

UVB-Induced Activation of PARP-1 in Cultured Human Lens Epithelial Cells

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Abstract

According to the World Health Organization, cataract is one of the leading causes of blindness in the world. Photo-oxidation by ultraviolet (UV) light has been linked to cataract development with evidence suggesting that UVB irradiation may cause cortical (the periphery of the lens) and posterior subcapsular (the back of the lens) cataract by damaging DNA within lens epithelial cells. Therefore we irradiated cultured human lens epithelial cells with UVB light and investigated the effects of the radiation on a DNA repair enzyme (PARP-1) located in the cell nucleus. We found that after cells were treated with UVB light and allowed to re-incubate at 37°C, PARP-1 activation increased within the nucleus of the cells. We conclude that PARP-1 may play a role in protecting human lens epithelial cells from UVB-induced DNA damage.

Introduction and Background

The lens of the eye is responsible for focusing light and images onto the back of eye where the retina is located. Normally, the lens is perfectly transparent so that light can be transmitted through it without scattering. The function of the lens is disrupted when a cataract forms. Cataract formation is a condition that involves the gradual clouding of the lens within the eye (Pascolini, 2004). When the lens becomes cloudy during cataract formation, light is scattered and unable to be processed correctly. As the disease progresses, it leads to increased opacity until vision is completely lost.

Studies have shown that photo-oxidation by ultraviolet (UV) light has been linked to cataract development. Solar radiation reaching the earth contains UVA (320-400 nm wavelength), UVB (295-320 nm wavelength), and visible light (400-800 nm wavelength) (Balasubramanian, 2000) (Fig. 1). Evidence suggests that UVB irradiation may cause cortical

(the periphery of the lens) and posterior subcapsular (the back of the lens) cataract (Li & Spector, 1996).

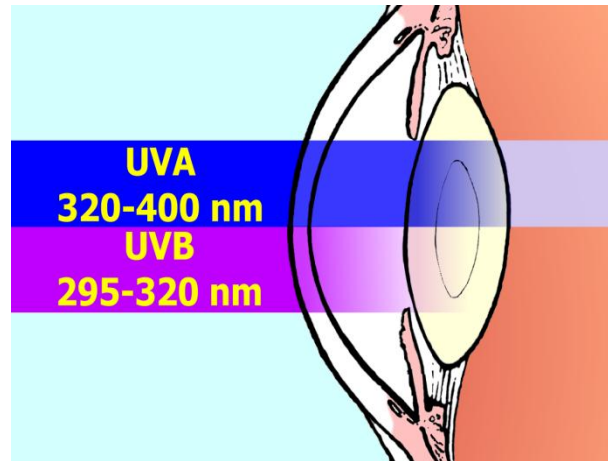


Figure 1: Wavelengths of Ultraviolet Light

UVB light is absorbed by the cornea and aqueous humor, and only about 1% reaches the lens. However, even the small amount of UVB light can damage lens epithelial DNA.

UVB radiation is known to cause both direct and indirect damage to cells within the body by different methods. As an exogenous agent, UVB radiation causes extensive cross linking of nucleic acids to occur (thymine dimers) which in turn leads to single strand breaks of DNA. UVB radiation can also cause endogenous damage by generating reactive oxygen species that can damage DNA and proteins within the lens resulting in loss of transparency (Wölfle, 2011). These types of DNA damage have been linked with human maturity-onset cataract (Kleiman, 1993).

Poly (ADP-ribose) polymerase-1 (PARP-1) is an enzyme present in the cell nucleus (Uchida et al., 2001). One key role of PARP-1 involves DNA repair. When DNA damage occurs (for example, during lens UV exposure) PARP-1 is activated and binds to DNA single strand breaks and utilizes NAD^+ to synthesize poly (ADP-ribose) polymers (PAR) on itself and

other nuclear proteins (Szabo & Virag, 2002) (Fig. 2). Although PARP-1 does not participate directly in DNA repair, the negative charge created by poly ADP-ribosylation results in electrostatic repulsion between DNA and histones which may play a role in chromatin remodeling, DNA repair, and transcriptional regulation (Szabo & Virag, 2002). In certain cases, over-activation of PARP-1 can lead to ATP depletion and eventually cell death (Alano et al., 2004). To prevent this from happening, the protein poly (ADP-ribose) glycohydrolase (PARG) catabolizes PAR units found within the nucleus. PARP-1, and particularly PARP-1 inhibitors, are actively being studied in non lens tissues (Szabo & Virag, 2002). It has been found (counter-intuitively) that inhibition of PARP-1 can be used to aid in the treatment of diseases such as diabetes and certain cancers.

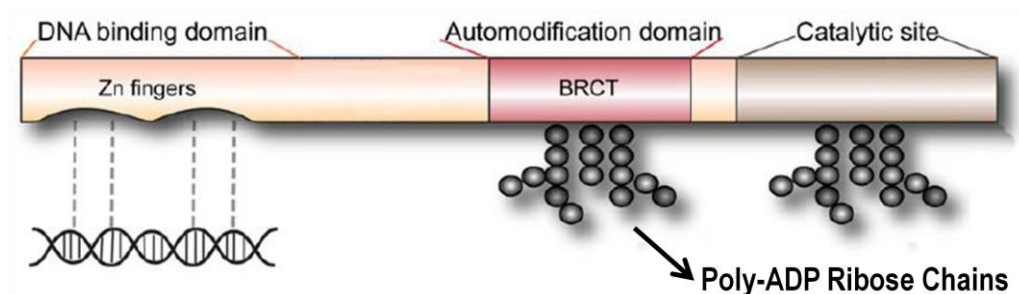


Figure 2: Structure of PARP-1 Protein

PARP-1 enzyme is comprised of three domains, shown above. The “Automodification domain (NAD⁺ Domain)” and the “Catalytic site” are the only sites where the PAR polymers bind, while the “DNA binding domain” contains the necessary zinc fingers to bind to damaged DNA.

This study will provide knowledge of how UVB light contained in sunlight can damage lens DNA and lead to cataract, which will help to educate the public on how to delay the onset of age-related cataract. New information will be obtained on the role of PARP-1 in repairing UVB-induced DNA single strand breaks in the lens epithelium. The work may lead to possible use of PARP-1 inhibitors as a means of protecting against radiation damage to the lens.

