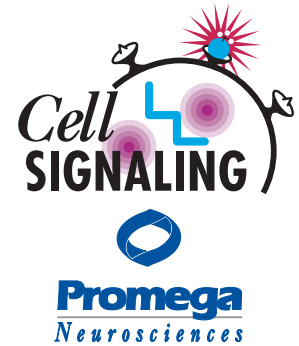


# Cleaved PARP as a Marker for Apoptosis in Tissue Sections



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The TUNEL assay, while popular for the detection of apoptotic cells, can yield highly variable results when used for apoptosis detection on research samples from various disease states. Use of a second, more specific marker of apoptosis, such as Promega's Anti-PARP p85 Fragment pAb<sup>(a)</sup>, significantly enhances identification of apoptosis in pathology research specimens.

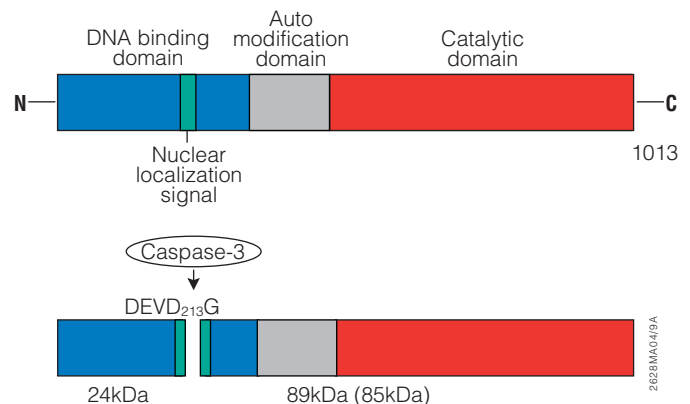
## INTRODUCTION

The detection of DNA fragmentation by the use of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay or in situ nick translation has become a standard method for the study of apoptosis in tissue sections. This technique appears particularly suited for research of diseases that are characterized by decreased cell replication and, in fact, cell death. However, the TUNEL technique can result in wide variations in the percentage of apoptosis reported for diseases such as atherosclerosis, cardiomyopathy and Alzheimer's disease. One reason for the inconsistent results using the TUNEL assay, is that small non-nuclear calcium-containing vesicles present in certain lesions can bind nucleotides (1). In addition, TUNEL staining is not specific for apoptotic cells; it was recently demonstrated in human atherosclerotic plaques that nonapoptotic nuclei showing high levels of RNA synthesis/splicing can be labeled by the TUNEL technique (2).

In the present study we colocalized TUNEL-labeled nuclei with an antibody that was developed by Promega against the C-terminal cleavage fragment ("p85" fragment) of PARP, or poly(ADP-ribose) polymerase. Full-length PARP is a 116kDa protein involved in the repair of DNA, in differentiation and in chromatin structure formation. During apoptosis this protein is cleaved by caspase-3, and possibly other caspases, into an 89kDa fragment (3). (The fragment was originally calculated to be 85kDa in size, hence the persistence of the p85 naming convention; Figure 1). Promega's Anti-PARP p85 Fragment pAb (Cat.# G7341) specifically recognizes the p85 fragment of PARP resulting from caspase cleavage and does not detect the 116kDa intact protein. The unique properties of this antibody make it a useful marker for immunohistochemical detection of apoptotic cells in research samples (4).

## IN SITU DNA END-LABELING

Tissue samples were fixed in formalin, embedded in paraffin and sectioned using standard techniques. After deparaffinization and rehydration, tissue sections were incubated with 3% citric acid to remove small calcium containing vesicles that could be responsible for nonspecific binding of the nucleotides (1). TUNEL staining was performed as described in the legend for Figure 2. Negative controls were generated by omitting TdT from the labeling mixture. Tissue from tonsils was used as a positive control (data not shown).



**Figure 1. Structure of PARP and cleavage by caspase-3.** Anti-PARP p85 Fragment pAb is directed against the C-terminal, 89kDa cleavage product of full-length PARP.

## IMMUNOHISTOCHEMICAL STAINING FOR RNA SPLICING FACTOR AND CLEAVED PARP

In order to use the same tissue sections for immunohistochemical staining (Figures 2 and 3), the TUNEL-labeled sections were destained with 1% hydrochloric acid in 70% ethanol, followed by antigen retrieval with trypsin digestion and citrate buffer treatment in a microwave oven. The sections were incubated with a mouse mAb against splicing factor (SC-35) diluted 1:200 (Figure 2) and with Anti-PARP p85 Fragment pAb (Figure 3). Anti-mouse and anti-rabbit peroxidase conjugates were used as secondary antibodies, respectively.

