Tr. J. of Medical Sciences
29 (1999) 529–534

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The Intracellular Concentration of Components of the Ubiquitin System in A549 Culture Cells During UV Treatment

Received: December 10, 1998

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Abstract: This study attempts to determine a link between the intracellular components of the ubiquitin (Ub) system and the process of DNA repair in eukaryotes. It is possible to investigate this link in two ways:

i. studying the concentration of mRNA for specific Ub components in DNA damaged and controlled cells.

ii. studying the concentration of proteins of the Ub system in both DNA damaged and control cells.

The specific aim of this study was to try to establish such a link between repair and Ub, by studying and making a quantitative analysis of the intracellular concentrations of both the Ub activating enzyme (E1) and Ub conjugating enzymes (E2) in human A549 tissue culture cells at specific times after UV treatment. Using the techniques of SDS PAGE, western blotting and autoradiography, the result presented here show that levels of components of the Ub sysetm do fluctuate and, in fact, increase after UV treatment. The most noticeable increases occurred for E1 and E2–17kDa two hours after UV treatment. It was also demonstrated that this E2–17kDa can actually become conjugated to Ub, even under normal situations, but the reasons for this still remain unclear.

If Ub does play a role in this repair system, it may be possible to detect changes in the concentration of E1 and E2 at specific times after UV treatment.

Key Words: UV Treatment, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2).

Introduction

Ub is a small protein of 76 amino acids, involved in several important regulatory processes through its ATP–dependent, covalent ligation to a variety of eukaryotic target proteins (1, 2). The Ub conjugation process itself is composed of several consecutive steps. In the first, Ub is activated by ATP. This activation process involves the adenylation of the C–terminal glycine residue of Ub, and is catalyzed by the Ub–activating enzyme (E1). In all eukaryotic cells there is ony one species of E1 and it is required for all conjugation reactions. This 100 kDa protein has been sequenced in many species and shows considerable sequence homology (3–5). In the second step, activated Ub is transferred from the E1 thiol site in an internal cysteine residue of one of a family of Ub conjugation enzymes (E2) (6, 7). In the final step, Ub is transferred to target protein via the action of E3. E3 has been shown to play a key role in transferring Ub to proteins that are destined to be degraded by the Ub dependent proteolytic pathway (8). In addition to protein degradation, Ub has a role in cell cycle control (9), DNA repair (10) and heat–shock response (11).

UV treatment is probably the most extensively used model system for investigating the biological consequences of DNA damage and its repair. When cells are exposed to this treatment, adjacent pyrimidine residues become covalently attached to form pyrimidine dimers. This sort of structural disorder may provide a physical impediment to replication and transcription, and therefore must be removed immediately by the cell repair system. This study investigated the intracellular concentration of the E1 and E2 in tissue culture cells at specific times after UV treatment.

Materials and Methods

A549 Tissue Culture Cells

The A549 tumour line was initiated from a human alveolar cell carcinoma. The cell line was maintained as a

monolayer culture in plastic 90mm culture plates in Dulbeccos Modification of Eagles minimum essential medium (GIBCO). When confluent, the cell line was passaged by dissociating the monolayer in trypsin EDTA. The suspended cells were distributed on culture plates and fresh medium was added.

UV Treatment of A549 Cells

10 plates of confluent A549 cells were subjected to UV treatment. For each sample 2x90 mm plates were used.

Sample	Treatment
Control (A)	Exposed to normal light for 20 secs, followed by incubation at 37°C for 20 mins.
1 Hour Samples (B)	Exposed to UV treatment for 20 secs., followed by incubation at 37°C for 1 hr.
2 Hour Samples (C)	Exposed to UV treatment for 20 secs., followed by incubation at 37°C for 2hrs.
3 Hour Samples (D)	Exposed to UV treatment for 20 secs., followed by incubation at 37°C for 3hrs.
5 Hour Samples (E)	Exposed to UV treatment for 20 secs., followed by incubation at 37°C for 5 hrs.

Before treatment, the medium was removed, stored in sterile tubes and replaced immediately after UV treatment.

When harvesting A549 tissue culture cells, the medium was removed. Sterile 3ml 0.9%NaCl was added and the cell sheet was scraped off using a plastic scraper. The suspended cells were transferred with a pipette into a tube. Cell preparations were made immediately. Suspended A549 cells were spun down for 10 mins. at 4°C. The pellet was resuspended in 150µl of RSB (10mM Tris pH6, 10mM NaCl, 3mM MgCl). After centrifugation, to produce cell lysate the cell pellet was resuspended in 200–500 µl of lysis buffer (0.1mM EDTA, 0.2mM DTT, 50mM Tris pH7.5). The solution was spun down for 5 mins. The pellet was discarded and the supernatant stored at –20°C.