Objectives

1. Determine whether PARP-1 is active in human lens epithelial cells (LECs)
2. Evaluate activity of PARP-1 in LEC's at various times after a dose of UVB light that produces substantial DNA single strand breaks.
3. Investigate if PARP-1 protects LECs from UVB-induced DNA damage.

Specific Aims

Determine the following at various times after exposure of LECs to UVB radiation:

1. LEC DNA single strand breaks by DNA single strand break assay.
2. Viability of LECs after various re-incubation times.
3. Levels of LEC poly ADP-ribose (PAR) using immunoblotting and immunocytochemistry.
4. Mass spectrometry to confirm protein identity.

Methods

Cell Culture

The immortalized human lens epithelial cell line SRA 01/04 established by Dr. Venkat Reddy of Oakland University's Eye Research Institute was chosen to mimic the cells within a human lens (Reddy et al, 1998). Approximately 1.5 million cells were seeded onto a 60mm Petri dish and allowed to grow to confluence in Modified Eagle Medium (MEM) medium containing 15% Fetal Bovine Serum (FBS), gentamicin and Fungizone[®] (Fig. 3).

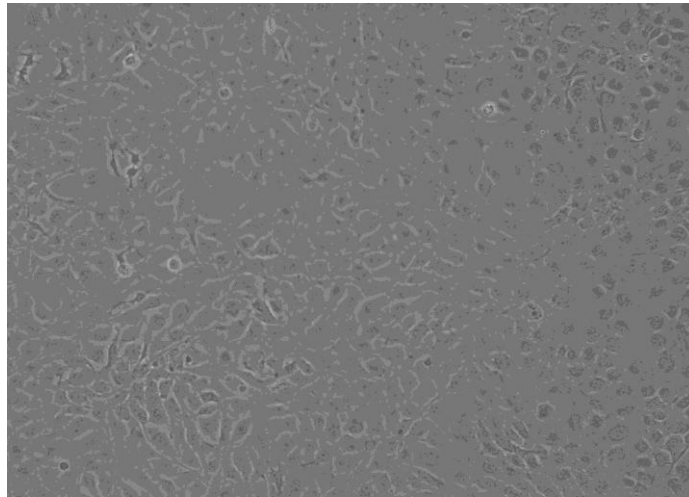


Figure 3: SRA 01/04 Cultured Human Lens Epithelial Cells

The immortalized human lens epithelial cell line grown to confluence in MEM containing 15% FBS, gentamicin and Fungizone[®]. Magnification: 20x.

Ultraviolet Light and Exposure

A "Medium Wave" UV lamp (Spectroline[®], New York) was used to produce light within the UVB spectrum (peak at 312 nm). The LECs were irradiated with 3.6 mW/cm² of UVB light for 5 minutes in serum-free PBS. Using a comet assay, we have found that this dose produces substantial DNA single strand breaks immediately after exposure. Cell culture plates were individually treated by placing the dish above the UV lamp at a distance that achieved the

desired dosage but avoided ambient heat given off by the lamp (Fig. 4). A series of filters were placed in the path of the incoming light to ensure that only ultraviolet light from the UVB spectrum was reaching the cells (lamp had a range of 270 nm-340 nm). The Intensity of the dosage was measured before and after treatment of each dish with a UVX-31 radiometer (UVP[®], California).

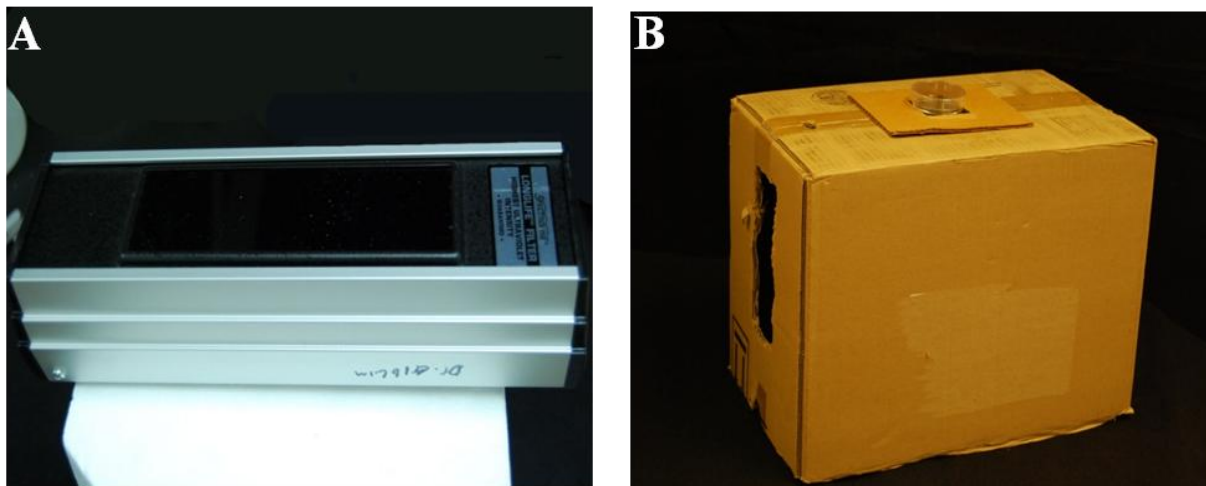


Figure 4: UVB- Irradiation Setup

A Spectroline[®] “Medium Wave” UV lamp (A) was used to produce light within the UVB spectrum (peak at 312 nm) that would hit the cells stationed on top of a box (B) at a height that would avoid excess heat given off by the lamp.

DNA Single Strand Break Assay

A comet assay was employed to demonstrate that the UVB dosage caused significant DNA single strand breaks that were repaired after re-incubation for 24 hours. After treatment with UVB, cells were harvested and suspended in 0.5 % low melting temperature agarose. This mixture was then allowed to polymerize in between two layers of normal agarose and cell membranes were lysed by a solution containing EDTA and Triton X-100. The prepared slides

then underwent electrophoresis and were stained with ethidium bromide. DNA migration was measured with a fluorescence microscope (Singh et al., 1988).

