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NAPO as a novel marker for apoptosis

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Apoptosis or programmed cell death plays a pivotal role in embryonic development and maintenance of homeostasis. It is also involved in the etiology of pathophysiological conditions such as cancer, neurodegenerative, autoimmune, infectious, and heart diseases. Consequently, the study of apoptosis is now at center of both basic and clinical research applications. Therefore, sensitive and simple apoptosis detection techniques are required. Here we describe a monoclonal antibody–defined novel antigen, namely *NAPO* (negative in apoptosis), which is specifically lost during apoptosis. The anti-*NAPO* antibody recognizes two nuclear polypeptides of 60 and 70 kD. The antigen is maintained in quiescent and senescent cells, as well as in different phases of the cell cycle, including mitosis. Thus, immunodetection of *NAPO* antigen provides a specific, sensitive, and easy method for differential identification of apoptotic and nonapoptotic cells.

Introduction

During the last decade, apoptosis has become a major focus of interest for many fields of biomedical research. Programmed cell death is required for proper embryonic development as well as for the maintenance of homeostasis in adult tissues (Vaux and Korsmeyer, 1999; Wyllie and Golstein, 2001). Moreover, apoptosis is involved in the etiology and pathophysiology of a variety of diseases, such as cancer, neurodegenerative, autoimmune, infectious, and heart diseases (Chervonsky, 1999; Roulston et al., 1999; Mattson, 2000; Narula et al., 2000; Reed, 2000). Apoptotic cell death is characterized by a series of morphological changes, including cell shrinkage, nuclear condensation, chromatin segregation, membrane blebbing, formation of membrane-bound apoptotic bodies, and internucleosomal DNA cleavage (Saraste and Pulkki, 2000). These morphological changes result from a series of genetically programmed biochemical changes initiated by either the activation of death receptors or intracellular stress conditions such as DNA damage. These proapoptotic signals are conveyed to mitochondria to cause the release of caspase-activating factors from this organelle, followed by a cascade of caspase activation which leads to cell death (Earnshaw et al., 1999; Gottlieb, 2000).

Apoptosis, as a critical component of life in multicellular organisms, is a target subject for understanding cellular mechanisms of many diseases, as well as for developing new drugs that interfere with either proapoptotic or antiapoptotic molecular networks. Consequently, it has become important to develop reliable assays to measure cell death. Techniques currently available for apoptosis detection are based on the study of morphology of apoptotic cells (light and fluorescence microscopy coupled to nuclear staining with specific dyes and electron microscopy), DNA fragmentation detected by terminal transferase-mediated dUTP nick-end labeling (TUNEL)* and similar techniques, membrane changes detected by annexin V in vivo labeling, and on immunological assays using antibodies directed to apoptosis-related proteins (Stadelmann and Lassmann, 2000). Essential requirements for apoptosis detection techniques include high sensitivity for apoptotic cells, the ability to differentiate between apoptosis and other forms of cellular changes, as well as distinction between different stages of the cell death process. However, we are facing a relative paucity for simple techniques fulfilling these requirements, and furthermore allowing quantitative analysis (van Heerde et al., 2000). Immunological detection of apoptosis-related proteins is probably the best approach to overcome this obstacle, but there are only a few known apoptosis marker antigens (Stadelmann and Lassmann, 2000).

Here we describe a mouse monoclonal antibody–defined nuclear antigen composed of two polypeptides that we call *NAPO* (for negative in apoptosis), which is strongly expressed in cells under many conditions (proliferation, quiescence, mitosis, and senescence) except apoptosis. The immunoreactivity of the antigen, as tested by immunofluorescence technique,

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Key words: apoptosis; apoptotic cell death; apoptotic marker; quiescence; senescence

*Abbreviations used in this paper: TUNEL, terminal transferase–mediated dUTP nick-end labeling.

