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On the use of an appropriate TdT-mediated dUTP-biotin nick end labeling assay to identify apoptotic cells.

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Abstract

Apoptosis is an essential cellular mechanism involved in many processes such as embryogenesis, metamorphosis and tissue homeostasis. DNA fragmentation is one of the key markers of this form of cell death. DNA fragmentation is executed by endogenous endonucleases such as caspase-activated DNase (CAD) in caspase dependent apoptosis. The TUNEL (TdT-mediated dUTP-biotin nick end labeling) technique is the most widely used method to identify apoptotic cells in a tissue or culture and to assess drug toxicity. It is based on the detection of 3'-OH termini which are labeled with dUTP by the terminal deoxynucleotidyl transferase. Although the test is very reliable and sensitive in caspases-dependent apoptosis, it is completely useless when cell death is mediated by pathways involving DNA degradation which generates 3'-P ends like in the LEI/L-DNase II pathway. We propose here a modification in the TUNEL protocol consisting of a dephosphorylation step prior the TUNEL labeling. This allows the detection of both types of DNA breaks induced during apoptosis caspases-dependant and independent pathways, avoiding to underestimate the cell death induced by the treatment of interest.

Keywords : Apoptosis, Caspases-Independent Cell Death, Endonuclease, LEI/L-DNase II, TUNEL assay

Abbreviations :

DNAse : endonuclease, **TUNEL** : TdT-mediated dUTP-biotin nick end labelling, **CAD** : Caspase Activated Dnase, **LEI** : Leucocyte Elastase Inhibitor, **L-DNase II** : LEI-Derived DNase II**, DNAse I**: endonuclease type I, **DNAse II**: endonuclease type II, **BHK**: Baby Hamster Kidney cells, **EDTA**: Ethylenediaminetetraacetic Acid, **CIAP**: Calf Intestinal Alkaline Phosphatase, **HMA**: Hexamethyleneamiloride, **Phtase (or Ph):** Phosphatase

Introductory statement.

DNA degradation is one hallmarks of the apoptosis. This process is executed by endogenous endonucleases and occurs in several steps. First, DNA is degraded into high molecular weight fragments (50 to 300 kbp), then into oligonucleosome-sized fragments (multiple of 180 bp). Many endonucleases have been involved in the DNA cleavage during apoptosis [1]. They can be classified according to the ionic requirement for their activation in the following groups: Ca^{2+}/Mg^{2+}-dependent and Mg^{2+}-dependent endonucleases generating DNA fragments with 3'-OH/5'-P ends, and cation independent DNases producing breaks with 3'-P ends [2]. The TUNEL (TdT-mediated dUTP-biotin nick end labelling) assay [3] that reveals DNA breaks is the most widely used method to identify apoptotic cells in a tissue or cell culture, and by consequence, it is commonly used to evaluate drug toxicity.

The TUNEL technique is based on the detection of 3'-OH termini labelled with dUTP by the terminal deoxynucleotidyltransferase. This method is very sensitive in labelling breaks induced by caspase activated DNase (CAD) or other apoptotic endonucleases generating 3'-OH ends. For this reason, it has been included in many commercially available apoptosis detection kits like Apopotag (Millipore) or Dead End (Promega), among others.

Although the test is very reliable and sensitive in caspases-dependent apoptosis, it is completely useless when cell death is mediated by pathways involving DNA degradation which generates 3'-P instead of 3'-OH ends. There are many examples of these situations in which extensive DNA degradation does not produce TUNEL positive nuclei. For instance, in the rat retina, in which exposure of the animal to a continuous bright light induces photoreceptor degeneration no TUNEL positive cells are seen [4]. This model is particularly striking because the cornea cells on the same eye section present TUNEL positive cells indicating that the lack of TUNEL labelling in the retina is not a methodological failure. In this paradigm, but also in others presenting the same behaviour [5], we found that the enzyme involved in DNA degradation is L-DNase II [6-7].