Protein Analysis – Lowry Method

The assay was performed in a 96–well, flexible polyvinylchloride plate. Each sample of unknown protein concentration was assayed in duplicate.

The samples were incubated at room temperature and absorbance was measured at 620nm. A standard curve was constructed (0–100µg, BSA) using mean standard absorbances, and thus protein concentrations in the samples were calculated.

Ubiquitin Thiolester and Conjugation Assay

Ubiquitin thiolester and conjugate formation was investigated for all UV treatment lysate preparation. In this assay, 50–100µg of protein lysate (20µl) was incubated at 37°C together with 50mM ATP (2.5µl), 50mM $MgCl_2$ (2.5µl), 50mM DTT (2.5µl) and 5µl of I–125 ubiquitin ($1x10^6$ cpm). For the formation of Ub–thioesters the incubation time was 20 mins, after which the reaction was stopped by adding 10µl of the gel application buffer (62mM Tris pH6.8, 10% SDS, 0.5M sucrose, bromophenol blue). Parallel reaction mixtures were stopped by adding mercaptoethanol (2µl). These samples served as the control, the mercaptoethanol reducing the thioesters. The samples were applied to 10% polyacrylamide gel and after running, the gel was dried. Bands were visualized with autoradiography.

Western Blotting

Samples containing 50–100µg of protein were run on 10% gel, until the dye front had reached the bottom of the gel. Proteins were transferred onto a nitrocellulose membrane. Membranes were treated with the antibodies (rabbit antisera raised to human 17kDA E2) to see the staning on the nitrocellulose.

Results

UV treated cellular preparation of lysates and total cell pellets were subjected to protein analysis by the Lowry Method, as follows:

Preparations And Samples	µg Protein/µL
LYSATE	
Control (A)	5.2
1 hour (B)	8.1
2 hour (C)	5.9
3 hour (D)	5.9
5 hour (E)	6.8
TOTAL CELL PELLET	
Control (A)	18.3
1 hour (B)	17.2
2 hour (C)	19.4
3 hour (D)	19
5 hour (E)	19.1

Figure 1 shows a picture of 10% polyacrylamide gel, in which protein samples from lysates and total cell pellets of A549 tissue culture cells were electrophoresised. The