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## Please Note:

The images reprinted as Panels A–D of Figure 2 were originally published in the *American Journal of Pathology* (1998) 152, 885, by the *American Society of Investigative Pathology*. Promega was only granted permission to reprint the images in the print version of *Promega Notes 72*.

Please refer to [www.amjpathol.org/](http://www.amjpathol.org/) for information about accessing these images.

**Figure 2. Human atherosclerotic plaques stained by the TUNEL technique or with antibody to splicing factor (SC-35).** For TUNEL staining, sections were incubated for 1 hour at 37°C in a solution containing 25mM Tris, 200mM sodium cacodylate, 1.25mg/ml BSA, 1.25mM CoCl<sub>2</sub>, 10μM dATP (Sigma Chemical Company), 2.5μM fluorescein-dUTP (Amersham Pharmacia Biotech AB) and 50U/ml TdT (Boehringer Mannheim); the pH was adjusted to 6.6. Fluorescein-dUTP incorporation was demonstrated with a sheep anti-fluorescein peroxidase-conjugated anti-serum (Boehringer Mannheim) diluted 1:300 for 45 minutes. The peroxidase conjugate was visualized using the substrate amino ethylcarbazol, resulting in a reddish-brown product. Sections were lightly counterstained with hematoxylin and mounted in glycerin-gelatin. **Panel A:** TUNEL-positive cells (reddish-brown) are present in the upper left of the micrograph. **Panel B:** The section used for Panel A was destained (see text for details) and reacted with the antibody to splicing factor (SC-35). The cells that stained TUNEL-positive in Panel A also stained with SC-35 (note stained cells in upper left of micrograph). **Panel C:** A fibrous cap of an atherosclerotic plaque stained by the TUNEL technique; several reddish-brown cells demonstrate TUNEL staining. However, these cells are negative for SC-35 staining in **Panel D**.

As a negative control, the primary antibody staining step was omitted, resulting in the complete disappearance of staining for the splicing factor or cleaved PARP (data not shown). This control indicates that the antibody used in the last step of the TUNEL assay does not interfere with the secondary antibody used to detect the splicing factor or cleaved PARP. In addition, serial sections that had not previously been stained by TUNEL were used as controls. In this case, Anti-PARP p85 antibody staining was identical to that of the sections previously stained by the TUNEL technique (Figure 3, Panel B). This was true for SC-35 antibody staining as well (Figure 2).

## IMAGE PROCESSING

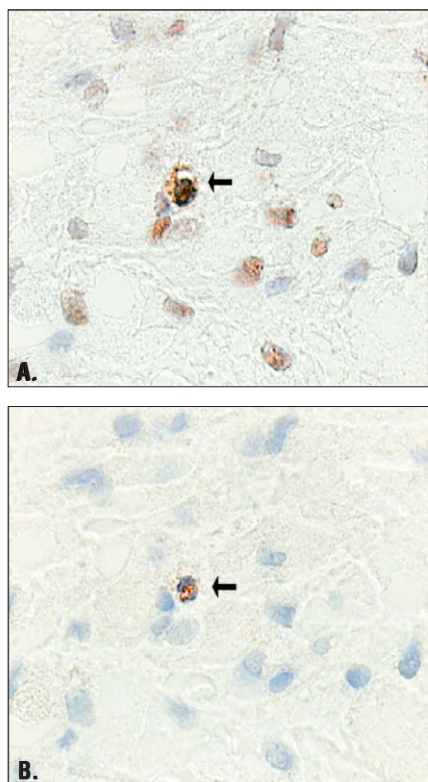
We were able to identify and compare the same tissue section using two different staining techniques through digital image processing. Digital images of the TUNEL-positive sections were saved on an optical disk using PC-Image (Fostler Findlay Associates, Newcastle-upon-Tyne, UK) and Adobe® Photoshop® 4.0 (Adobe Systems, Inc., San Jose, California). Each image was mapped in the tissue section, using a digital coordinate system (Märzhäuser, Wetzlar, Germany). The sections were then destained and restained, as noted above. To retrieve the identical tissue section regions that had been captured in the TUNEL samples, the digital X and Y coordinates used were noted and revisited for the immunohistochemical staining. The original cells in the first sections were found by opening the images from the first stain, followed by thresholding of all cells. These nuclei were placed in a bitmap and were projected as a binary overlay on the videorecorder for the exact fitting of the images. Using this technique, the TUNEL-stained cells could be analyzed further with Anti-PARP p85 staining.

