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Lipopolysaccharide-caused fragmentation of individual microtubules in vitro observed by video-enhanced differential interference contrast microscopy

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Abstract Microtubule disassembly is commonly believed to be a process of endwise tubulin dimer release. The present study demonstrates by video interference contrast microscopy that *Escherichia coli* lipopolysaccharide (LPS) caused microtubule disassembly in vitro by both endwise shortening and fragmentation. In contrast, the microtubules were only shortened from their ends in the presence of DNA, used as another example of a macromolecular microtubule effector. LPS-caused microtubule fragmentation was confirmed by transmission electron microscopy. Because of its ability to induce both fragmentation and endwise shortening, LPS, which is involved in sepsis pathogenesis, has to be regarded as a highly active microtubule-destabilizing agent.

Key words: Microtubule; Lipopolysaccharide; Disassembly; Severing; In vitro; Real-time observation

1. Introduction

Microtubules are intracellular protein assemblies required for various vital processes in eukaryotic cells including separation of genetic material and intracellular transport. They are dynamic structures, permanently undergoing processes of disassembly (shortening) and reassembly (growth). Their disassembly, which may be induced e.g. by cooling or addition of calcium ions, is commonly regarded to be realized exclusively by endwise microtubule shortening. However, some proteins have been characterized that are able to destroy microtubules by fragmentation [1–3]. Using rhodamine-labelled tubulin and real-time fluorescence microscopy, Shiina et al. [3] demonstrated that elongation factor 1α severs microtubules in vitro. Therefore, fragmentation may now be considered to be an additional mechanism by which a cell can more efficiently disrupt its microtubules, usually stabilized by binding with their minus end to a microtubule-organizing center.

The present paper deals with the in vitro interaction of microtubules with lipopolysaccharide (LPS). LPS, which is the major toxic product of the cell wall of Gram-negative bacteria and is responsible for many manifestations of septic shock (see e.g. [4]), was found to bind to microtubules in vitro [5] and to inhibit microtubule formation [6,7]. Parallel to turbidity measurements, we followed the effects of LPS on microtubules by real-time video differential interference contrast microscopy. By this procedure, the fragmentation of microtubules could be directly observed for the first time in a non-protein compound.

2. Materials and methods

In all experiments, we used chromatographically pure LPS from *Escherichia coli* (Sigma, Deisenhofen, Germany). It was added from stock solutions of 1 mg/ml in assembly buffer (see below). The DNA (Boehringer, Mannheim, Germany), obtained from calf thymus, was added from a 10 mg/ml stock solution made in assembly buffer.

The microtubule protein was isolated from porcine brain homogenates by three cycles of temperature-dependent disassembly/reassembly [8,9], using a buffer containing 20 mM PIPES, 80 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.8 (assembly buffer).

Microtubule formation was induced by 20 min incubation of 1.2 mg/ml microtubule protein, containing about 85% tubulin and 15% microtubule-associated proteins (MAPs), with 0.5 mM GTP at 37°C. After reaching the steady-state level, disassembly was started by addition of LPS (200 μg/ml final concentration). The kinetics of assembly and disassembly were monitored by time-dependent turbidity measurements at 360 nm with a Cary 1E spectrophotometer (Varian, Darmstadt, Germany).

To follow the disassembly dynamics of individual microtubules, microtubule suspensions were diluted 20-fold with assembly buffer containing 10 μM taxol (Sigma), and filled into a narrow glass channel, enabling the exchange of buffer additives under microscopic control (see e.g. [10]). The microtubules bound to the glass surfaces were visualized by video-enhanced differential interference contrast microscopy, using a Provis AX70 microscope (Olympus, Hamburg, Germany) equipped with a Newicon camera (Hamamatsu, Herrsching, Germany) and the image processing system Argus 50 (Hamamatsu). After selecting the observation field, the assembly buffer within the chamber was changed for a fresh one containing additionally 200 μg/ml LPS or DNA.

For electron microscopy, diluted microtubule suspensions were transferred to formvar-carbon-coated copper grids. After adhesion, the microtubules were incubated for 30 min with 200 μg/ml LPS in assembly buffer, negatively stained with 0.5% uranyl acetate, and inspected in an EM 902 transmission microscope (Zeiss, Oberkochen, Germany).

3. Results

Turbidity measurements showed that addition of LPS to microtubule suspensions caused a complete disassembly (Fig. 1). Within 15 min, nearly all microtubules were found to be destroyed.

