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Oxidation of thiol in the vimentin cytoskeleton

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Sublethal doses of H_2O_2, which induces oxidative stress, cause substantial alteration to the vimentin cytoskeleton in various cell types. We have used a thiol-blot assay to assess thiol status in individual proteins from cell extracts. Vimentin thiol is oxidized in preference to other cytoskeleton proteins. Immunoblot analysis also demonstrated a loss of reactivity to an anti-vimentin monoclonal antibody under non-reducing conditions, possibly due to thiol-group oxidation. During induced oxidative stress a number of proteins become associated with the cytoskeleton extracts.

INTRODUCTION

The cytoskeleton of the eukaryotic cell is made up of three major classes of filaments: microtubules (25 nm diameter), intermediate filaments (IF) (10 nm diameter) and microfilaments (7 nm diameter) [1]. IF can be classified into five groups based on sequence similarity: Type I (acidic keratins), Type II (neutral–basic keratins), Type III (vimentin, glial fibrillary acidic protein and desmin), Type IV (neurofilaments) and Type V (lamins) [2,3].

These protein are developmentally regulated. The function of IF remains unclear, although it has been proposed that they function as mechanical integrators of space [4]. However, many observations suggest they have a more subtle function [5,6]. Of particular interest is the finding that vimentin binds with high affinity to naturally occurring DNA sequences, which has given rise to the proposal that they are involved in gene expression or replication [7,8]. They are not obligatory for cell survival, since some cells do not possess this class of filament [6].

We have previously shown that certain fibroblastic cell types have an increased susceptibility to vimentin collapse around the nucleus on exposure to oxidative stress of 25 μM-H_2O_2 [9]. Vimentin contains a single cysteine residue, and it is known that these residues are highly susceptible to oxidation *in vitro*. It has been shown, for example, that oxidation of thiol groups in cytoskeleton proteins occurs when hepatocytes are exposed to lethal doses of quinone-induced superoxide production [10,11]. However, thiol groups show different levels of reactivity according to their location within a protein. Thiol groups that are exposed on the protein surface are more reactive. Also, if there is a basic amino acid in close proximity, then this favours the dissociation of the proton, again increasing its reactivity. It is possible that some cytoskeleton proteins are more susceptible to thiol-group oxidation than others. Furthermore, microtubule oxidation has been shown to affect their stability *in vitro* [12,13]. By using a thiol-blot technique we have assayed the cytoskeleton extracts for the relative susceptibility of each protein to oxidation.

METHODS AND MATERIALS

Cell culture

Fibroblast-like (Type B) synoviocytes were cultured from synovial tissue lining removed from rheumatoid joints at operation. This was achieved by digestion with collagenase type 1A (Sigma, Poole, Dorset, U.K.) for 3 h, followed by a 1 h digestion with trypsin to release the cells. These were then maintained in Dulbecco's minimal essential medium with 10% (v/v) foetal-calf serum, 1 mM-glutamine and streptomycin/penicillin (Gibco). Cells were used between passages 3 and 8, when little morphological de-differentiation was seen.

Oxidative stress

Cell monolayers were exposed to sublethal oxidative stress using H_2O_2 at 25 μM by substituting it directly into the medium. The cells were exposed for various times up to 6 h.