L-DNase II stands for LEI-Derived DNase II. This endonuclease is derived by post-translational modification from Leukocyte Elastase Inhibitor (LEI). LEI, also known as serpin B1, has an anti-protease activity. In its native form, it inhibits elastase proteinase 3 and cathepsins G and D [4, 8]. As all serpins, LEI is a suicide inhibitor and may be cleaved by these proteases, leading to a loss of its anti-protease activity. However, unlike other serpins, the conformational modification leads to the exposure of a preexisting DNAse activity site. The change in the enzymatic activity is followed by a change in its cellular localization because the conformational modification also unveils a Nuclear Localization Sequence (NLS) that enables its nuclear translocation [6]. The activation of this nuclease has been reported in

the retina as well as different cell lines [9-10] and animal models [4, 10-18]. Different proteases can transform LEI into L-DNase II: intracellular elastases, AP-24 (apoptotic protease 24 kDa) [9] or other serine proteases [19]. Subsequently, L-DNase II can also be activated by cathepsin D as consequence of lysosomal membrane permeabilization [20]. L-DNase II, like most acid endonucleases, generates 3'-P ends [21]. Consequently, when activated, even with an extensive degradation of DNA, the TUNEL labelling remains negative, since the terminal transferase is unable to bind a d-UTP to 3'-P ends.

As a consequence, in many models where endonucleases, such as L-DNase II, are activated, dying cells remain undetectable if the TUNEL assay is used. In this paper, we describe a modification of the classical TUNEL assay revealing TUNEL negative dying cells bearing 3'-P ends DNA fragments through a few examples.

Materials and Methods

Mice retinas

Wild type (WT) C57/bl6 mice were sacrificed at 5 weeks. Eyes were mounted in Tissue Tek O.C.T. and frozen with liquid nitrogen immediately after enucleation. Then, 10 µm frozen sections were cut on a Leica CM3050S freezing microtome. Sections were incubated in 4% formaldehyde in PBS for 15 min and in 0.3% Triton X-100/PBS for 15 min. Enzymatic digestion with either DNase I or DNase II were then performed. For DNase I digestion, sections were incubated 30 min at 37°C with 2 units of DNase I in associated buffer (#EN0521, Thermo Scientific). For DNase II digestion, sections were incubated 30 min at 37°C with 10 units of L-DNase II (Worthington) in 120 mM Tris-HCl, 120 mM EDTA buffer (pH 5,5). Some sections were then dephosphorylated with 10 units of calf intestinal alkaline phosphatase (CIAP) in the associated buffer (Invitrogen, 8009-019) for 30 min. Finally, the TUNEL assay was performed on all sections following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Cell culture

Baby Hamster Kidney cells (BHK) cells were seeded at a density of 20000 cells/cm^2 and grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum, 4mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin and 5 mg/ml fungizone (all from Life Technology), at 37°C in a humidified atmosphere containing 5% CO2. They were maintained in culture for 2 days and then treated for 18 h with 40 µM Hexamethyleneamiloride (HMA).

HeLa cells (S3 clone) were grown in D-MEM with GlutaMAX TM I supplemented with 10% fetal calf serum, 4,5g/L (25mM) D-Glucose, 0,11g/L (1mM) Sodium Pyruvate, 200 U/ml penicillin and 0.2 mg/ml streptomycin (all from Life Technology), at 37°C in a humidified atmosphere containing 5% CO_2. They were seeded at a density of 60,000 cells/cm^2, maintained in culture for 2 days and then treated for 24 h with 350 µg/ml ciprofloxacin.

