Early Reporting of Apoptosis by Real-time Imaging of Cancer Cells Labeled with Green Fluorescent Protein in the Nucleus and Red Fluorescent Protein in the Cytoplasm

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Abstract. *Background/Aim: We previously developed PC-3 human prostate cancer cells expressing red fluorescent protein (RFP) in the cytoplasm and green fluorescent protein (GFP) linked to histone H2B expressed in the nucleus. We demonstrate in the present report the use of these dual-color cells for early detection of apoptosis in the presence of cancer chemotherapy agents. Materials and Methods: Induction of apoptosis was observed by real-time imaging of cytoplasmic and nuclear size and shape changes and nuclear fragmentation using fluorescence microscopy. Apoptosis was also detected by measuring DNA fragmentation. The cancer chemotherapy agents paclitaxel and vinblastine were used for induction of apoptosis. Results: When the PC-3 dual-color cells were treated with paclitaxel or vinblastine, cytoplasmic and nuclear size and shape changes and nuclear fragmentation were observed by 24 hours. The paclitaxel-treated PC-3 dual-color cells exhibited ring-like structures formed by the fragmented nuclei, which could be brightly visualized by H2B-GFP fluorescence. Apoptosis was also detected by the dual-color PC-3 cells by 24 hours when treated with vinblastine. However, no nuclear ring-like structures were formed in the PC-3 cells by vinblastine treatment. In contrast, DNA fragmentation could not be observed in PC-3 cells until 48 hours after exposure to paclitaxel. Conclusion: Dual-color PC-3 cells can serve as a simple real-time early reporter of apoptosis and as a screen for novel cancer therapeutics or genotoxic agents. The dual-color cell real-time imaging assay is a more sensitive and earlier reporter for apoptosis than the DNA fragmentation assay.*

Apoptosis is a major pathway of cellular death. Current apoptosis assays are tedious involving multiple steps and are not in real-time, such as assays for caspases (1) and annexin V (2). Fluorometric caspase assays have been developed (3), but they also involve numerous steps and are not in real-time. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay (3) can label DNA fragments; however, this approach also involves many steps that are not in real-time.

The use of the histone H2B-green fluorescent protein (GFP) and red fluorescent protein (RFP) to differentially label the nucleus and cytoplasm of cancer cells has allowed the visualization of nuclear-cytoplasmic dynamics in real-time (4). Dual-color cancer cells have been used to demonstate cellular behavior in real-time, including nuclear and cytoplasmic destruction by tumor-targeting *Salmonella typhimurium* (5); nuclear and cytoplasmic deformation by cancer cells in narrow capillaries (6, 7); clasmocytosis (destruction of the cytoplasm) of cancer cells *in vivo* in real-time (8-10); nuclear-cytoplasmic behavior in real-time of cancer cells trafficking in blood vessels (11) and lymphatic vessels (12); nuclear and cytoplasmic separation during cancer cell death (9, 13); real-time apoptosis *in vitro* (4, 14) and *in vivo* (15-17); real-time mitosis *in vitro* (4) and *in vivo* (18, 19); macrophage ingestion and digestion of cancer-cell nuclei and cytoplasm (20, 21) and the role of nuclear rigidity in cancer-cell migration (6, 7, 22).

In the present report, we demonstrate the facile use of PC-3-GFP-RFP human prostate carcinoma cells to indicate the onset of early apoptosis visualized by cytoplasmic and nuclear size changes and nuclear fragmentation in real-time, readily imaged on a standard fluorescence microscope.

This paper is dedicated to the memory of A. R. Moossa, MD.

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Key Words: GFP, histone H2B, fusion gene, RFP, imaging, paclitaxel, vinblastine, nuclear fragmentation, apoptosis, DNA fragmentation, real-time, imaging.