Sample Preparation for SDS-PAGE and Western Blot Analysis

After each plate was irradiated for 5 minutes, the serum free PBS was suctioned off and MEM media plus 15% serum reintroduced to the plate. The cells were then re-incubated at 37°C for 0, 5, 30, 60, 90, or 120 minutes. Following re-incubation, cells were harvested with trypsin and immediately centrifuged into pellets. Following centrifugation, the supernatant was discarded and the pellet resuspended into a buffer containing urea, glycerol, SDS and mercaptoethanol. To ensure complete permeabilization of the nuclear membrane, sonication on ice was also performed. The samples were kept on ice whenever possible to minimize breakdown of PAR by PARG (Poirier et al., 2003).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

A uniform gel of 7.5% was used to separate proteins. The low acrylamide content for the gels allowed PARP-1, which is a high molecular weight protein (113 kDa), to be resolved. After preparation, samples were loaded onto the polyacrylamide gel and an electric current was applied. The SDS gives proteins a negative charge which allows each protein to move through the semi-porous gel to the positive end. During the migration, the larger proteins will be deposited at a higher location, while smaller proteins are free to migrate further down. After the smallest protein reaches the bottom of the gel, the current is stopped and the gel is removed. Some of the gels were stained for total protein using Commassie blue stain and some were used to transfer the protein to a membrane for immunoblotting. The Commassie blue stained gel was used to demonstrate an equal distribution of protein loaded into each gel and also allowed for excised protein to be further studied by mass spectrometry.

Western Blot Analysis

Western blotting was used to detect increased levels of PAR (the polymers made by PARP-1) that were produced following UVB-induced DNA single strand breaks. Proteins from the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting. To detect PAR, blots were blocked in whole milk and goat serum for 1 hour and incubated overnight in rabbit anti-PAR polyclonal antibody. Next, the blots were washed with Tween-20 and incubated in goat anti-rabbit IgG HRP conjugate antibody. Immunoreactivity was visualized by incubating blots in chemiluminescence substrate (Poirier et al., 2003).

Immunocytochemistry

Immunocytochemistry was done to visualize the amount of PAR that was being produced within the intact cells and to confirm that PARP-1 activation was occurring within the cell nucleus. Following treatment and re-incubation, cells were immediately fixed in formaldehyde. The nuclear membrane was then permeabilized with Triton X-100 and samples were blocked with a solution containing bovine serum albumin (BSA). The same rabbit anti-PAR polyclonal antibody was used to tag PAR in cell samples while a fluorescently labeled anti-rabbit secondary antibody was used to visualize the protein under a fluorescence microscope. Each cell's nucleus was stained with DAPI and colocalization was observed (Kupper, 1996).

Mass Spectrometry

To confirm the identity of the protein found in the Western blot analysis, mass spectrometry was done on protein excised from SDS-PAGE gel and compared to a commercially

bought PARP-1 protein. To do this, the excised protein band was destained with an ammonium bicarbonate solution and dried by vacuum centrifugation. The dried gel piece and the commercial PARP-1 were then incubated with a Trypsin Gold (Promega[®]) solution to digest the protein. To elute the tryptic peptides, the gel piece was incubated in an ammonium bicarbonate solution overnight. The following day the tryptic peptides of both the gel piece and the commercial PARP-1 were loaded into an Agilent 6520 Q-TOF mass spectrometer (located in the Oakland University Chemistry Department) in a formic acid buffer (Lee, 2009). The masses of these tryptic peptides were then compared to predicted weights generated by the Protein Analysis Work Sheet (PAWS) software.

Results

UVB Exposure

To ensure that the 5 minute dose used was not sufficient enough to kill the cultured cells, samples were examined under a microscope after being treated and left overnight at 37°C. The epithelial lens cells that were exposed to 5 minutes of UVB light showed no major morphological change when compared to the control cells (Fig. 5).

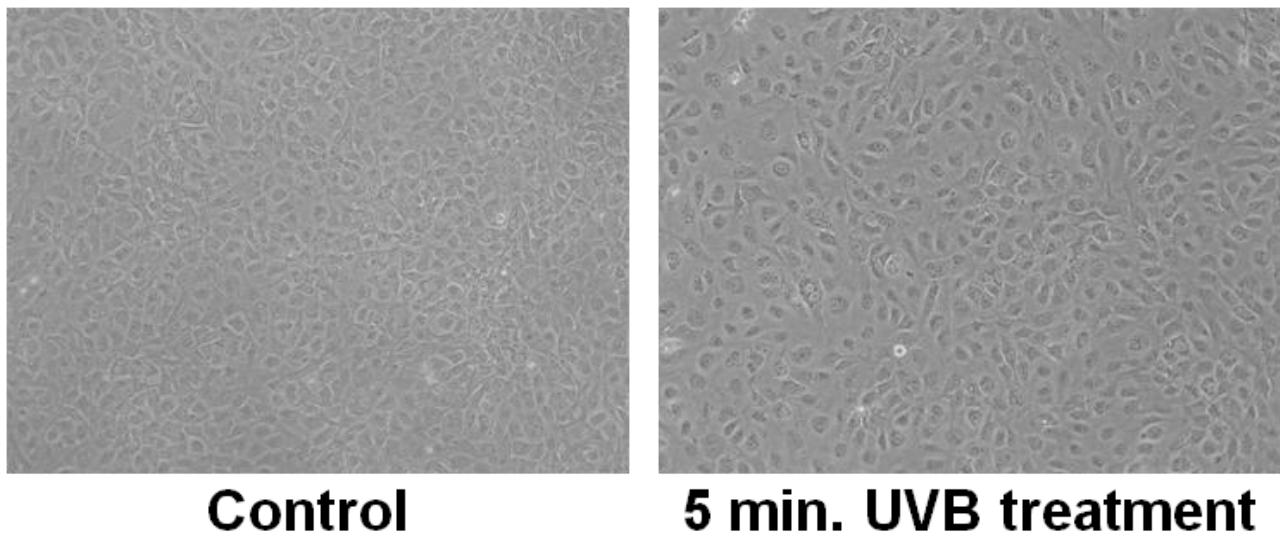


Figure 5: Human Lens Epithelial Cells exposed to UVB light

Photograph of treated cells after a 24 hour incubation. No major morphological changes occurred after the cells were exposed to 5 minutes of UVB light (intensity of 0.9 mW/cm²).

Comet Assay

Cells that underwent electrophoresis immediately after UVB exposure showed large tails of DNA migration, which is a sign of DNA single strand breaks (Fig. 6A). After 24 hours of normal re-incubation at 37 °C, the assay showed complete repair (lack of any DNA migration) of the previous DNA damage (Fig. 6B).