Both cell lines were fixed with 4% paraformaldehyde for 20 min and permeabilized with 1% Triton X-100 in PBS for 15 min. In some assays, DNases were used to treat the cells. For DNase I treatment, cells were incubated with 25 units of Deoxyribonuclease I (Sigma) for 30 min at room temperature in 50 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM $CaCl_2$ buffer (pH 7,4). For DNase II treatment, cells were incubated with 25 units of L-Deoxyribonuclease II (Worthington) for 30 min at room temperature in 120 mM Tris-HCl, 120 mM EDTA buffer (pH 5,5). For phosphatase treatment, cells were incubated for 30 min at 37°C with 20 units of CIAP in the associated dephosphorylation buffer (Invitrogen). Then the TUNEL assay was

performed following the manufacturer's instructions (Roche Diagnostics).

Rat Retinal Explants

All experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and approved by local ethical committees of University Paris Descartes. Adult males Lewis rats 6-8 weeks old (Janvier, Le Genest-Saint-Isle, France) were sacrificed by carbon dioxide inhalation. After enucleation, retinas were immediately isolated under aseptic conditions. They were flattened by 4 orthogonal incisions, then transferred onto a Cyclopore 0.2 µm polycarbonate membrane (Whatman, Maidstone, England), the vitreal side up. The membranes were then placed in 6-well tissue culture plates containing 2 ml D-MEM supplemented with 10% steroid-free FCS, 1% penicillin-streptomycin and 0.1% Amphotericin-B. Explants were treated with 0.5 mg/ml Hydrocortisone in media supplemented with 2% Decomplemented steroid-free FCS for 24 h. The retinas were then washed and fixed with 4% paraformaldehyde for 15 min, permeabilized with 1% Triton X-100, in PBS and incubated with anti vonWillebrand or anti-LEI primary antibodies at a 1:100 dilution overnight. Retinas were then incubated accordingly with one of the following secondary antibodies for 1 h: Alexa Fluor® 405-conjugated goat anti-rabbit IgG and Alexa Fluor® 594-conjugated Donkey anti-mouse IgG or Alexa Fluor® 568-conjugated goat anti-chicken IgG and Alexa Fluor® 405-conjugated goat anti-rabbit IgG (1:200). For phosphatase treatment, retinas were incubated for 30 min at 37°C with CIAP in dephosphorylation buffer then washed 3 times. Retinas were finally fixed with 4% paraformaldehyde, incubated for 1 h with 80 µl of TUNEL reaction mixture at 37°C (Roche Diagnoctics). At the end of the assay, retinas were flat mounted using Fluoromount. Images were taken using a confocal laser scanning microscope Zeiss LSM 710 (Oberkochen, Germany).

Results and Discussion

We have previously reported that stress activates apoptosis as a cell death outcome without caspase activation pathways [4, 22]. This is the case of cells treated with HMA, an inhibitor of the Na-H exchanger. In this situation cell death is mediated by a caspase-independent cell death pathway, the LEI/L-DNase II. L-DNase II is an endonuclease degrading DNA and generating 3'-P ends that are not labelled by the Terminal transferase used in this assay. Figure 1 shows BHK cells treated with 40 µM HMA for 18 h and HeLa cells treated with 350 µM ciprofloxacin for 24 h. As expected, the nuclei of control cells were TUNEL negative, however the nuclei of treated cells also remained TUNEL unstained, even in cells with highly condensed DNA or with clear apoptotic morphology (arrows). To test the proper functioning of the assay, we incubated both cell types with DNase I before the TUNEL assay in order to generate 3'-OH ends. These cells indeed showed positive TUNEL staining (Figure 1, DNase I row).

Becaus we knew that L-DNase II is activated in these cells [23], and this enzyme produces 3'-P ends, we introduced a dephosphorylation step prior to the TUNEL labelling, in order to transform 3'-P into 3'-OH ends. This dephosphorylation can be done by any phosphatase. We usually use calf intestine alkaline phosphatase (CIAP) according to the manufacturer's instructions. As shown on Figure 1, the addition of this dephosphorylation step turns TUNEL negative HMA or cyprofloxacine treated cells into TUNEL positive cells. As done before, to show that this step is unmasking 3'-P ends, we incubate untreated cells with commercial DNase II (the DNase II from Worthighton is, as we have shown by protein sequencing, L-DNase II [7]); Figure 1, row DNase II shows a typical result of these experiments. After DNase II incubation, both HeLa and BHK cells remain TUNEL negative. The use of the dephosphorylation step unmasks 3'-P breaks induced by this endonuclease.