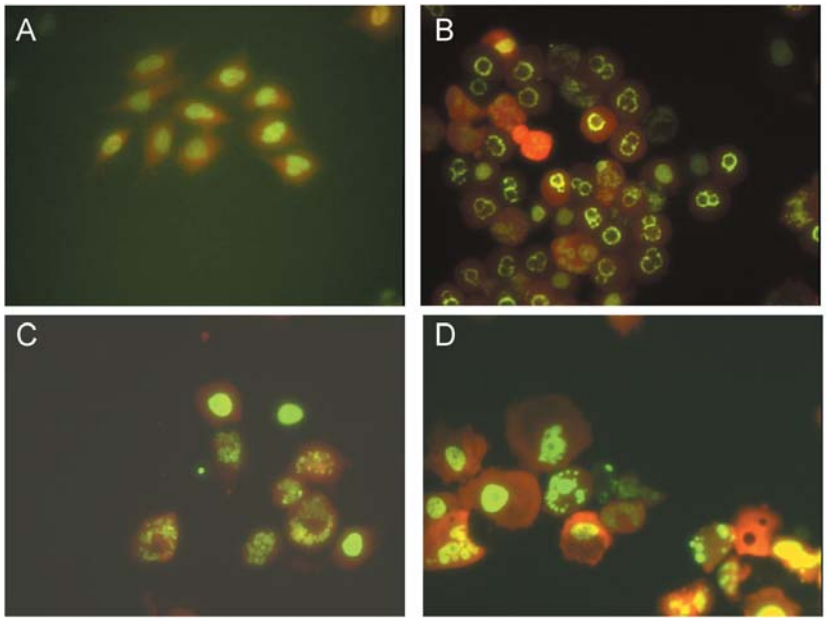

Figure 1. *Real-time imaging of cytoplasmic and nuclear events of apoptosis of PC-3 prostate cancer cells, expressing GFP in the nucleus and RFP in the cytoplasm, treated with paclitaxel. A: Time zero (T0); B: Twenty-four hours exposure to paclitaxel (0.8 μg/ml) (T24); C: Forty-eight hours exposure to paclitaxel (0.8 μg/ml) (T48); D: Ninety-six hours exposure to paclitaxel (0.8 μg/ml) (T96). All magnification: 200×*

Materials and Methods

Production of RFP retroviral vector. The Hind III/NotI fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA, USA), containing the full-length *RFP* cDNA, was inserted into the *Hind* III/*Not*I site of pLNCX2 (Clontech Laboratories, Inc.) containing the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. PT67 packaging cells (Clontech Laboratories, Inc.) were cultured in DMEM (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-products, Calabasas, CA, USA). PT67 cells, at 70% confluence, were incubated with a precipitated mixture of LipofectAMINE reagent (Life Technologies, Inc. Grand Island, NY, USA) and saturating amounts of pLNCX2-DsRed2 plasmid for 18 h. DsRed2 cells were cultured for 7 days in the presence of 200-1,000 μg/ml G418 (Life Technologies, Inc.), increased stepwise, in order to produce high amounts of pLNCS2-DsRed2 (4, 23-25).

Production of histone H2B-GFP vector. The histone H2B-GFP fusion gene was inserted at the *Hind*III/ClaI site of pLHCX (Clontech Laboratories, Inc.) that has the hygromycin resistance gene. The pLHCX histone H2B-GFP plasmid was transfected in PT67 cells, which were cultured in the presence of 200-400 μg/ml hygromicyn (Life Technologies, Inc.), increased stepwise, in order to produce high levels of pLHCX histone H2B-GFP plasmid (4, 23-25).

RFP and histone H2B-GFP gene transduction of human prostate cancer cells. To establish dual-color cells, clones of PC-3 were first established expressing RFP in the cytoplasm (PC-3-RFP). Retroviral supernatants of PT67-RFP cells and PC-3 cells were incubated in RPMI 1640 (Mediatech, Inc., Herndon, VA, USA) containing 10% FBS and selected in G418 (200-400 μg/ml), which was increased stepwise (4, 23-25). PC-3-RFP cells were then incubated with supernatants of PT67 H2B-GFP cells and then incubated with hygromycin, which was increased stepwise up to 400 μg/ml (4, 23-25).