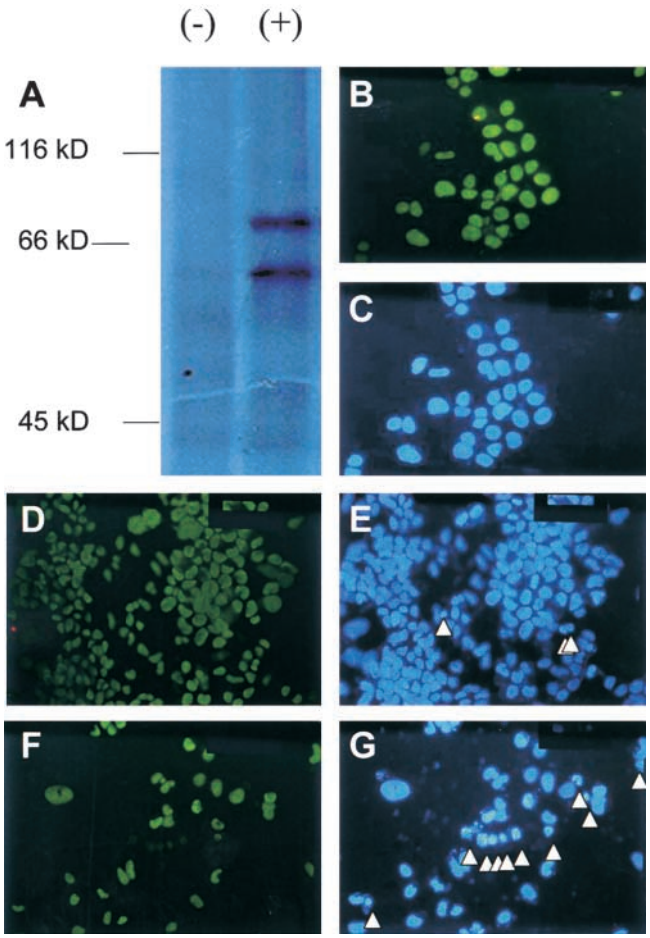

Figure 1. **Initial characterization of *NAPO* antigen.** Anti-*NAPO* monoclonal antibody recognizes two bands migrating at ~60 and 70 kD. [^{35}S]methionine-labeled Huh7 cells were subjected to immunoprecipitation with anti-*NAPO* antibody (+). (−) is a negative control (A). Immunofluorescence staining of Huh7 cells with anti-*NAPO* antibody indicates that *NAPO* is a nuclear antigen (B). Hoechst 33258 counterstain for nuclear DNA (C). *NAPO* immunofluorescence staining of SNU 398 cells growing in standard culture medium indicates that the majority of cell nuclei are positive, but occasionally some cells with small nuclei (presumably apoptotic) are negative (D), as indicated by white arrows in nuclear DNA staining (E). *NAPO* antigen is negative in apoptotic SNU 398 cells which are induced by growth in serum-free medium (F). Apoptotic cells are indicated by white arrows in Hoechst 33258 counterstaining (G).

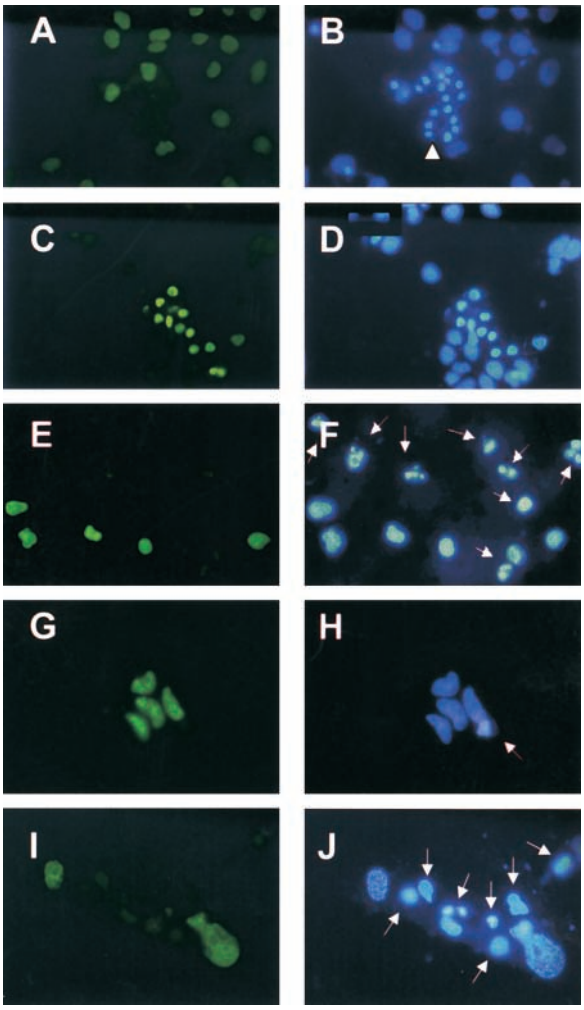

Figure 2. **Identification of *NAPO* as a common apoptosis marker.** *NAPO* is negative in 100 μM H_2O_2-treated apoptotic Huh7 cells (A), in contrast to positive staining with TUNEL (C). NAPO is also lost in Fas-mediated apoptosis in Jurkat cells (E), H_2O_2-mediated apoptosis in 293 cells (G), and UV-C–mediated apoptosis in MRC-5 cells (I). B, D, F, H, and J show Hoechst 33258 counterstaining.