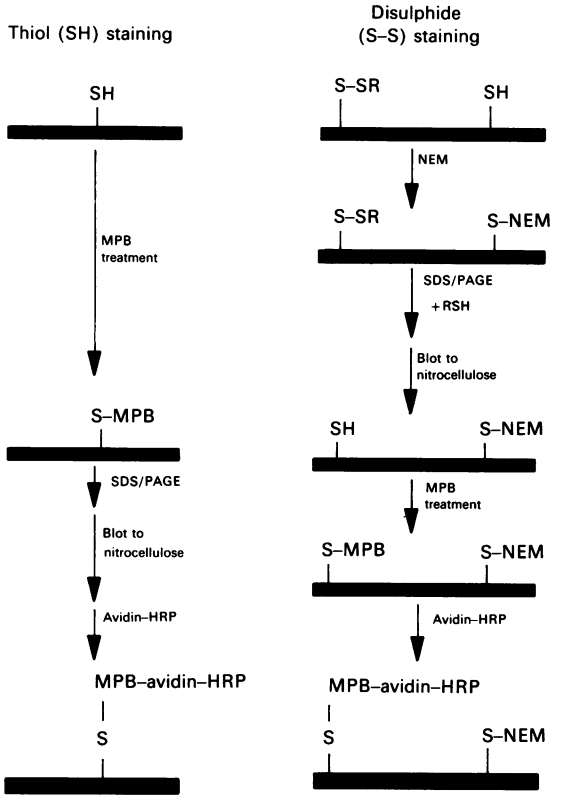
Electrophoresis

For electrophoretic studies, subconfluent cell monolayers were exposed to the stress stimuli and were removed with 2.5% trypsin, which was then inactivated by addition of medium. The cells were pelleted, counted, and the cytoskeleton proteins were extracted by suspension in buffer containing 1% (w/w) Triton X-100, 7 mM-$MgCl_2$, *N*-α-*p*-tosyl-L-arginine methyl ester hydrochloride (1 mg/ml) and 0.1 mM-phenylmethanesulphonyl fluoride. DNAase 1 (Sigma; type 4) was then added at 0.3 mg/ml and at 37 °C for 10 min. The sample was centrifuged at 1600 *g* at 4 °C for 10 min, and the pellet was suspended in SDS sample buffer and run on 10%-(w/v)-polyacryamide gels by using the Laemmli method [14] under either reducing (with mercaptoethanol in the sample buffer) or non-reducing (without mercaptoethanol) conditions.

Immunoblotting

After electrophoresis the proteins in the gel were transferred to nitrocellulose (0.45 μm pore size; Schleicher und Schüll, Dassel, Germany) using semi-dry blotting [15]. Gels were equilibrated in blotting buffer (20% methanol made up in SDS running buffer) for 20–30 min before blotting, and transfer was carried out at a current of 1–1.5 mA/cm^2 of gel area for 2–3 h. After transfer, the position of the protein bands was revealed by staining with Ponceau S and then the filters were blocked with 5% (w/v) BSA in Dulbecco's phosphate-buffered saline, pH 7.3 (PBS) for 1 h. Vimentin was revealed by using an anti-vimentin monoclonal antibody (V9; Dakopatts, High Wycombe, Bucks., U.K.) which was diluted in 1% BSA in PBS and the filter incubated for 2 h, followed by a 40 min wash in 0.1% Tween 20 in PBS. The filter was then incubated with a rabbit anti-mouse horseradish peroxidase (HRP)-labelled antibody diluted as described above and again incubated for 2 h and washed. The filters were developed with diaminobenzedine (1 mg/ml) and 50 μl of 30% (v/v) H_2O_2.

Abbreviations used: NEM, *N*-ethylmaleimide; MPB, 3-(*N*-maleimidopropionyl)biocytin; HRP, horseradish peroxidase; IF, intermediate filaments; PBS, Dulbecco's phosphate-buffered saline, pH 7.4.



Scheme 1. Thiol blot

Thiol blot (see Scheme 1)

3-(*N*-Maleimidopropionyl)biocytin (MPB) (Sigma) was used as a specific thiol-binding compound which can be detected by using avidin–HRP [16]. This method has been adapted and extended by Freeman & Meredith [17].

Thiol-group labelling. Cytoskeleton extracts were suspended in SDS sample buffer (without mercaptoethanol), 37 μg (per mg of protein) of MPB was added and left for 1 h at 37 °C to react with thiol groups. The sample was then boiled for 3 min and subjected to 10%-(w/v)-PAGE for 2 h at 20 mA. The proteins were then transferred on to nitrocellulose by using a semi-dry system at 45 mA/gel for 3 h. The filters were then blocked with 5% BSA in PBS for 1 h. Avidin–HRP was then added at 2 μg/ml for 1 h with agitation. This was followed by washing with 0.2% Tween 20 for 2 h. The filter was then developed with diaminobenzidine (0.1 mg/ml) and 12 μl of 30% H_2O_2.