Visualization of the apoptotic process in live PC-3-GFP-RFP cells. To visualize the apoptotic processes in real-time, PC-3 GFP-RFP were cultured with RPMI-1640 with 10% fetal bovine serum (FBS). Paclitaxel (Pharmaceutical Buyers Intl., Cedarhurst, NY, USA) was added at 0.8 μg/ml or vinblastine (Pharmaceutical Buyers Intl.) was added at 62 μg/ml, the next day. The cells were incubated for 24, 48 or 96 hours with the chemotherapeutic drugs. The cells were visualized under standard fluorescence microscopy to observe nuclear size changes and fragmentation.

DNA fragmentation assay. DNA fragmentation was observed in PC-3-GFP-RFP cells. Cells were treated with 0.8 μg/ml paclitaxel for 24 and 48 hours. The cells' DNA was extracted with TRI Reagent™ (Sigma-Aldrich, St. Louis, MO, USA) at different time points. DNA was electrophoresed through a 1.8% agarose gel and stained with ethidium bromide.

Results and Discussion

Real-time visualization of apoptosis of PC-3-GFP-RFP cells treated with paclitaxel. Apoptosis, after paclitaxel treatment, was visualized by 24 hours. The cytoplasm and nuclei greatly swelled and many nuclei fragmented and formed ring-like structures of fragmented chromatin, which were brightly visualized by histone H2B-GFP fluorescence against a background of RFP-expressing cytoplasm, which showed

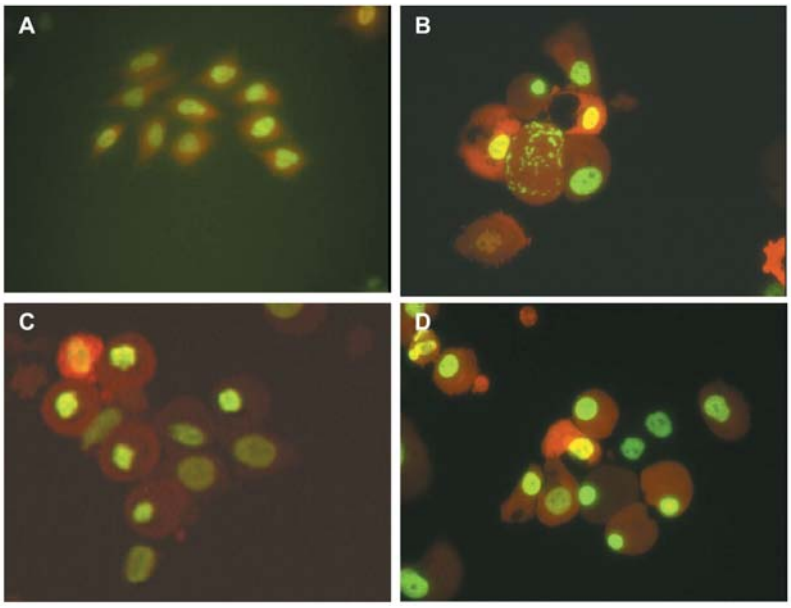

Figure 2. *Real-time imaging of cytoplasmic and nuclear events of apoptosis of PC-3 prostate cancer cells, expressing GFP in the nucleus and RFP in the cytoplasm, treated with vinblastine. A: Time zero (T0); B: Twenty-four hours exposure to vinblastine (62 μg/ml) (T24); C: Forty-eight hours exposure to vinblastine (62 μg/ml) (T48); D: 96 hours exposure to vinblastine (62 μg/ml). All magnification: 200×.*

