Original Article

OBSERVATIONS OF INTERFACE BETWEEN TAIL MICROTUBULES AND OUTER DENSE FIBERS IN HUMAN NECROZOOSPERMIC SPERMATOZOA

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Summary

It is well known that outer dense fibers (ODF) of mammalian spermatozoa are associated with axonemal doublets, but the mechanisms and molecules responsible for this interaction have not been identified yet. We studied naturally degenerating human necrozoospermic spermatozoa in order to find out whether the ODF-microtubule association in these cells is preserved. Electron microscopy revealed consistent binding of ODF to the corresponding microtubules. In some cells ODF had ruptured and the bulk of their material had moved to the periphery. Nevertheless, a small part of it had remained connected to the doublet. Immunogold microscopy for alpha-tubulin showed that, when microtubules of degenerating spermatozoa were absent, gold granules were observed occupying their place, at the inner surface of ODF. This finding suggested that even after complete disintegration of microtubules some of their alpha-tubulin remained attached to the corresponding ODF. To our knowledge, this study has demonstrated for the first time that attachment of ODF to microtubules is more permanent and stable than bonds between components within any of these two cytoskeletal structures, and alpha-tubulin is involved in this attachment. It could be concluded that reliable association between ODF and axonemal doublets is of vital importance for sperm function.

Key words: axoneme, immunoelectron microscopy, spermatozoa, tubulin, ultrastructure

Introduction

Outer dense fibers (ODF) are periaxonemal cytoskeletal elements of mammalian sperm tail contributing to its stability and progressive motility by increasing stiffness [1]. Starting at the neck, a ring of nine ODF encircles the axoneme throughout the middle piece and in the proximal part of the principal piece. During spermiogenesis, they are formed in proximal-distal direction, each in close association with a microtubule doublet [2, 3]. This association has implications for the types of morphological abnormalities observed in cases of sperm pathology. When a doublet is absent, the corresponding ODF is usually also missing. ODF are rarely seen alone [4].

The connections between microtubules and ODF have been addressed by a number of studies. An ODF component, Spag4, also localizes to the

axoneme during sperm development [5]. A major ODF protein - ODF2, has been shown to have affinity for microtubules [6]. Tektin 4, previously thought to be a microtubule component, has been recently localized at the concave surface of ODF [7]. Despite these advances, the mechanisms responsible for ODF – microtubule interaction and the molecules directly involved in it still remain identified. Experimental systems in which sperm tail structure has been disrupted could provide valuable data. For that reason, some investigators have used various chemical dissection protocols [7]. We focused on naturally degenerating necrozoospermic spermatozoa and studied their ultrastructure and reaction with anti-alpha-tubulin antibody to find out whether the ODF – microtubule association in these cells is preserved.

Materials and Methods

Ejaculates were obtained from infertile patients undergoing semen analysis. To select samples for the study, we used an approach similar to that in [8]. We started with a group of ejaculates with absent or severely reduced sperm motility, and then selected a subgroup based on the ultrastructural features of cells. First, we prepared the samples for routine transmission electron microscopy as described in [9]. Five samples, in which high percentage of cells with apparent necrotic degeneration had been seen (from about 70% to over 90%), were selected for further study.

An aliquote of each sample was processed for pre-embedding immunogold electron microscopy. The reaction was carried out as described in [10]. Monoclonal antibody TU-01 (Institute of Molecular Genetics, Prague) against an N-terminal "cryptic" alpha-tubulin epitope [11], diluted 1:100, was used as 1st antibody and anti-mouse IgG-10 nm gold conjugate (Sigma Co.), diluted 1:20, as 2nd antibody. After that, sperm cells were fixed and embedded in the same way as those processed for routine electron microscopy. Staining specificity was assessed by control tests. In negative controls, TU-01 antibody was omitted or replaced with an unrelated monoclonal antibody.

Results

Electron microscopic observation of the majority of spermatozoa in the selected ejaculates revealed clear signs of cell degeneration such as swollen mitochondria and damaged membranes, which allowed us to characterize the samples as necrozoospermic. Additionally, a wide range of defects presumably originating in spermiogenesis was observed, e.g. disorganization or abnormal number of axonemal and periaxonemal structures. Nevertheless, ODF showed consistent association with the corresponding microtubule doublets, even when the latter were displaced very far from their normal location (Figure 1).

In some cells, ODF seemed detached from their doublets. However, careful observation revealed that these ODF had apparently undergone rupture, after which a small part of their material had remained connected to the doublet while the bulk had moved to the periphery (Figure 2).

Microtubules in many cases had retained their normal appearance, but in other cells from the same ejaculate they showed varying degrees of structural damage. In some tails they were altogether absent, leaving an "empty" ODF ring and a fibrous sheath.