is lost in apoptotic cells in a way opposite to TUNEL and annexin V staining. Thus, *NAPO* antigen may serve as a reliable marker for apoptosis.

Results and discussion

Biochemical characterization of the *NAPO* antigen

A mouse IgG monoclonal antibody (named anti-*NAPO* antibody) was generated against a nuclear antigen after immunization with human colorectal cell line COLO 320. Detergent-soluble proteins were prepared from metabolically labeled Huh7 cells and subjected to immunoprecipitation with anti-*NAPO* antibody. As shown in Fig. 1 A, anti-*NAPO* antibody recognized two proteins migrating at ~60 and 70 kD, respectively. Immunofluorescence studies revealed with the same cell line indicated that *NAPO* was a nuclear antigen (Fig. 1, B and C). This antibody was specific for human and monkey *NAPO*, since all human cell lines and monkey COS-7 showed positive immunostaining, but mouse (HC 11), rat (IAR-6), and hamster (CHOK-I) cell lines were negative (unpublished data).

Although *NAPO* antigen was ubiquitously present in all human cell lines so far tested, small nuclear fragments which are occasionally observed with some cell lines under normal culture conditions were negative (Fig. 1, D and E as an example). This suggested to us that *NAPO* antigen could be lost during apoptosis.

Identification of *NAPO* as an apoptotic marker

Hepatocellular carcinoma–derived SNU 398 cells, which undergo apoptosis when grown under serum-free conditions were serum starved for three days and tested for *NAPO* antigen immunoreactivity. Cells displaying morphological char-

Table I. **List of cell lines tested for loss of *NAPO* immunoreactivity after induction of apoptosis by various stimuli**

Cell line	Origin	Morphology	Apoptosis stimuli
Huh 7	HCC	Epithelial	H_2O_2
SNU 398	HCC	Epithelial	Serum starvation
MCF7	Breast cancer	Epithelial	TNF-α, UV-C
HeLa	Cervix cancer	Epithelial	UV-C
SW480	Colon cancer	Epithelial	UV-C
LNCaP	Prostate cancer	Epithelial	UV-C
U2OS	Osteosarcoma	Epithelial	UV-C
A375	Melanoma	Epithelial	UV-C
Jurkat	TCL	Lymphoid	Anti-Fas, UV-C
MRC-5	Lung	Fibroblastic	UV-C
293	Embryonal kidney	Epithelial	Cisplatin, H_2O_2

HCC, hepatocellular carcinoma; TCL, acute T cell leukemia.

acteristics of apoptosis (cell shrinkage, nuclear condensation, and fragmentation) displayed negative *NAPO* staining in contrast to positive nuclear staining of all nonapoptotic cells (Fig. 1, F and G).

To confirm the loss of *NAPO* antigen during apoptosis in another cellular system, hepatocellular carcinoma–derived Huh7 cells were used. H_2O_2 (100 μM) treatment of these cells induce apoptosis under serum-deficient (0.1% FCS) conditions (unpublished data). As shown in Fig. 2 A, *NAPO* antigen was negative in apoptotic Huh7 cells that are identified as cells with small nuclei by Hoechst 33258 counterstaining (Fig. 2 B). To test whether the loss of *NAPO* expression is specific to this antigen, rather than a common feature shared by nuclear proteins, we also tested Huh7 cells for p53 protein immunoreactivity under similar conditions. Huh7 cells express a mutant p53 protein that accumulate in their nuclei (Volkmann et al., 1994). Both apoptotic and nonapoptotic Huh7 cells displayed positive staining for p53 protein. Indeed, apoptotic cells displayed a stronger p53 immunoreactivity when compared with nonapoptotic cells (unpublished data). This indicated that the loss of *NAPO* immunoreactivity in apoptotic Huh7 cells was specific to this antigen rather than a common feature of nuclear proteins.