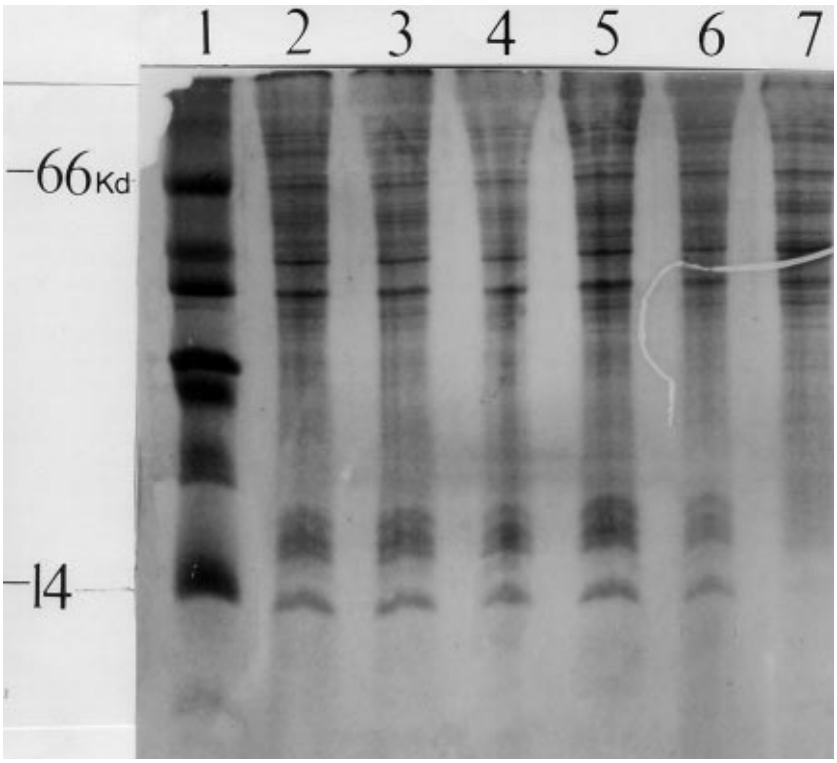

Figure 1. The electrophoretic Pattern (10% PAGE) of proteins in A549 Tissue Culture Cells (see results section for details).
Lane 1. MM standards (66, 45, 36, 29, 24, 20 and 14 kDA)
Lane 2. Total Cell Pellet (TCP) (A)
Lane 3. TCP (B)
Lane 4. TCP (C)
Lane 5. TCP (D)
Lane 6. TCP (E)
Lane 7. Lysate (A).

samples loaded and the protein content of each sample were as follows:

LANE	SAMPLE	PROTEIN CONTENT
1	MM Standards	25µl
2	Non UV TCP (A)	55µg
3	UV treated TCP (B)	51µg
4	UV treated TCP (C)	58µg
5	UV treated TCP (D)	57µg
6	UV treated TCP (E)	57.4µg
7	Non UV treated lysate (A)	52µg

TCP: Total Cell Pellet

In all lanes except lane 7, proteins of molecular mass 12–16 kDa are clearly visible. These proteins are probably histone proteins. In lanes 2–7, bands are clearly present with apparent molecular masses of 36, 44, 47 and 54kDa. These bands are likely to be intermediate filaments which are key determinants of cellular structure.

Figure 2 shows the results of a thiolester assay in which I–125 Ub was incubated with lysate preparation in the presence of ATP, $MgCl_2$ and DTT for 20 min. These samples were run on a 10% polyacrylamide gel before being autoradiographed. The samples were loaded and their protein concentrations were as follows:

LANE	SAMPLE	PROTEIN CONTENT
1	Non–treated lysate (A) + mercaptoethanol	36µl
2	Non–treated lysate (A)	36µg
3	UV treated lysate (B)	56.7µg
4	UV treated lysate (C)	41µg
5	UV treated lysate (D)	41.6µg
6	UV treated lysate (E)	47.6µg

The sample in lane 1 served as a control, as any thioester bonds formed are cleaved with the mercaptoethanol. A graph was constructed from the log molecular masses of the protein standards against their electrophoretic mobility in the gel. This was then used to estimate the molecular mass of both unknown protein samples in the gel, and bands on the autorad. Molecular masses of Ub thiolesters on the autorad were calculated, taking into account the molecular mass of Ub, which obviously appears to increase the molecular mass of proteins, to which it is bound. From the results, a protein of molecular weight 105 kDa is present in lanes 2–6 but not in lane 1. This protein is most probably the Ub