Disulphide-group labelling. Cytoskeleton extracts were suspended in SDS sample buffer. Any free thiol groups were blocked by the addition of 10 mM-*N*-ethylmaleimide (NEM). Mercaptoethanol was then added to 2% to produce reducing conditions that break any disulphide bond to give a free thiol group that can then be labelled. The sample was subsequently electrophoresed and transferred to nitrocellulose as described above. The membranes were then treated with MPB at 1 μg/ml and, from the 5%-BSA-blocking step, treated sequentially as above.

RESULTS

During exposure to H_2O_2 at 25 μM there is no visible change in cell visibility as assessed by Trypan Blue exclusion. However, above this concentration there is a rapid fall in cell viability (results not shown).

Cytoskeleton extracts of cells exposed to H_2O_2 for 4 h, when run under non-reducing conditions, show a number of new protein bands at 50, 66 and 76 kDa, but there is no change in the vimentin band (Fig. 1). When the corresponding immunoblot

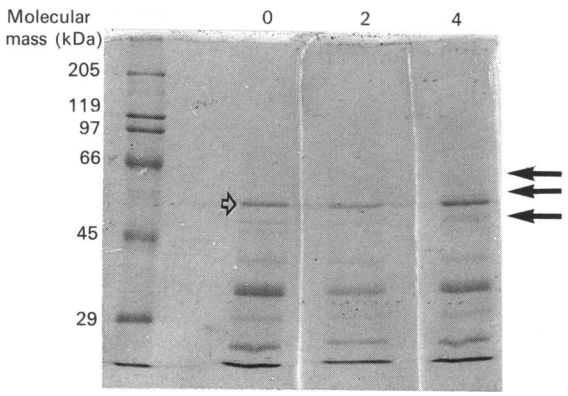


Fig. 1. Coomassie Blue-stained gel of cytoskeleton extracts separated by PAGE run under non-reducing conditions as described in the Methods and materials section

Lanes 0, 2 and 4 correspond to time (h) after exposure to 25 μM-H_2O_2. The open arrow points to the 57 kDa vimentin band. Closed arrows point to new associated proteins. Note that the 68 kDa band was visible before drying the gel, but not after.

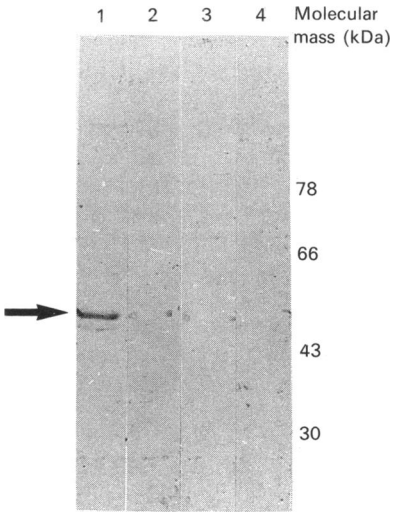


Fig. 2. Western blot of cytoskeleton extracts separated by PAGE under non-reducing conditions as described in the Methods and materials section

Lane 1 is the control without H_2O_2 (25 μM) exposure; lanes 2, 3 and 4 correspond to time (h) after H_2O_2 exposure. The arrow points to the 57 kDa vimentin band.

was performed on these extracts, only the unexposed ones showed any reaction with the anti-vimentin antibody (Fig. 2). There was, however, no change in the mobility of the band. Under reducing conditions all the extracts reacted with the antibody. The thiol blot is shown in Fig. 3. The 57 kDa vimentin band shows a substantial reduction when the thiol is directly labelled. However, when the extracts are run under reducing conditions to label both disulphide and thiol residues, there is no change in this band intensity. These results suggest a blocking of the thiol group. Under non-reducing conditions addition of NEM blocked all of the bands, as expected.