For further characterization of NAPO as an apoptosis marker, additional studies were performed in different cell lines treated with different apoptosis stimuli. For all experiments NAPO tests were run in parallel to TUNEL or annexin V staining (TUNEL data for Huh7 shown in Fig. 2 C as an example). To show whether NAPO antigen is lost during death receptor–mediated apoptosis, TNF-α–treated MCF 7 and anti-Fas antibody–treated Jurkat cells were used. NAPO was lost in apoptotic Jurkat (Fig. 2 E) as well as MCF-7 cells (unpublished data). To test whether *NAPO* loss during apoptosis was common to cells of different origin, additional tumor-derived (HeLa, U2OS, A375, SW480, LNCaP) as well as normal tissue–derived (293 and MRC-5) cell lines were induced to undergo apoptosis by H_2O_2, UV-C, or cisplatin treatment (Table I). NAPO staining was lost in all apoptotic cells in contrast to strong nuclear staining of the nonapoptotic counterparts (example data on 293 and MRC-5 cells are shown in Fig. 2, G and I, respectively).

These results demonstrate that NAPO is ubiquitously expressed in living cells, but lost during apoptosis independent of the apoptosis activating pathway (Table I). The loss of

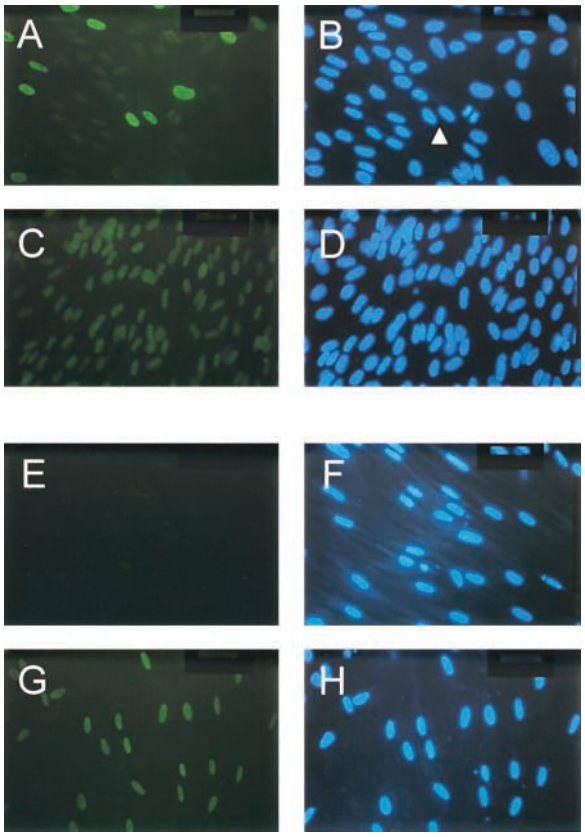

Figure 3. ***NAPO* antigen is positive in quiescent cells.** MRC-5 cells were tested in parallel for BrdU incorporation (A and E) or *NAPO* antigen (C and G). Cells in A–D were grown under standard culture conditions. Cells in panels E–H were serum starved for 3 d to induce a quiescent state, as indicated by negative BrdU staining in E. Note that both actively growing (C) and quiescent cells (G) are positive for *NAPO*. B, D, F, and H show Hoechst 33258 counterstaining.

NAPO during apoptosis strongly suggests that this antigen is a nuclear caspase substrate. The epitope recognized by anti-*NAPO* antibody on this antigen is probably lost as a result of caspase-mediated protein cleavage. However, it is presently unclear whether any of 60- and 70-kD polypeptides of the *NAPO* antigen are known caspase substrates. To our knowledge, proteins with similar molecular weight have not been described previously as apoptosis-associated proteins. Thus, *NAPO* appears to be a novel marker for apoptosis that could serve to distinguish apoptotic from nonapoptotic cells. However, it was important to know whether the antigenic reactivity is modified under different growth conditions such as quiescence, cell cycle (especially mitosis), and senescence.