## RESULTS

In human atherosclerotic plaques, regions with high percentages of TUNEL-positive nuclei were detected (Figures 2 and 3). However, as previously reported (2), most of the TUNEL-labeled nuclei are also positive when stained with an antibody directed against RNA splicing factor (SC-35; Figure 2, Panel B). The presence of splicing factor indicates a high rate of transcription in these cells. Only a small fraction of the TUNEL-stained nuclei were negative when subsequently stained for the splicing factor (Figure 2, Panel D). These TUNEL-positive and splicing factor-negative nuclei often had an apoptotic morphology (chromatin condensation and nuclear fragmentation) and showed a signal with the Anti-PARP p85 Fragment pAb (Figure 3, Panel B).

## SUMMARY

The present study supports our recent finding that a large fraction of TUNEL-positive nuclei in atherosclerotic plaques have active gene transcription, indicated by immunostaining for RNA splicing factor (2). As such, these cells do not appear to be apoptotic. The cell is still active and is transcribing genes that may or may not be related to the apoptotic process. These cells were negative when stained with Anti-PARP p85 Fragment pAb. However, the cells that were TUNEL-positive and RNA splicing factor-negative were positive when stained with the Anti-PARP antibody.



**Figure 3. Analysis of apoptosis in tissue sections by detecting genomic DNA fragmentation and PARP cleavage. Panel A: TUNEL assay.** Human atherosclerotic plaques were stained using the TUNEL technique as described in Figure 2. Numerous TUNEL-stained nuclei (reddish-brown) are visible. **Panel B: PARP cleavage.** The same tissue section in Panel A was destained (see text for details) and reacted with Anti-PARP p85 Fragment pAb (Cat.# G7341), which recognizes the C-terminal cleavage fragment only. Most of the TUNEL-positive cells in Panel A are not labeled with the antibody against cleaved PARP. This indicates that these TUNEL-positive cells are not in the final, “execution” phase of apoptosis (characterized by irreversible cleavage of chromosomal DNA) but could be TUNEL-positive as a consequence of high levels of RNA synthesis and splicing (see reference 2). Only the indicated cell (arrow), in which the nucleus is TUNEL-positive in Panel A, is reactive to the antibody against the p85 PARP cleavage product. Hematoxylin counterstaining is shown as blue in both panels.

The fact that the TUNEL technique is not specific for the late, “execution” phase of apoptosis is not surprising. This end stage of apoptosis is largely characterized by the irreversible fragmentation of chromosomal DNA. The TUNEL technique is selective but not specific for apoptotic nuclei, which contain a far greater degree of DNA fragmentation than nonapoptotic nuclei. The present study showed that high TUNEL values can be found in regions that show signs of RNA transcription and splicing. Therefore, the combination of TUNEL with a marker of RNA synthesis/splicing factor is a good technique for exclusion of nonspecific TUNEL labeling.

Staining of TUNEL-labeled tissues with Anti-PARP p85 antibody provides additional evidence that the TUNEL technique stains nonapoptotic cells in atherosclerotic plaques. Anti-PARP p85 Fragment pAb has been shown to be a useful immunohistochemical marker for specific detection of apoptotic cells. The specificity of Anti-PARP p85 Fragment pAb for apoptotic cells adds a more specific weapon to the arsenal of techniques for confirming apoptosis in research tissue specimens.

## REFERENCES

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3. Lazebnik, Y.A. *et al.* (1994) *Nature* **371**, 346.
4. *Anti-PARP p85 Fragment pAb Technical Bulletin #TB273*, Promega Corporation.



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## Ordering Information

Product	Size	Cat.#
Anti-PARP p85 Fragment pAb	50µl	G7341

## Related Products

Product	Size	Cat.#
Apoptosis Detection System, Fluorescein	60 reactions	G3250
DeadEnd™ Colorimetric Apoptosis Detection System	20 reactions	G7360
	40 reactions	G7130
CaspACE™ Assay System, Colorimetric	50 assays	G7351
	100 assays	G7220
CaspACE™ Assay System, Fluorometric	160 assays	G3540

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