DISCUSSION

Our results show that oxidation at the thiol group can occur preferentially in the vimentin IF when compared with other proteins in our extracts. Human vimentin contains a single thiol group (cysteine-326) [18]. This effect may explain the vimentin cytoskeleton collapse around the nucleus via a striated structure

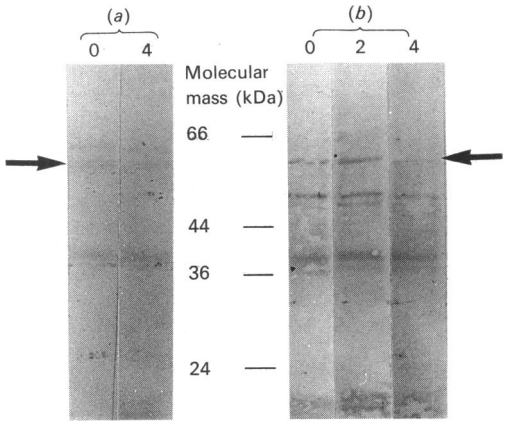


Fig. 3. Thiol blot on cytoskeleton extracts (see the Methods and materials section)

(*a*) Disulphide staining (–S–SR) 0 and 4 h after exposure to 25 μM-H_2O_2; (*b*) thiol (–SH) staining 0, 2 and 4 h after exposure to 25 μM-H_2O_2. The arrow points to the 57 kDa vimentin band.

that we have previously described [9]. IF structure and formation are complex and have been reviewed extensively elsewhere [19,20]. Recent work has indicated that the *N*-terminal region is important in filament formation [21]. The thiol residue on the protein is located in the *C*-terminal region, which may possess a site to which the *N*-terminal region binds. Most studies have concentrated on '*in vitro*' assembly assays where reducing conditions are maintained (by addition of mercaptoethanol), thus maintaining the thiol group in a reduced state. Such studies have not therefore addressed the role of this thiol group [22,23]. Furthermore, these simple '*in vitro*' systems do not reflect the complex assembly mechanisms that exist *in vivo* [24]. We propose that, during oxidative stress in the cell, the cysteine becomes blocked by GSH by formation of a disulphide bond. This is supported by the fact that there is no visible change in the mobility of the vimentin band, so the results cannot be explained by the formation of mixed disulphide with another protein. It should be noted that, in our assay, disulphide labelling occurred under denaturing conditions, whereas free-thiol labelling occurred before denaturation; this, however, does not affect the result, because no internal disulphide can be present as there is only one cysteine residue in the protein. Thiol blocking may then perturb IF structure by causing a steric inhibition or a conformational change of the site important for *N*-terminal binding. There is one recent report that the *C*-terminal thiol residue is important in at least one type of IF, since in lamins it is essential for binding to the nuclear membrane [25]. It should be stressed that, at least *in vitro*, the *C*-terminal region is not required for vimentin or desmin assembly; however, vimentin lacking the *C*-terminus region shows an increased tendency to form lateral aggregates [26,27]. In the cell, therefore, this could lead to an increase in bounding.

It is an intriguing possibility that other cellular events may lead to an altered thiol status and therefore cause oxidation at this site. It is known that during mitosis there is a substantial reduction in the GSH/GSSG ratio and a concomitant collapse in the vimentin cytoskeleton [28,29], although it should be stressed that vimentin phosphorylation also occurs at this time. Meister has suggested that GSH levels play a role in the cell [30].

The proteins (at molecular masses of 50, 66 and 76 kDa) that become associated with the cytoskeleton may possibly indicate that IF have a scavenging role for denatured proteins. This has been suggested in other systems, and the fact that such proteins change mobility under reducing as compared with non-reducing conditions suggests that disulphide bridges are involved [31,32]. This may be caused by the oxidative stress, leading to denaturation of the protein and binding to vimentin, owing to the hydrophobic interaction. Association of heat-shock protein 73 (β-internexin) to vimentin IF supports this hypothesis, as these proteins are thought to function by influencing protein folding [33–35]. Overall our results show that the thiol group in vimentin can become oxidized in the cell. This may be of significance in reactive-oxygen-species toxicity and also in other cellular events.

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