This type of result is not limited to cells in culture but also obtained in tissues. In Figure 2, slices from WT mice retina are treated with either DNase I or DNase II. Then, a TUNEL staining with or without the CIAP step is performed. On untreated retinas (Control row), no DNA cleavage was detected by TUNEL labelling. With DNase I digestion (DNase I row), DNA cleavage was detected in all nuclear layers of the retina since this endonuclease generates 3'-OH breaks recognized by the TUNEL enzyme. On the contrary, very few apoptotic nuclei are detected by a simple TUNEL assay after DNase II digestion (DNase II row, -Ph). If this was a real situation in which a toxic agent was used, the result would lead to the wrong conclusion that there was no DNA cleavage in the section. Therefore misleading us to believe that there were no dying cells, even though DNA is heavily cleaved, as revealed with a dephosphorylation preceding the TUNEL labeling.

Even more troubling,, this non-detection of apoptotic cells by the TUNEL technique can occur in pathology studies (Figure 3). Here we show an example with genetic retinal degeneration [5]. In the SCA7 mice model, we observe a reduction of the photoreceptor nuclear layer leading to a decrease in retinal functionality {Yefimova, 2010 #631}. In this degeneration, dying cells can only be detected with a dephophorylation step (SCA7, +Ph). Otherwise, we would conclude that no cell death occured (SCA7, -Ph), exactly like what happened on WT littermate mice retina (not shown). It is important to note that, without this step, no “apoptotic” cells would be identified and the retina might be considered as non-degenerating.

Erroneously concluding the lack of cell death because of the lack of TUNEL labelling could be a very important issue when evaluating the toxicity of a compound on a tissue, since the TUNEL assay is widely used to establish the disclosure of a safe compound. The results presented in this paper show that the activation of cell death effectors like L-DNase II are misrepresented in classical TUNEL assay. It can then be falsely assumed that cells are not dying because of the lack of TUNEL labelling. An example of this situation is seen on figure 4. In this figure, a flat mount of a rat retina has been treated with hydrocortisone and endothelial cells death has been investigated. Glucocorticoids are commonly used to treat various ocular pathologies, such as macular oedema. Although they are sufficient to treat this condition, our laboratory has shown that they can be toxic for some retinal cells [24]. In this example, we used a triple labelling: anti Von Willebrand factor for vessels (blue), anti LEI/L-DNase II antibody for activation of this pathway (red) and TUNEL labelling (green), pre or post dephosphorylation. The amount of TUNEL positive cells is clearly increased if a dephosphorylation step is included (+Ph column). Without this step and based only on TUNEL labelling results, we would conclude the non-toxicity of hydrocortisone on endothelial cells (-Ph column).

What we described in these examples is commonly seen in the retina, a neural tissue in which caspases are tightly controlled and where cell death frequently goes through caspase-independent pathways [4]. This is also the case of Triamcinolone treatment for macular edema where it has been shown that there is no caspase activation and TUNEL labelling remains negative [10]. To note, this procedure has already been used by ourselves and others to study DNA breaks in the lens [25-27]. but as a marginal tool, without understanding that it indeed reflects , the activation of an alternative pathway of apoptosis. Hayashi et al have already explored the concept using dephosphorylation to uncover 3’-P ends [28]. However, they matched this procedure with in situ nick end labelling technique (ISEL), not used as frequently as the TUNEL technique in regular laboratory practice.

In conclusion, we show here that a dephosphorylation step prior to TUNEL technique reveals

cells that are dying through non-caspase mediated cell death. These results are extremely important for toxicological purposes, mostly with tissues in which it is very common to evaluate the toxicity of a compound by assessing the number of TUNEL positive cells. If caspases are activated, the classic TUNEL technique is appropriate. However, if alternative pathways are activated, a misconclusion about cell death may be made.