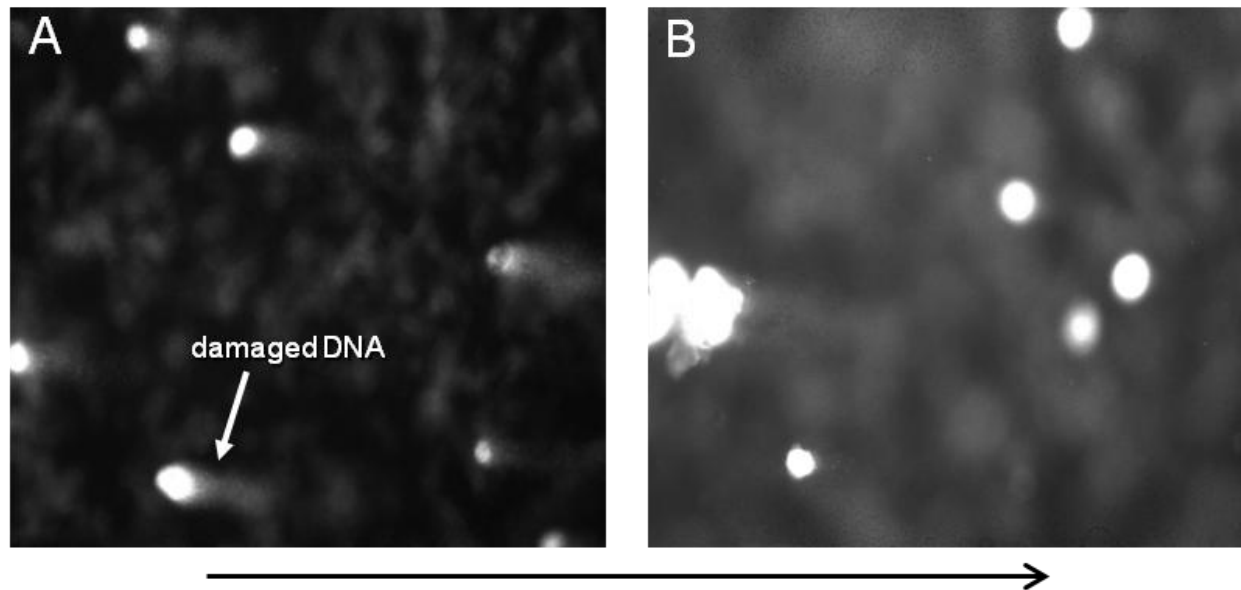


Figure 6: Comet Assay Analysis

Electrophoresis immediately following irradiation of cells exhibits long tail of damaged DNA (A). Electrophoresis following 24 hours of re-incubation at 37°C no longer shows signs of DNA damage (B). The arrow beneath the figures shows the direction of the electrophoresis. Smaller, damaged DNA travels faster than the undamaged DNA.

SDS-PAGE

To ensure equal loading of protein for each sample well, one of the SDS-PAGE gels was stained with Coomassie blue dye. The presence of the bands at the same levels and intensity confirms that equal amounts of protein were loaded into each well (Fig. 7). The band with a molecular weight (M.W.) of approximately 113 kDa was excised and used for mass spectrometry analysis.

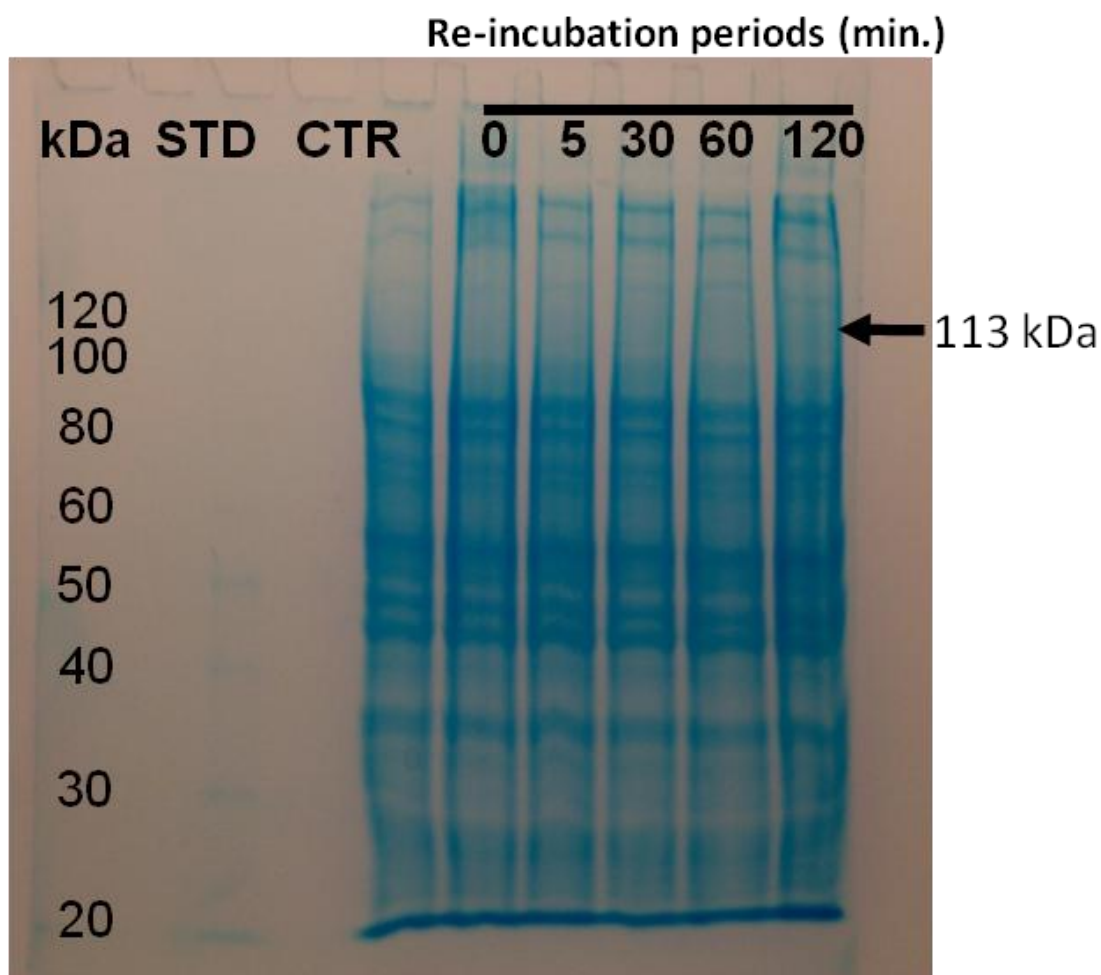


Figure 7: SDS-PAGE of treated samples (Coomassie staining)

Cells were exposed to UVB light for 5 min and then reincubated normally for 0, 5, 30, 60 and 120 minutes. Intensity of bands from each sample confirms equal amounts of protein loaded into each well. The indicated band (M.W. of 113 kDa) was excised from this gel for further mass spectrometry.