This result was confirmed by following the course of disassembly on individual microtubules by video-enhanced interference contrast microscopy. Moreover, this method revealed that the microtubules were not only shortened from their ends but additionally showed inside breaks (Fig. 2a–c). The number of such breaks increased with incubation time. In contrast to LPS, DNA, used as a reference biomacromolecular sub-

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Abbreviations: LPS, lipopolysaccharide; MAP, microtubule-associated protein

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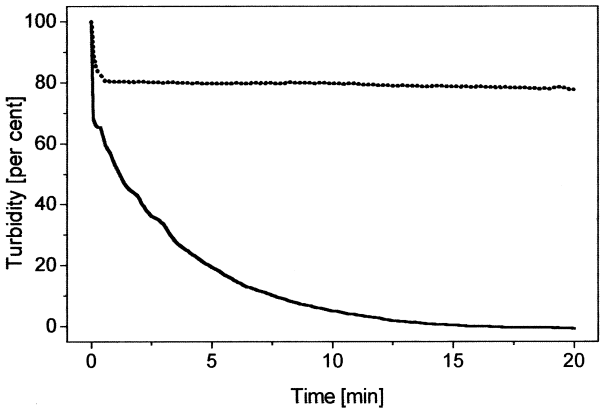

Fig. 1. LPS-caused microtubule disassembly followed by turbidity measurements. Dashed line: 50 μl LPS (1 mg/ml) was added to 500 μl of a microtubule suspension (1.2 mg/ml microtubule protein); solid line: control with buffer instead of LPS. The starting level of turbidity was set as 100%. The turbidity increase resulting from adding the LPS solution was subtracted.

stance with microtubule-destabilizing activity [11,12], induced only endwise disassembly (Fig. 2d,e).

Beside video-enhanced interference contrast microscopy, electron microscopy was used to study LPS-caused microtubule disassembly. It was found that in many cases the microtubule wall was spliced inside (Fig. 3a,b). The spliced parts had the appearance of uncompleted microtubules or protofilament ribbons (see [13]). The protofilament number of such parts usually did not reach the protofilament number of complete microtubules (commonly 13 under the assembly conditions used [14]). The frequency of splicing depended on the LPS exposure time. In numerous cases, single protofilaments were broken (Fig. 3a).

4. Discussion

LPS-caused microtubule disassembly was already observed by Risco et al. [6]. However, studying the mechanism of disassembly by video-enhanced interference contrast microscopy, we found that LPS is a unique macromolecule able to cause both endwise microtubule shortening and microtubule fragmentation. Fragmentation can be regarded as an additional tool of microtubule disruption. By increasing the number of free ends, it markedly enhances the efficiency of endwise microtubule disassembly, suggesting that LPS is an especially highly active exogenous microtubule-poisoning compound.

The question arises whether the cell itself is able to synthesize special compounds with microtubule-fragmenting activity, involved in the reorganization of stable microtubules during the cell cycle. Indeed, the occurrence of such compounds (e.g. katanin [2] and elongation factor 1α [3]) has been already confirmed. Both are proteins which were shown to sever microtubules under in vitro conditions. Serial reconstruction analyses of branching axons [15] corroborate the possible cell physiological relevance of microtubule fragmentation. A similar conclusion was drawn studying the breakdown of microtubules in a heliozoan [16]. Fragmentation is not only known for the microtubule system. This mechanism of disassembly, also induced by special proteins, e.g. severin [17], is involved in microfilament dynamics, too. Our study based on real-time observation of microtubule dynamics shows for the first time that non-protein compounds can also possess microtubule-fragmenting activity.

For a cell-free system, the fragmentation of individual microtubules has been already demonstrated by means of real-time fluorescence microscopy, using elongation factor 1α as a disassembly-inducing agent [3]. Our results confirm the observation of these authors who used rhodamine-labelled tubulin.