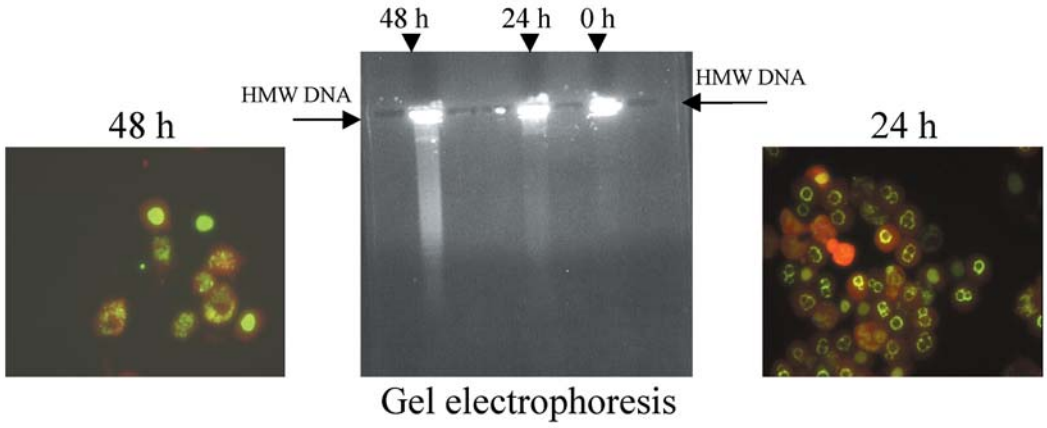

Figure 3. *Real-time imaging of cytoplasmic and nuclear events of apoptosis of PC-3 prostate cancer cells, expressing GFP in the nucleus and RFP in the cytoplasm, treated with paclitaxel compared to gel-electrophesis of DNA fragments. PC-3 cells were treated with 0.8 μg/ml paclitaxel and imaged as above for 24 or 48 hours. The PC-3 cells' DNA was also extracted with TRI ReagentTM (SIGMA) at 24 or 48 hours. The extracted DNA was electrophoresed through a 1.8% agarose gel and stained with ethidium bromide. The DNA fragmentation assay was carried out at time zero (T0) and after 24 and 48 hours exposure to paclitaxel. HMW=high molecular weight DNA.*

swelling and blebbing. This process was visualized to progress over 96 hours with subsequent cell destruction (Figure 1).

Real-time visualization of apoptosis of PC-3-GFP-RFP cells treated with vinblastine. Vinblastine also caused apoptosis of the PC-3-GFP-RFP cells (Figure 2). However, although nuclear and cytoplasmic swelling and nuclear fragmentation could be observed by 24 hours by RFP and histone H2B-GFP fluorescence, no ring-like structures were formed as with paclitaxel (Figure 2). Thus, paclitaxel and vinblastine caused very different types of nuclear fragmentation and chromatin rearrangement.

Detection of apoptosis by DNA fragmentation in PC-3-GFP-RFP cells treated with paclitaxel. DNA fragmentation visualized by gel electrophoresis could be only visualized by 48 hours and not at 24 hours treatment of PC-3 cells with paclitaxel (Figure 3).

The results of the present study demonstrate the simplicity and usefulness of cancer cells expressing H2B-GFP in the nucleus and RFP in the cytoplasm for early detection of apoptosis, in which images of cancer-cell nuclei and cytoplasm undergoing apoptosis in real-time can be readily visualized by fluorescence microscopy. The present results demonstrate that paclitaxel may be a stronger apoptotic agent than vinblastine and that paclitaxel may have a special apoptotic mechanism that causes the formation of ring-like structures of the fragmented nuclei. The results also demonstrate that imaging of cytoplasmic and nuclear shape changes and nuclear fragmentation provides earlier detection of apoptosis than gel-electrophoresis of DNA fragmentation. Thus, living cells expressing H2B-GFP in the nucleus and RFP in the cytoplasm enable the visualization of the onset and progression of apoptosis in real-time at early time points. The real-time two-color, nuclear-cytoplasmic imaging assay of apoptosis is superior than caspase and other apoptosis assays currently in use, which require multiple steps, reagents and are not in real-time. The real-time cellular assay for apoptosis described in the present report can be used to screen for more effective cancer chemotherapeutics and genotoxic agents as well as to study nuclear-cytoplasmic behavior in real time (4-26).

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