Western Blot Analysis

Western blot analysis was employed to verify the presence of UVB-induced PARP-1 (M.W. of 113 kDa) within the lens epithelial cells. All the samples showed a band at the 113 kDa M.W., indicating that the enzyme is present within the cells. The 120 minute band at 113kDa showed a substantial increase in intensity, compared to the control (Fig. 8). Quantification of the immunoblot supported this observation, indicating that there was 3 times the amount of ADP-ribose polymers in the 120 minute experimental sample compared to the control (Fig. 9).

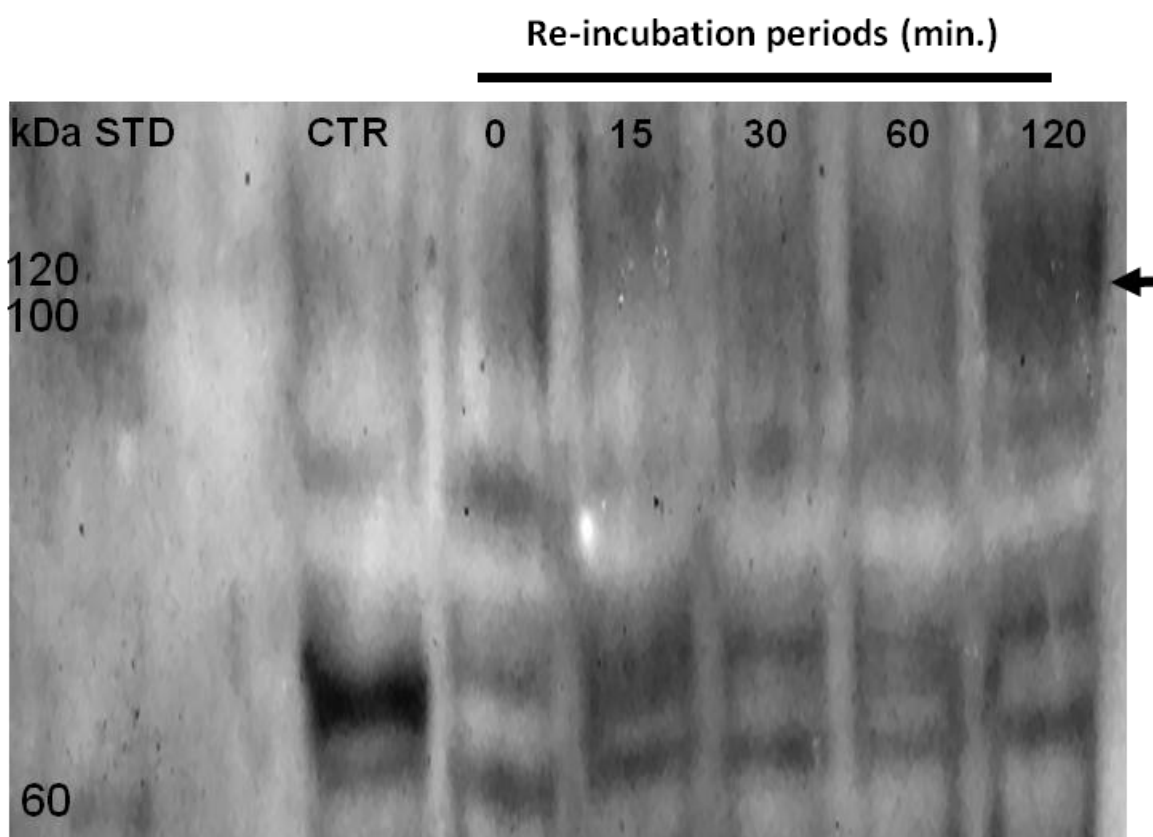


Figure 8: Western Blot Analysis

Samples were treated with 5 minutes of UVB light and re-incubated for 0, 15, 30, 60 and 120 minutes. A significant increase in activation of the 113 kDa PARP-1 enzyme can be seen in the 120 minute sample compared to the control.