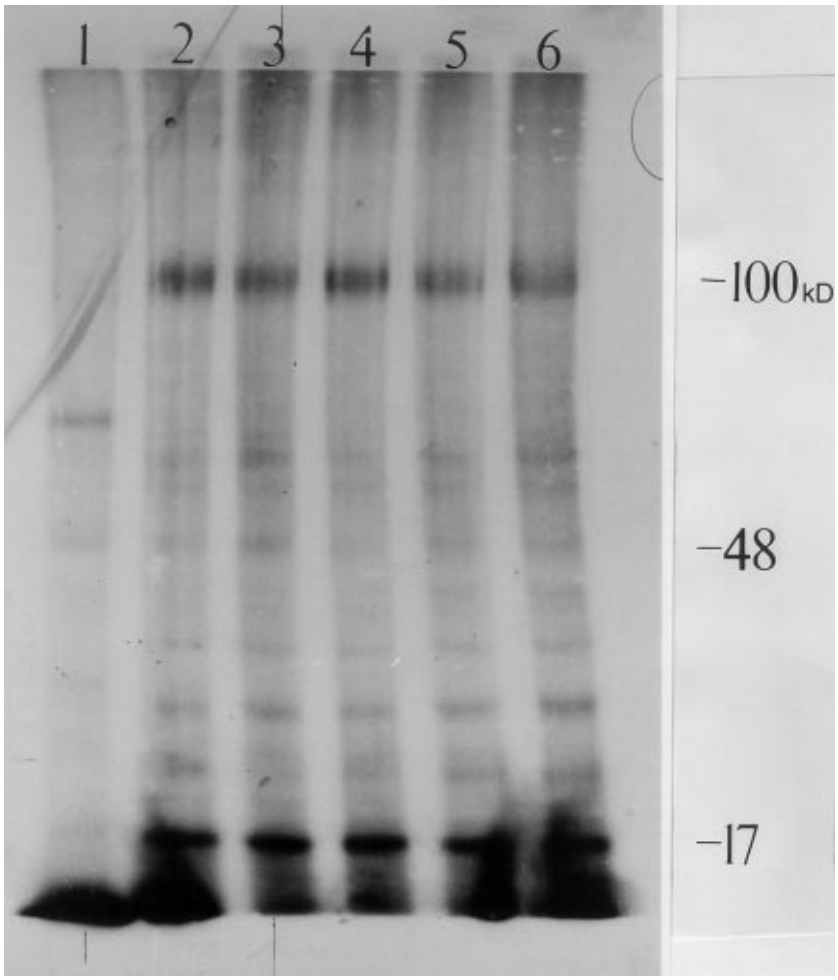

Figure 2. The autoradiography of the thiolester assay using a 10% gel system (see results section for details).
Lane 1. Lysate (A) + marcaptoethanol
Lane 2. Lysate (A)
Lane 3. Lysate (B)
Lane 4. Lysate (C)
Lane 5. Lysate (D)
Lane 6. Lysate (E)

activating enzyme (E1), which has a MM of 100kDa. Bands corresponding to molecular masses of 17, 26, 33, 40, 48 and 59 kDa are also visible, the most prominent band being the 17kDa band, which is obviously assumed to be the human 17 kDa Ub carrying enzyme (E2). The amounts of protein varied slightly in each sample. The intensities of the bands in each of the lanes could not be correlated efficiently to each other.

Figure 3 shows the results of a western blot, whereby electrophoretically separated components were transferred from the gel to nitrocellulose membrane and probed with rabbit antisera against human 17 kDa E2 which were provided by Dr. M.A. Billet (QMC. Biochem., Nottingham). The E2 was detected by horseradish peroxidase linked to swine anti–rabbit IgG, which forms a brown precipitate in the presence of 3,3–diaminobenzedine (DAB). The samples and their protein content are given as follows; 25µl sample was used for each lane.

LANE	SAMPLE	PROTEIN CONTENT
1	Non–treated lysate (A)	130µl
2	UV treated lysate (B)	202µg
3	UV treated lysate (C)	147.5µg
4	UV treated lysate (D)	148µg
5	UV treated lysate (E)	170µg

Discussion

The main points of this study (thiolester assay using a 10% gel system) are that thiolester bonds from between Ub and proteins with apparent molecular masses of 17, 26, 33, 40, 48, 59 and 105 kDa (calculated allowing for the 8.5 kDa provided by Ub).

The largest of these species is proposed to be the Ub activating enzyme E1, of which there is only one type found in all eukaryotic cells. The smaller molecular mass species are proposed to be Ub conjugating enzymes E2,

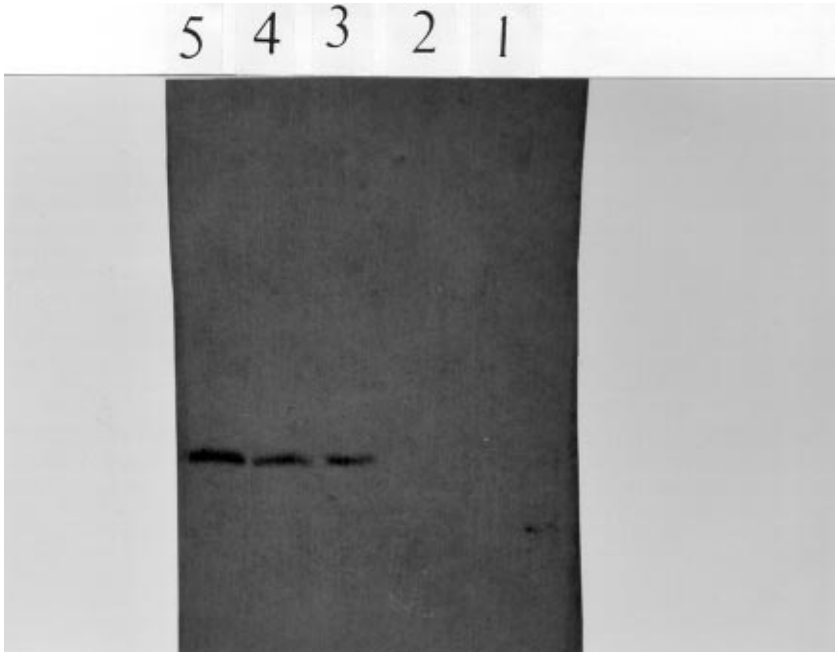

Figure 3. Western Blot analysis for detection of the 17 kDa E2 (see results section for details).
Lane 1. Lysate (A)
Lane 2. Lysate (B)
Lane 3. Lysate (C)
Lane 4. Lysate (D)
Lane 5. Lysate (E)