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Captions to figures:

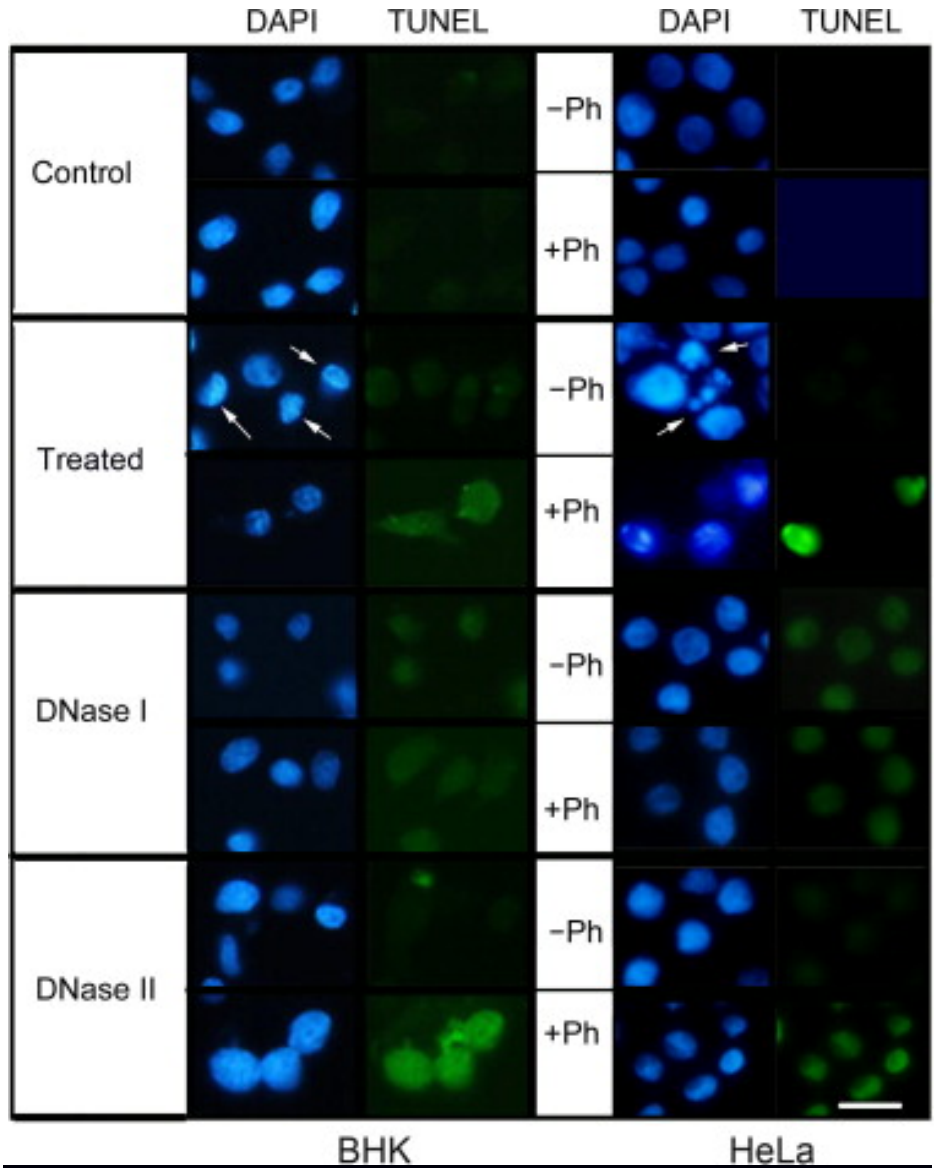

Figure 1: TUNEL assay on BHK and HeLa cells. Cells nuclei are stained with DAPI (blue) and apoptotic cells are labeled with TUNEL (green). HMA-treated BHK cells and ciprofloxacin-treated HeLa cells (Treated row) present positive TUNEL labeling only with a dephosphorylation step prior to the TUNEL assay (+Ph). Otherwise, they do not show any labelling (-Ph), such as non treated cells (C). Arrows indicate cells presenting a clearly condensed nucleus that should be labelled by the TUNEL assay. When cells are treated with DNase I (DI), we can clearly see TUNEL-labelled cells with the TUNEL assay. In the case of DNase II treated cells (DII), Cleaved DNA can only be detected with a dephosphorylation step prior to the TUNEL assay (DII, +Ph). Scale bar represents 15 µm.