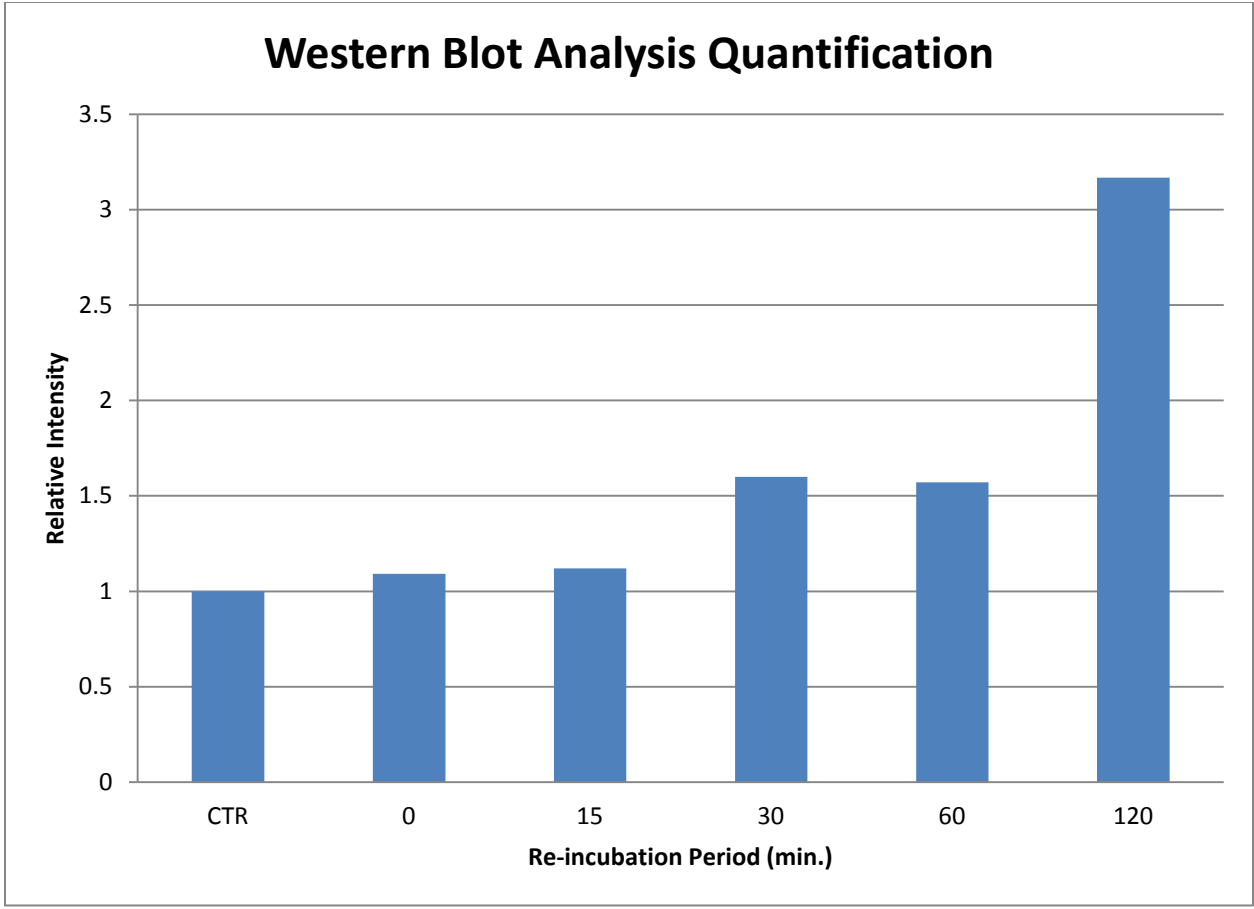


Figure 9: Western Blot Analysis Quantification

Quantification of the immunoblot shows a 3 fold increase in UVB-induced activation of PARP-1 at the 120 minute time period compared to the control.

Immunocytochemistry

Immunostaining using fluorescent antibodies was conducted to confirm the location and levels of PARP-1 activation within UVB-exposed LECs. The ADP-ribose units were stained with a green fluorescent tag attached to the secondary antibody to the anti-PAR polyclonal antibody, while the nucleus of each cell was stained red with DAPI fluorescent stain. Colocalization of the ADP-ribose polymers within the nucleus resulted in a yellow staining and was quantified using the ImageJ[®] software. The images show a strong increase in ADP-ribose polymers located in the nucleus of the experimental cells after 5 and 30 minutes of re-incubation followed by a slight decrease at 60 minutes re-incubation. The production of ADP-ribose in the nucleus is lowest at 90 minutes but then shows the largest spike after 120 minutes of re-incubation (Fig. 10). These observations are supported by quantification that shows almost 4 times the intensity of colocalization at 120 minutes of re-incubation (Fig. 11).

