the nucleus of the spermatid bent on one side and the asymmetrical pattern was intensified. Strong specific fluorescence was recognized on the concave side of the nucleus, but little fluorescence was seen on the convex side (Figs. 7 and 8). In the late spermatid, the nucleus showed the same shape as that of the mature spermatozoon, and specific fluorescence was recognized only on the concave side of the nucleus (Fig. 9). In the mature spermatozoon, no specific fluorescence was noticeable around the nucleus (Fig. 10).

FIG. 11. The rat spermatid which has a round nucleus. Specific fluorescence is recognized in the symmetrical pattern on the posterior half of the nucleus. $\times 1400$

FIG. 12. The rat spermatid which is in a similar stage to Fig. 11. Specific fluorescence appears to deviated slightly to one side of the nucleus. $\times 1400$

FIG. 13. The rat spermatid which has a fishhook-like nucleus. The concave side of nucleus shows more intense fluorescence than convex side does. $\times 1400$

FIG. 14. The rat spermatid in the late stage. The specific fluorescence is recognized only on the concave side. $\times 1400$

Distribution of tubulin in rat spermatids:

When the rat spermatids were stained with anti-tubulin serum, the early spermatid having a round nucleus showed specific fluorescence around the posterior half of the nucleus, as in the case of mouse spermatid (Fig. 11). Occasionally, there were some spermatids which still had round nuclei but already showed specific fluorescence deviating to one side of the nuclei (Fig. 12). In accordance with the elongation of the nucleus, the sites showing specific fluorescence became restricted to only one side of the nucleus. Then, the nucleus tended to bend toward the area where specific fluorescence was recognized (Figs. 13 and 14). No fluorescence was noticed around the nucleus of the mature spermatozoon.

Distribution of tubulin in dog spermatids:

The spermatid having a round nucleus showed fluorescence on the posterior half of the nucleus. Bundles of fibers ran in a tangential direction, and this figure was of the same pattern as those of the early spermatids of mouse and rat (Figs. 15 and 16). However, in the advanced spermatid, the distribution pattern of the specific fluorescence was quite different from that of the rodent. Namely, in the late spermatid of the dog, which had ovoid- or rod-shaped nucleus, the specific

FIGS. 15, 16. The dog spermatid which has a round nucleus. The symmetric specific fluorescence is recognized on the posterior half of the nucleus. $\times 1400$

FIGS. 17, 18. The dog spermatid which has an ovoid nucleus. The specific fluorescence is seen in a symmetrical pattern on the posterior half of the nucleus. $\times 1400$

fluorescence was recognized symmetrically on the posterior half of the nucleus (Figs. 17 and 18). As described before, the nucleus of the late spermatid of the rodent became elongated, and the specific fluorescence showing the sites of tubulin was recognized on one side of the nucleus.

DISCUSSION

The tubulin prepared by the method described by Shelanski *et al.* (5) contained both tubulin subunit and other high molecular components. These high molecular components were separated from the tubulin subunit by column chromatography on Sephadex G-100 eluted with PBS containing SDS. The SDS-acrylamide gel electrophoretic pattern of the protein in the second peak showed a single band, and the molecular weight was estimated about 52,000. Therefore, the protein of the second peak used as the antigen in this study is believed to be pure tubulin subunit. Moreover, the antiserum prepared against this protein reacted to the spindle fibers and cytoplasmic fibers of the mouse fibroblast (L-cell). Since these components are said to be composed of tubulin subunits (1, 7), the antiserum used in this experiment must be the specific antiserum against tubulin.

By using this anti-tubulin antiserum, the microtubules of the spermatids of mouse, rat and dog were stained immunohistochemically, and the distribution patterns of microtubules during spermatogenesis were compared among them. In the case of mouse and rat, the microtubules were distributed symmetrically around the posterior half of the nucleus in the spermatid of the early stage. However, as the shape of the nucleus changed to the fishhook-shape, the distribution of the microtubules changed to an asymmetrical pattern. In the spermatid of the dog, on the contrary, the microtubules were distributed symmetrically around the posterior half of the nucleus even in the late stage of spermatogenesis. These results suggest that the microtubules in the rodent spermatid may pull the nucleus from one side, and consequently, the nucleus changes to take an asymmetrical fishhook shape. While, in the dog spermatid, as the microtubules surround the nucleus evenly and pull it from all directions, the nucleus keeps the symmetrical shape until the late stages of spermatogenesis.

REFERENCES

1. Fuller, G. M., Brinkley, B. R. and Boughter, J. M.: Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science* 187; 948-950, 1975.
2. McDevitt, H. O., Peters, J. H., Pollard, L. W., Harter, J. G. and Coons, A. H.: Purification and analysis of fluorescein-labeled antisera by column chromatography. *J. Immunol.* 90; 634-642, 1963.
3. McIntosh, J. R. and Porter, K. R.: Microtubules in the spermatids of the domestic fowl. *J. Cell Biol.* 35; 153-173, 1967.
4. Nagano, T.: Observations on the fine structure of the developing spermatid in the domestic chicken. *J. Cell Biol.* 14; 193-205, 1962.
5. Shelanski, M. L., Gaskin, F. and Cantor, C. R.: Microtubule assembly in the absence of added nucleotides. *Proc. Nat. Acad. Sci. (USA)* 70; 765-768, 1973.
6. Weber, K. and Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244; 4406-4412, 1969.

7. Weber, K., Pollack, R. and Bibring, T.: Antibody against tubulin: The specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Nat. Acad. Sci. (USA)* 72; 459-463, 1975.
8. Weisenberg, R. C.: Microtubule formation *in vitro* in solutions containing low calcium concentrations. *Science* 177; 1104-1105, 1972.