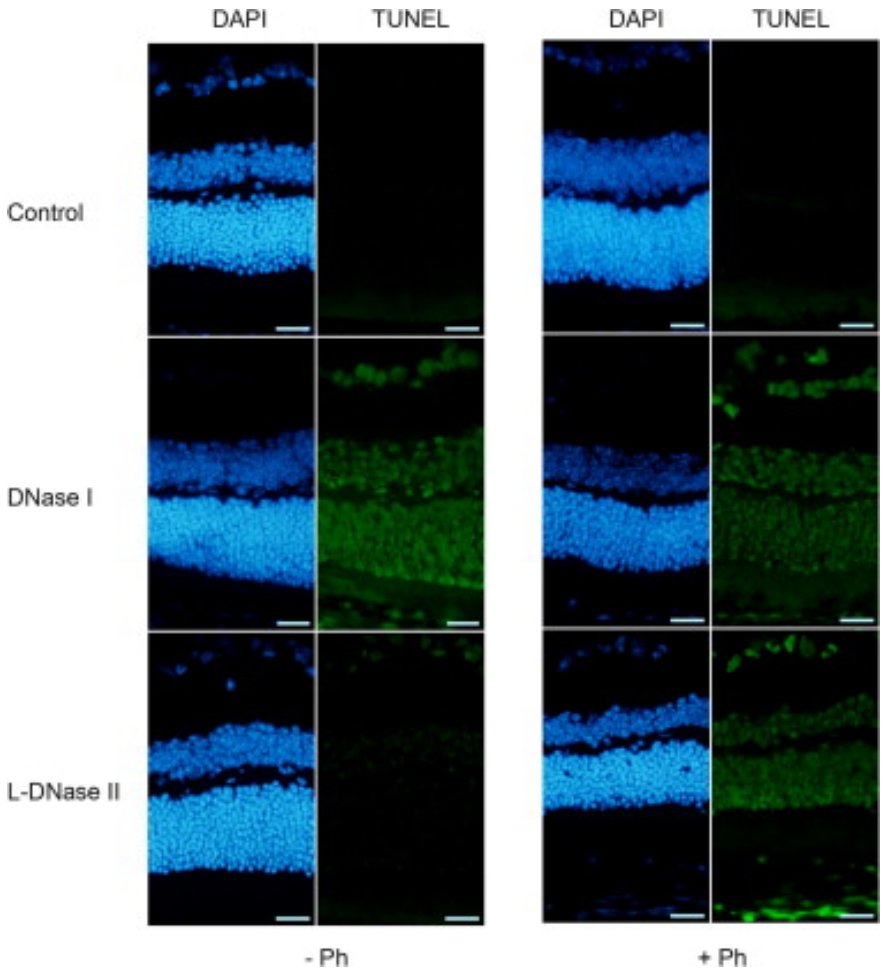

Figure 2: TUNEL assay on mice retina slices. Cells nuclei are stained with DAPI (blue) and cleaved DNA is stained with the TUNEL assay (green). Wild type mice retina sections do not present any labelled cell with the TUNEL assay whatever there is a pre phosphatase digestion or not (Control row). Inversely, DNase I treated sections showed positive cells in both conditions (DNase I, row). In the case of DNase II DNA degradation is only revealed with a previous dephosphorylation (DNase II row + Ph column). With the usual TUNEL assay, the labelling is dull or nonexistent (DNase II row-,+ Ph). Scale bar represents 10 µm.