Expression of the *NAPO* antigen in quiescent cells

MRC-5 human embryonic lung fibroblast cells (passage 18) were grown to confluency and serum starved for 3 d to induce quiescence. To show that these cells are indeed quiescent, BrdU incorporation was also tested. Our results indicate that ~15% of asynchronously growing MRC-5 cells are positive for BrdU i.e., in S phase (Fig. 3 A), whereas no BrdU labeling was observed in quiescent cells (Fig. 3 E). Under both conditions, all cells displayed a similarly positive nuclear staining for *NAPO* (Fig. 3, C and G). These obser-

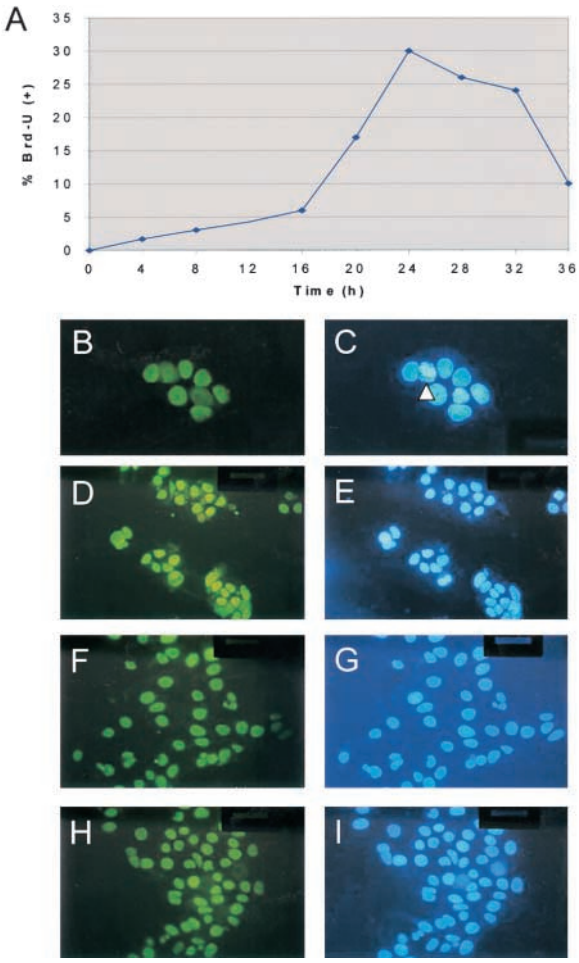

Figure 4. ***NAPO* expression during cell cycle.** Huh7 cells were synchronized by nocodazole treatment, followed by mitotic shake-off. Freshly collected cells were then grown in culture for up to 36 h. The S phase was identified by BrdU incorporation assay. Time points between 0–16, 20–32, and 36 h were evaluated respectively as G1, S, and G2 phases according to the BrdU incorporation index (A). *NAPO* and BrdU staining were performed at 4 h intervals. B, D, F, and H illustrate *NAPO* staining of cells at mitotic arrest (B), 8 h (D), 24 h (F), and 36 h (H), respectively. C, E, G, and I show Hoechst 33258 counterstaining. Note diffused *NAPO* staining in mitotically arrested Huh7 cells (B and C) which were digitally magnified threefold for better visualization.

vations indicated that *NAPO* expression is not lost in nondividing quiescent cells.

Expression of the *NAPO* antigen during cell cycle

We also analyzed the expression pattern of *NAPO* in synchronized cells in order to follow its positivity during different phases of the cell cycle. For this purpose Huh7 cells were treated with nocodazole and mitotic cells were collected by mitotic shake-off and plated onto coverslips. Synchronized Huh7 cells were tested every 4 h for 36 h of culture for both BrdU incorporation and *NAPO* staining. BrdU incorporation was minimal until 16 h after the release from mitotic arrest with a maximum of BrdU incorporation at 24 h, followed by a significant decrease at 36 h (Fig. 4 A). According to BrdU incorporation index, cells at time points before 16 h were evaluated as G1 phase cells, cells between time points 20 and 32 h as S phase cells, and those at time point 36 h as G2 phase cells. Mitotically arrested cells showed a diffusely positive (nuclear and cytoplasmic) *NAPO* staining (Fig. 4 B). *NAPO*-staining pattern was nuclear throughout the cell cycle, at all time points (time points 8, 24, and 36 h are shown in Figs. 4, D, F, and H, respectively). Thus, *NAPO* staining was always positive during the cell cycle, the only noticeable change being a diffuse staining during mitosis, in contrast to strictly nuclear staining in other phases of the cell cycle.