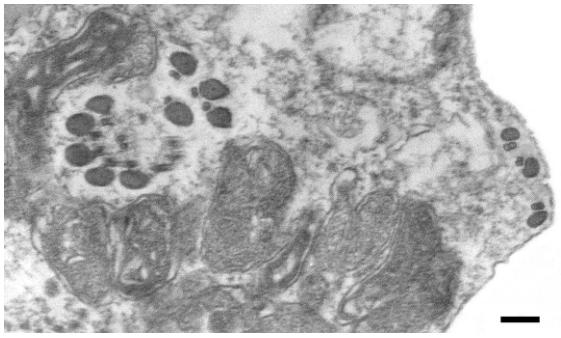

Figure 1. Transverse section of a middle piece with disorganized axoneme and supernumerary microtubule doublets and ODF. All ODF are associated with doublets, including the three ectopic ones under the cell membrane (right). Bar = 200 nm.

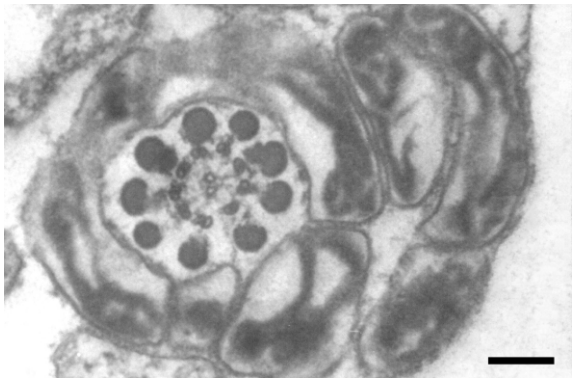

Figure 2. Transverse section of a middle piece with clear signs of degeneration and ruptured ODF. Bar = 200 nm.

Immunocytochemical reaction for alpha-tubulin yielded different results depending on the structural peculiarities of the cell. In tails with apparently normal axoneme, gold granules localized to its microtubules. In sections of damaged axonemes, the reaction was more intense and could be also seen in the surrounding cytoplasm. Such diffuse labelling was also detected when microtubules were completely disintegrated. However, when tails of this type were sectioned at middle piece or proximal principal piece level, an additional peculiarity was found: gold granules showed preferential binding to the inner (concave) surface of the ODF, at the site of missing microtubules (Figure 3). No labelling was detected in negative controls (data not shown).

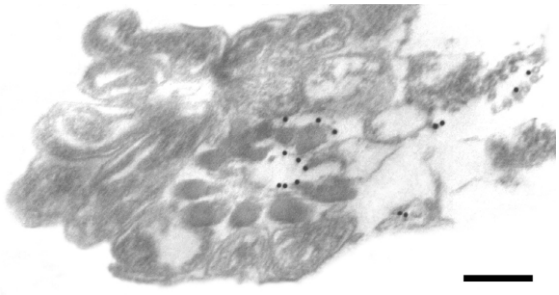

Figure 3. Immunoelectron microscopic reaction for alpha-tubulin in a transverse section of a middle piece with disintegrated axoneme. Gold granules are seen attached to the inner surface of ODF. The cytoplasm and a group of ectopic microtubules (right) are also labelled. Bar = 200 nm.

Discussion

The gradual loss of the *in vivo* structure after cell death is usually regarded as an obstacle in microscopic studies, and all efforts are aimed to preserve viability until proper fixation. However, changes accompanying necrosis, similarly to those resulting from chemical dissection, could provide valuable information about natural structure. Our observations of necrozoospermic human sperm tails showed that the association between microtubules and outer dense fibers is characterized by remarkable stability and fidelity. While at first glance some ODF seemed separated from their corresponding doublets, more careful examination revealed rupture and longitudinal splitting of the ODF, leaving their inner parts attached to the axoneme. This behaviour of ODF suggests that important differences in composition and structure may exist between their inner surface, outer surface and interior space.

The immunocytochemical reaction with anti-alpha tubulin antibody was carried out primarily to reveal the fate of tubulin dissociated from partially or completely disintegrated axonemes. TU-01 antibody was well suited for this purpose because it reacted with an N-terminal epitope exposed on damaged microtubules and soluble tubulin [11]. While some other investigators have reported negative immunocytochemical reaction for tubulin in necrozoospermia and have attributed it to post-necrotic protein degradation [8], it was positive in our samples. This difference could be due to variable preservation of different tubulin epitopes.

Apart from the expected binding to microtubules, we observed more diffuse labelling when they were damaged or absent, which could be explained with transition of tubulin to a soluble state. The most intriguing result was the decoration of inner surface of ODF with gold granules in tails with missing axonemes. This finding suggested that even after microtubules had collapsed and disappeared as ultrastructural objects, some of their alpha-tubulin molecules remained attached to the corresponding ODF. Because the N-terminal domain of alpha-tubulin *in vivo* binds with beta-tubulin to form the alpha-beta dimer [12], it seemed likely that beta-tubulin had dissociated to make the N-terminal epitope accessible to the antibody probe. We could hypothesize that the association between ODF and microtubule doublets is based on binding of unidentified proteins to alpha-tubulin. It could not be excluded, however, that beta-tubulin also takes part in the interaction.

Conclusion

To our knowledge, this study has demonstrated for the first time that attachment of ODF to microtubules is more permanent and stable than bonds between components within any of these two cytoskeletal structures, and alpha-tubulin is involved in this attachment. It could be concluded that reliable association between ODF and axonemal doublets is of vital importance for human sperm motility and function.

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