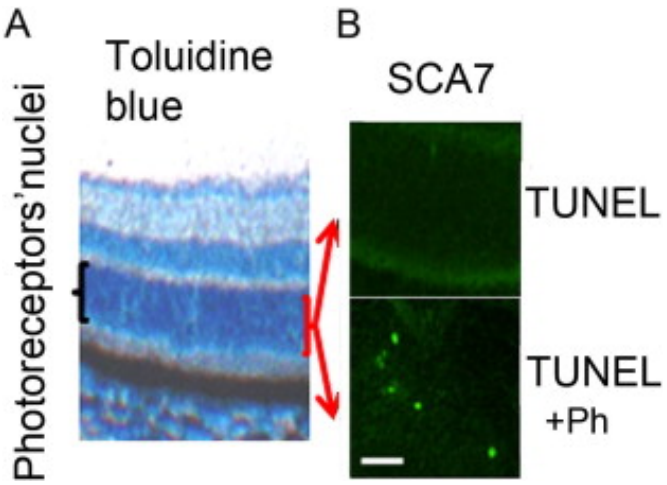

Figure 3: TUNEL assay on SCA7 mice retina slices

SCA7 mice retina section stained with Toluidine Blue (A). SCA7 mice retina section stained with TUNEL labeling (B). No apoptoic cells are detected retina labelled with a classical TUNEL assay (SCA7, TUNEL) even though they are visible when a dephophorylation is performed prior the TUNEL reaction (SCA7, TUNEL +Ph). Scale bar represents 30 µm.

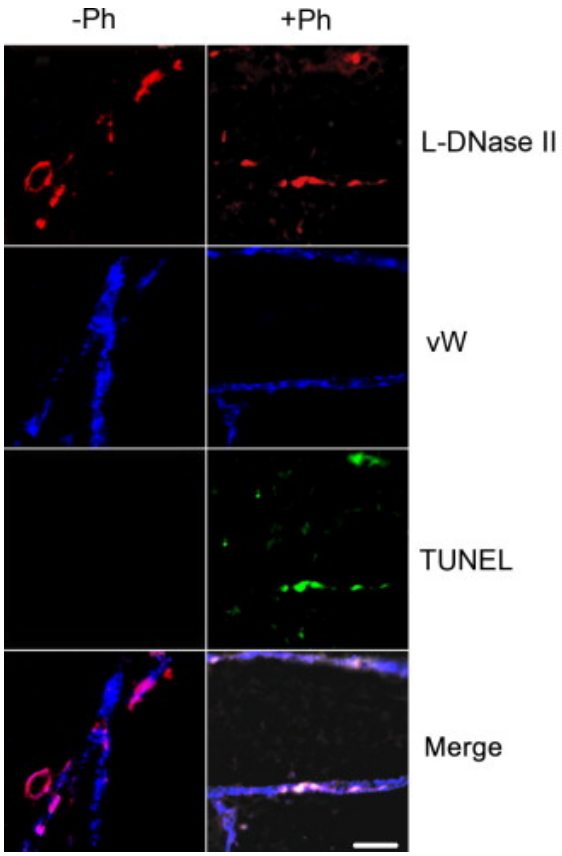

Figure 4: TUNEL technique on rat flat mounted retinas treated with hydrocortisone.

A triple labelling was performed: anti Von Willebrand factor for vessels (blue), anti LEI/L-

DNase II antibody for activation of this pathway (red) and TUNEL labelling (green) without (-Ph) or with a previous dephosphorylation step (+ Ph). LEI/L-DNase II-induced cell death is detected only with a dephosphorylation prior TUNEL staining (TUNEL +Ph). Scale bar represents 30 µm.