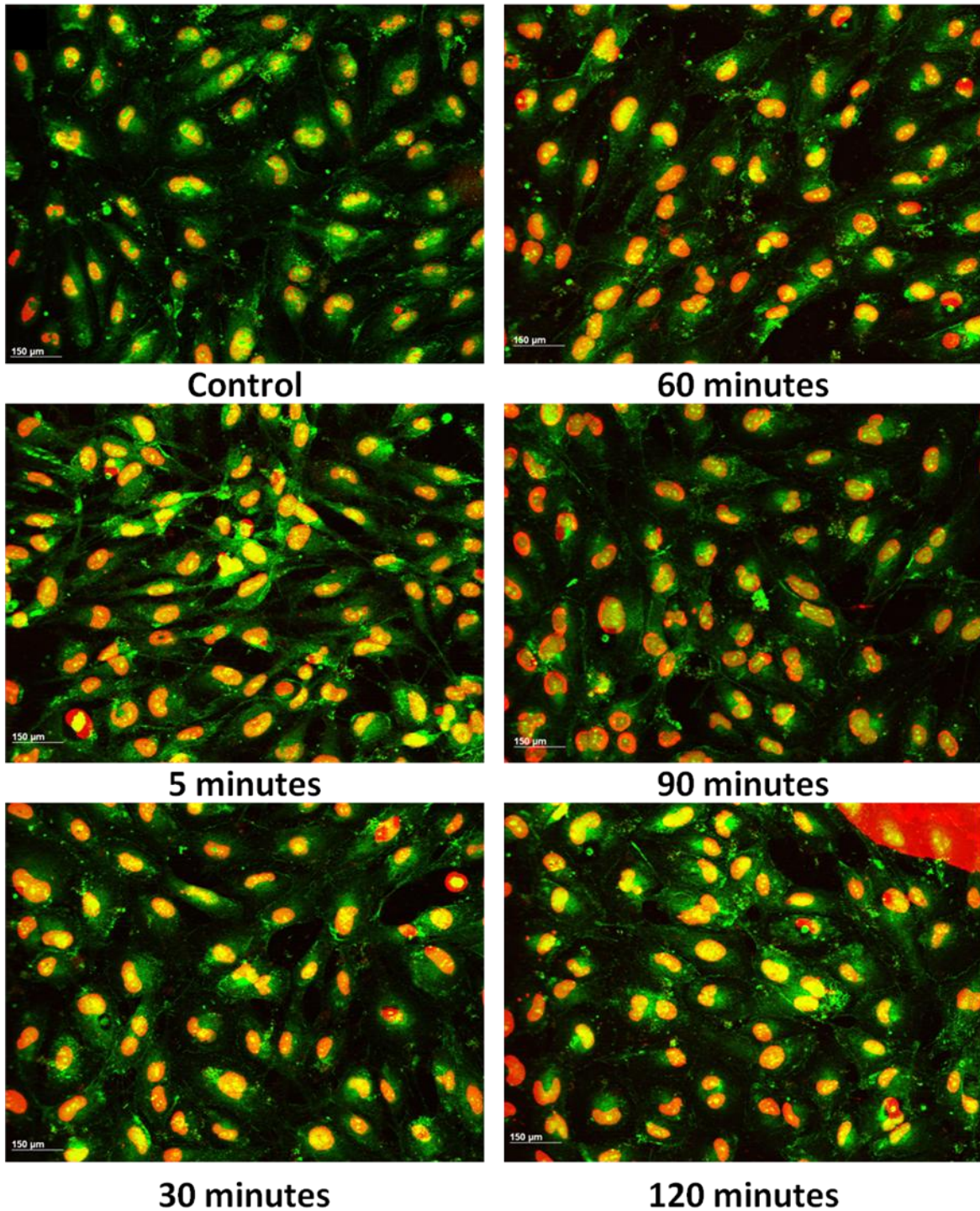


Figure 10: Immunocytochemistry of Human Epithelial Cells using PAR antibody

These images show the ADP-ribose polymers (green), the nucleus of each cell (red) and colocalization of the ADP-ribose polymers within the nucleus (yellow). The amount of ADP ribose polymers (PARP-1 activation) increases sharply in the 5, 30, and 120 minute re-incubation samples as compared to the control.

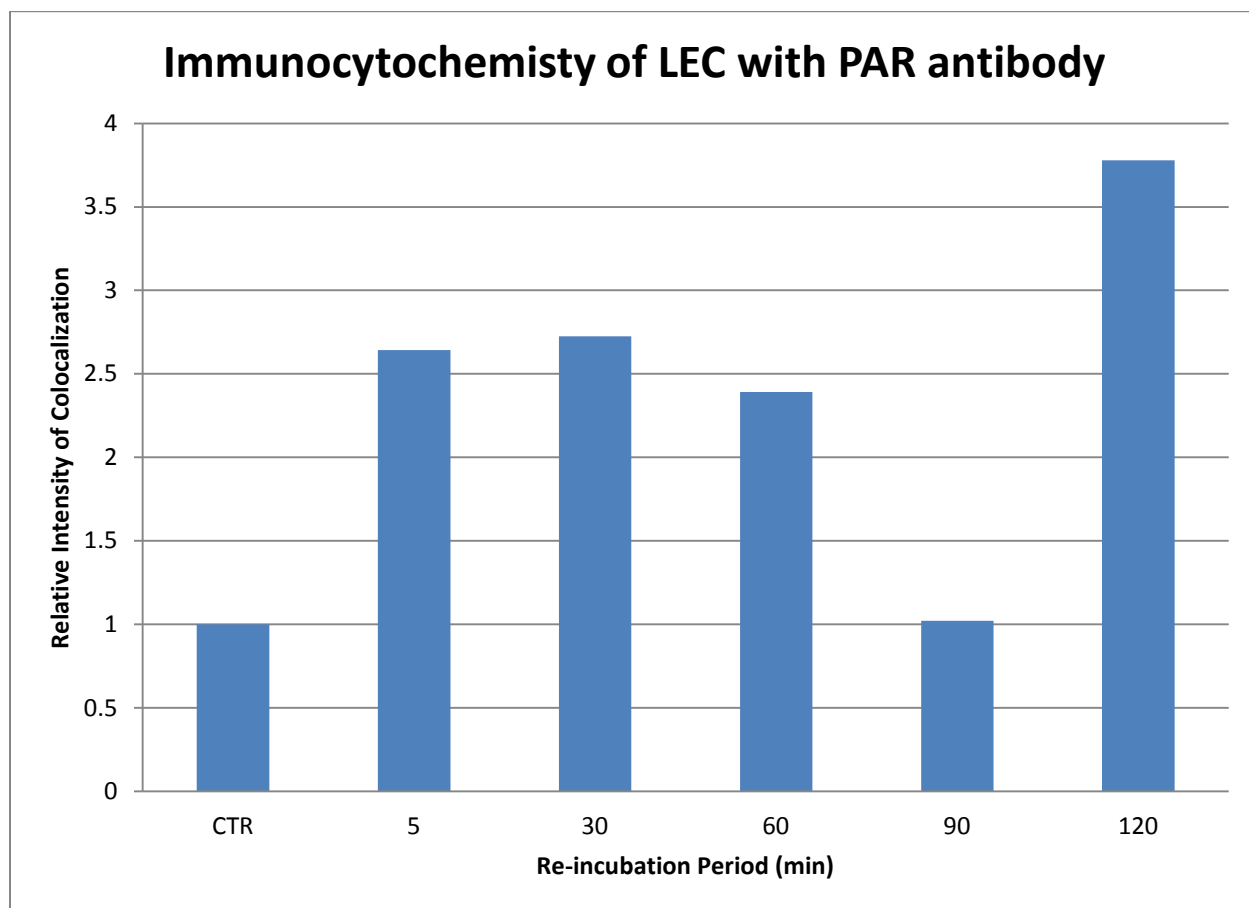


Figure 11: Quantification of Immunocytochemistry

Quantification of the immunostaining of Figure 10 shows that activation of PARP-1 subsides around 90 minutes, suggesting a biphasic activation of the PARP-1 enzyme.

Mass Spectrometry

Mass Spectrometry analysis suggested the presence of the whole PARP-1 enzyme (consisting of the automodification, catalytic and DNA binding domain) in the band that was excised from the SDS gel. The tryptic peptide weights from the commercial PARP-1 were first compared to the known sequence of the PARP-1 protein and showed strong correlation. The SDS-PAGE gel tryptic peptides were then compared to the commercial PARP-1 and also showed a strong correlation in similar molecular weights (Fig. 12 A & B). This result supports the Western blot findings (Fig. 8 & 9).