Expression of the *NAPO* antigen in senescent cells

To test whether *NAPO* antigen expression is modified during senescence, MRC-5 cells were grown until passage 40, at which point they remain alive and attached to cell plate, but they stop dividing, a characteristic feature of senescence (Fulder and Holliday, 1975).The senescence is often accompanied by a positive SA-β-gal activity, which is negative in presenescent cells (Dimri et al., 1995). As shown in Fig. 5, in contrast to presenescent MRC-5 cells at passage 18 (Fig. 5 A), senescent MRC-5 cells at passage 40 were positive for SA-β-gal activity (Fig. 5 B). Immunofluorescence data shown in Fig. 5, C and D, indicated that both presenescent and senescent MRC-5 cells were positive for *NAPO* antigen immunoreactivity, demonstrating that *NAPO* expression is not lost in senescent cells.

Our observations demonstrate that *NAPO* is present in living cells in all phases of the cell cycle as well as during senescence and quiescence, getting lost only during apoptosis. When compared with other available apoptosis detection systems, *NAPO* test is highly specific for apoptosis and offers the simplicity of antibody-based assays. The anti-*NAPO* antibody can be used for detection of apoptotic cells under different conditions, such as in situ staining of cells and tissue sections, and for flow cytometry. TUNEL assay (Gavrieli et al., 1992) is widely used for the identification of apoptotic cells, even though it requires several cumbersome experimental steps. As *NAPO* and TUNEL assays provide exclusive nuclear staining of alive and apoptotic cells, respectively, we believe that both assays may be combined for better identification of apoptosis. Moreover, *NAPO* assay may detect apoptotic cells before DNA fragmentation and it does not require special pretreatment of assay samples. *NAPO* may also be used in combination with annexin V staining (Martin et al., 1995). *NAPO* differs from previously identified and antibody-defined apoptosis markers (Grand et al., 1995; Zhang et al., 1996; Hammond et al., 1998; Srinivasan et al., 1998; Leers et al., 1999) by its exclusive loss in apoptotic cells, but not in quiescent, proliferating, senescent, or even mitotic cells. We believe that this antibody will be very helpful for development of simple and easy immunoassays for measurement of apoptosis in both cell lines and tissue samples.

Materials and methods

Monoclonal antibody production

10,000,000 COLO 320 cells were lysed in 2 ml PBS and 0.5 ml of lysate was injected into tail vein of Balb/c mice. 1 mo later, mice were immunized twice more at 1 wk intervals, hybridomas were prepared from splenic cells, and antibody-producing clones were selected as described previously (Ozturk et al., 1989). One of the antibodies of IgG isotype, named anti-*NAPO,* was used for further studies.

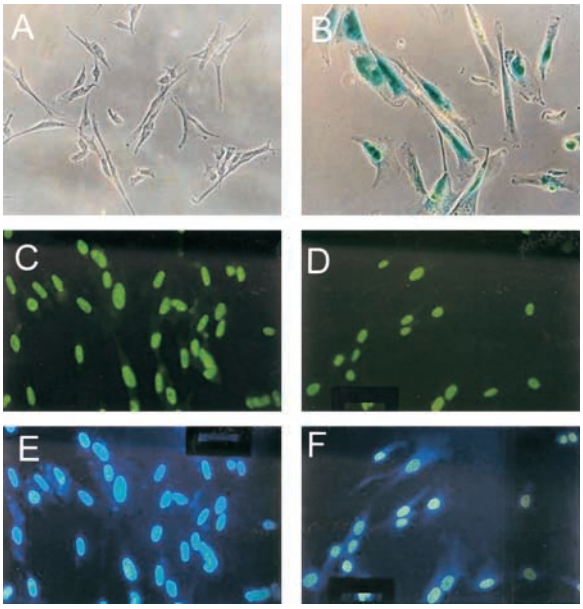

Figure 5. ***NAPO* antigen is positive in senescent cells.** Presenescent (A, C, and E) and senescent (B, D, and F) MRC-5 cells were stained for senescence-associated β-galactosidase activity (A and B), *NAPO* immunoreactivity (C and D), and Hoechst 33258 DNA staining (E and F). Note that senescence-associated β-galactosidase-positive cells are also positive for *NAPO* antigen.