the smallest of which, the 17 kDa species, has been shown to be homologous to RAD 6 (12). The proportion of E1 thiolesters in A549 tissue culture cells varies at certain time points after UV treatment. In this experiment, one sample was non–UV treatment (lane 1 in Fig. 2). If we assume that the intensities of the bands representing the Ub thiolesters of E1 are proportional to the amounts of E1 in the cell, then the value of intensity reached here represents the amount and possibly the activity of the E1 enzyme in normal situations, i.e. the non–stressed state. If we then consider the values achieved for E1 thiolesters 1 and 2 hours after UV treatment, there is an apparent increase in the value. Thus, we are seeing a rapid increase in the amount of E1 thiolesters after UV treatment. This increase could be due to an increase in gene expression or up–regulation of E1 activity. Alternatively, it could be due to both possibilities. This leads to the question of why there is such an increase. Obviously this result implies that Ub conjugation to proteins must increase in stressful situations, as E1 is required for the first step in this process.

It is unlikely that Ub is involved in the cell cycle at this time as the cells were confluent when treated, i.e. stuck in the Go phase. More probable is that Ub is being conjugated to proteins destined for degradation, or to nuclear proteins such as histones which may possibly mediate in the DNA repair process. Five hours after treatment, the levels of E1 thiolesters appeared to decrease, thus indicating that the cell may be returning to its normal state.

Thiolester conjugation experiments carried out in this study indicated a very high proportion of E2–17kDa in A549 tissue culture cells. The intensities of the bands representing this 17kDa E2 species, could be solely due to I–125 Ub being conjugated to it, or alternatively these bands could represent both conjugates and thiolesters as they would obviously migrate to the same position in the gel. Therefore an experiment where a control lane was used for every time point, i.e. with mercaptoethanol which cleaves thiolester bonds may show differences in intensities between lanes where E2–17kDa thiolesters and conjugates are running and in control lanes where only conjugates would be present. Obviously if the intensity of the band in the control lane was half that of the normal lane then it could be proposed that the remaining intensity of the band is due to thiolester formation. In this experiment only one control lane was used and this sample was non–UV treated.

The formation of a band in this lane shows that conjugation between Ub and E2–17kDa occur in cells under normal situations. The reasons for this remain unclear, although it may have some regulatory function, i.e. if E2–17kDa is conjugated, then it is unlikely that it could serve as a donor of Ub from E1 to protein, or the Ub protin ligase, therefore conjugation of Ub to E2–17kDa may prevent this process. With the findings of

C terminal hydrolases which specifically cleave Ub from substrates, there could be a reversible regulatory role of Ub analogous to protein phosphorylation mediated by kinases and phosphatases. In the thiolester assay, the highest amount of Ub attached to E2–17kDa via a thiolester or covalent linkage is highest two hours after treatment. If we assume that this indicates the highest amount of both conjugates as well as thiolester, then we can associate this finding indirectly with respect to DNA repair. As stated, E2–17kDa is capable of ubiquitinating histones (13, 14) and this conjugating enzyme shows 69% homology with RAD6 which is involved in DNA repair in yeast (12). When looking at the conjugation and the blot assays, E2–17kDa conjugates appear to be maximum two hours after UV treatment and the blot analysis of antibody against E2–17kDa (Fig. 3), intensity of the bands on the membrane increased further, five hours after with UV–treatment. The elevated levels of E2–17kDa once again could be due to increased levels of gene expression or up–regulation of the activity of this enzyme. In this case, however, it seems likely that increases in gene transcription are responsible. This comes from the fact that many of the conjugating enzymes found in yeast are found to be heat inducible and therefore regulated at the level of transcription. Alternatively there could be a decreased rate in turnover of these E2 species which may be due to lack of recognition by the 26S protease.

Acknowledgements

Tissue culture cell experiments of this study were performed in the Biochemistry Department of Nottingham Unversity, UK:

The author is indebted to Prof. Dr. J. Mayer, M. Landon and their staff in Notthingham for their help.

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