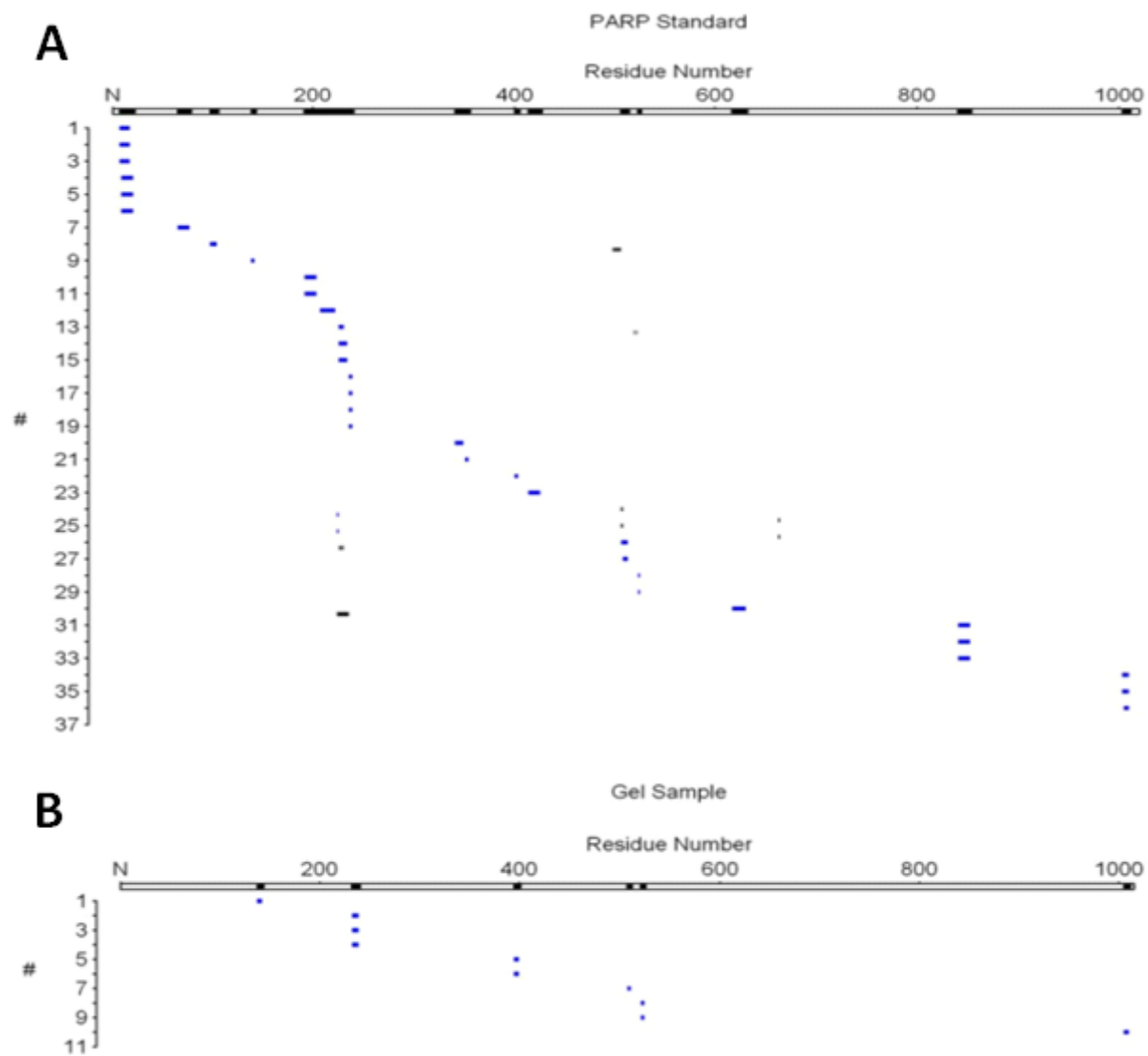


Figure 12: Mass Spectrometry Analysis

Trypsin digest of commercial PARP-1 enzyme (A). The analysis of the SDS gel protein band (B) shows matching molecular weights when compared to A.

Discussion and Conclusions

We have shown through the use of Western blot analysis and immunocytochemistry the nuclear protein poly (ADP-ribose) polymerase-1 (PARP-1) does exist in the nucleus of human lens epithelial cells. Using these techniques, we were able to show that the product of the protein is produced after the cells were challenged with UVB light and allowed to re-incubate for various time periods. After being produced, the poly (ADP-ribose) chains can either stay bound to the PARP-1 protein and facilitate repair of single strand breaks within the cell's DNA or be disassociated from the PARP-1 protein by PARG. Our Western blot shows that a band near 113 kDa contains ADP-ribose polymers that are still bound to a molecule weighing the same as PARP-1 (Fig 8). To confirm this, we used mass spectrometry to compare peptides of the sample to theoretical peptides and to the peptides of the commercial PARP-1 protein (Fig. 12). Furthermore, the location of the PAR within the cell was confirmed with the use of immunocytochemistry. The colocalization of the PAR (green dye) within the nucleus (red dye) shows that the ribose units were strongly associated with the nucleus in each experimental sample (Fig. 10).

Both the Western blot and immunocytochemistry studies show similar results after UVB treatment of the cells. While both assays show a greater than threefold increase in the activation of PARP-1 after 120 minutes of re-incubation, the immunostaining shows more of a biphasic activation of the protein over a course of time (Fig. 9 & 11). The biphasic activation of PARP-1 has been demonstrated in other cell lines, but has never been shown in human lens epithelial cells before (Vodenicharov, 2004). One explanation of this phenomenon has been attributed to the various ways in which DNA can be damaged. After the treatment of the cells, there is significant direct damage of DNA that occurs as a result of direct absorption of UVB radiation

by the DNA. This is indicated by the first large spike of PARP-1 activation seen in the immunostaining after 5 minutes of re-incubation. After this large spike, activation starts to level off and subside as the DNA is repaired and the PAR is digested by the PARG enzyme. As free radicals and reactive oxygen species accumulate in the cell due to the UVB treatment, they begin to also cause widespread DNA damage. This method of damage takes longer to occur and as such the PARP-1 activation accompanying it takes longer to show up as well. In the LECs it can be seen in the immunostaining after 120 minutes of re-incubation (Fig. 10).

By conducting the single strand break assay, we were able to demonstrate that UVB light was causing significant DNA strand breaks within the LECs. After treating the samples of cells to a measured dose of UVB light, we were able to observe the amount of DNA damage immediately after treatment and after a period of 24 hours. The results show that repair of heavily damaged DNA does take place after a period of re-incubation (Fig. 6). When compared to the Western blot and immunostaining, it is possible to conclude that PARP-1 has a significant role in facilitating that repair.

While this research supports the role of PARP-1 in the protection of DNA during UVB damage to LECs, further work needs to be completed to confirm the findings. Different protocols could be used to evaluate the presence of PAR in the cytoplasm of cells seen in the immunostaining images. The Western blot results need to be reproduced and more closely related to the immunocytochemistry findings before they can be considered completely valid. Also, the presence of multiple bands has been observed in repeated trials of the Western blots and should be further investigated to see what other proteins PAR may be interacting with.

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