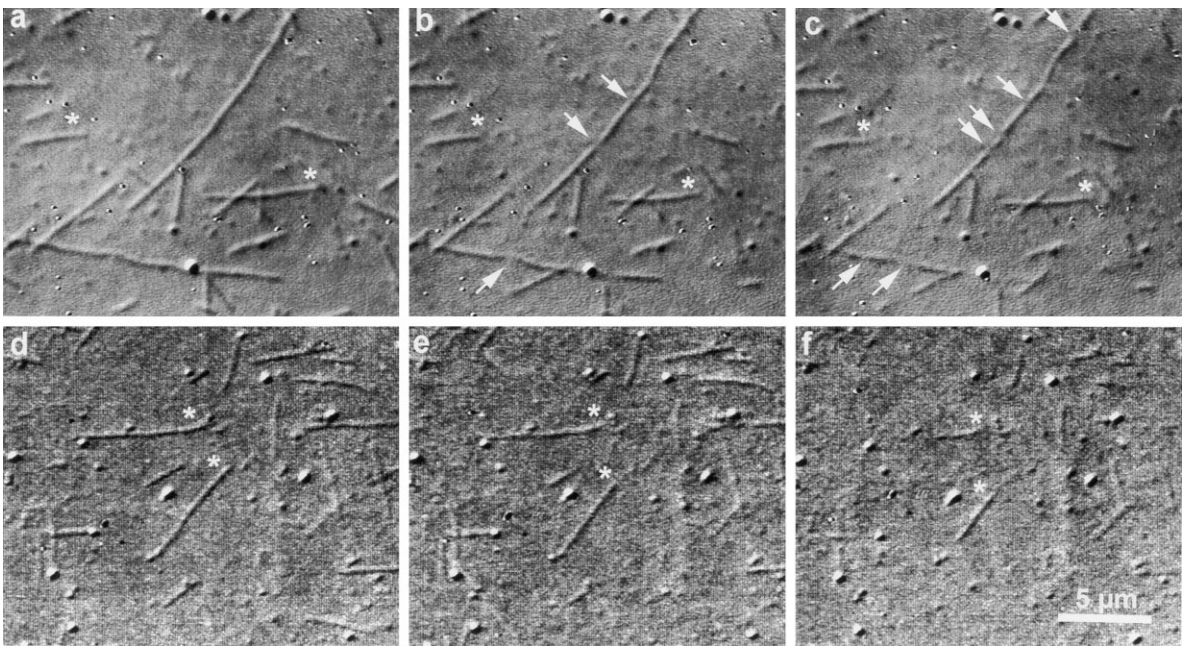

Fig. 2. Microtubule disassembly visualized by video contrast microscopy. a–c: Disassembly in the presence of 200 μg/ml LPS. The microtubules were found to be both endwise shortened (asterisks) and fragmented (arrows). d, e: Disassembly in the presence of 200 μg/ml DNA. Only shortening processes were observed. a, d: Control before adding LPS or DNA; b, e and c, f: about 15 min and 30 min after addition of LPS or DNA.

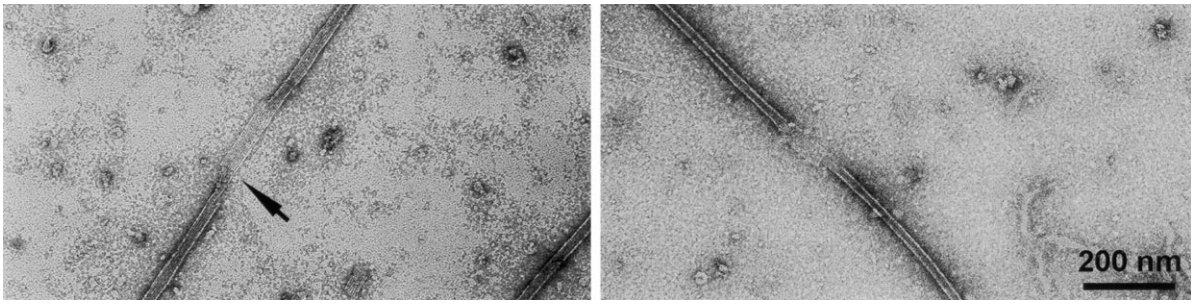

Fig. 3. LPS-caused disassembly of microtubules visualized by transmission electron microscopy after negative staining. In numerous cases (see arrow), single protofilaments were broken.

Now, it can be excluded that the fluorochrome labelling caused the weakening of longitudinal association between the dimers inside the microtubule.

Comparing our results obtained from video-enhanced interference contrast microscopy with those from electron microscopy, the question arises whether LPS caused a real fragmentation or only a splicing of the microtubule wall. It is known that video contrast microscopy is able to visualize not only microtubules, but also flat protofilament ribbons and zinc-induced sheets [18]. Therefore, we conclude that the breaks observed by video contrast microscopy on glass are complete interruptions of the microtubule wall and do not only result from a loss of contrast, due to the lower thickness of the object in the *z*-range at these sites (5 nm vs. 25 nm). We assume that the protofilaments contacting the carbon surface of electron microscopic grids are very tightly bound, so that their tubulin dimers cannot be removed during disassembly. That means that the LPS can remove only those tubulin dimers from the microtubule wall that are not bound to carbon. Unlike carbon surfaces, to which both the negatively charged tubulin and the positively charged MAP molecules are bound, glass surfaces are known to bind microtubules only via MAPs (or other proteins with basic domains, e.g. kinesin), which seem to act as a certain type of spacer. Consequently, in this case the LPS could attack all the protofilaments around the microtubule.

Parallel to LPS, disassembly was caused by DNA. Like LPS [6,7], DNA is also able to inhibit tubulin assembly and to cause microtubule disassembly by sequestration of MAPs [11]. However, in this case, only microtubule shortening was observed. We hypothesize that LPS binds not only to MAPs, but additionally to their complexes with tubulin dimers. Obviously, LPS removes MAP-tubulin complexes resulting in the appearance of gaps within the microtubule lattice. This hypothesis is corroborated by results of Risco et al. [6] who found by dot blotting that LPS bound to both tubulin and MAPs.

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