Tissue culture

Huh7, SNU 398, COLO 320, MCF-7, HeLa, U2OS, SW480, A375, 293, MRC-5, COS7, IAR-6, and CHOK-I cells were grown in DME (Biochrome or GIBCO BRL). HC11 was grown in RPMI 1640 (Biological Industries) supplemented with 10 ng/ml EGF (Sigma-Aldrich) and 5 μg/ml insulin (Sigma-Aldrich). Jurkat and LNCaP cells were grown in RPMI 1640. All cells were grown in media supplemented with 10% FCS, 1% nonessential amino acids, 100 μg/ml penicillin/streptomycin at 37°C and 5% CO_2.

Induction of apoptosis

Apoptotic cell death was induced by either serum starvation or treatment with H_2O_2, UV-C, cisplatin, anti-Fas antibody or TNF-α treatment. SNU 398 hepatocellular carcinoma cells were induced in serum-free medium for 3 d and tested for apoptosis. For oxidative stress–induced apoptosis, Huh7 cells were incubated in a culture medium containing 0.1% FCS for 72 h, and treated with freshly prepared 100 μM H_2O_2 for at least 4 h before apoptosis assay. 293 cells were treated with 200 μM H_2O_2 or 100 μM cisplatin. MCF-7, HeLa, U2OS, A375, SW480, LNCaP, Jurkat, and MRC-5 cells were treated with UV-C irradiation (60–120 mJ/cm^2). For physiologically induced apoptosis studies, TNF-α–treated (Boehringer; 50 ng/ml for 72 h) MCF-7 and anti-fas antibody–treated (Upstate Biotechnology; clone CH11, 25 ng/ml for 24 h) Jurkat cells were used.

Induction of quiescence

Presenescent MRC-5 cells (passage 18) were grown to confluency on coverslips and serum starved for 3 d. At the end of 3 d, one set of cells was tested for BrdU labeling and the other set was subjected to immunofluorescence for the expression of the *NAPO* antigen as described later. Asynchronously growing MRC-5 cells of the same passage were used as a control.

Mitotic arrest and cell cycle synchronization

Huh7 cells were grown to 60% confluency and incubated with 50 ng/ml nocodazole (Sigma-Aldrich) for 18 h. Mitotic cells were collected by mitotic shake-off and replated onto coverslips. At indicated time points (between 4 and 36 h), one set of cells was tested for BrdU labeling, and the other set was subjected to immunofluorescence for the expression of the *NAPO* antigen.

Immunoprecipitation

Huh7 cells grown to 70% confluency were starved in DME lacking methionine (Sigma-Aldrich) and labeled with 200 μCi [^{35}S]methionine (Amersham Pharmacia Biotech) per 4 ml medium for 2 h. Cells were scraped in ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris pH 8.0, protease inhibitor cocktail; Roche), and centrifuged at 13,000 rpm at 4°C for 30 min. The cell lysate was incubated with anti-*NAPO* antibody for 2 h and the *NAPO* antigen was immunoprecipitated by using protein G sepharose (Amersham Pharmacia Biotech).

Immunofluorescence

Cells were grown on coverslips and fixed with 100% ice-cold acetone for 1 min or by 4% paraformaldehyde for 1 h. When paraformaldehyde was used, cells were permeabilized for 3 min with 0.1% Triton X-100 in 0.1% sodium citrate. After saturation with 3% BSA in PBS-T (0.1%) for 15 min, fixed cells were incubated with anti-*NAPO* antibody for 1 h at room temperature. FITC-conjugated goat anti–mouse antibody (Dako) was used as the secondary antibody and diluted as recommended by the supplier. The immunofluorescence staining of Huh7 cells for p53 protein was tested using 6B10 monoclonal antibody (Yolcu et al., 2001). Nuclear DNA was visualized by incubation with 3 μg/ml Hoechst 33258 (Sigma-Aldrich) for 5 min in the dark. Cover slips were then rinsed with distilled water, mounted on glass microscopic slides in 50% glycerol, and examined under fluorescent microscope (ZEISS). Jurkat cells were cytospinned (Shandon) for 3 min at 200 rpm before immunofluorescence procedures.

TUNEL and annexin V stainings

The TUNEL assay was performed using an in situ cell death detection kit (Roche), according to manufacturer's recommendations. The annexin V assay was performed by annexin V-PE reagent (PharMingen), according to manufacturer's recommendations, and cells were fixed in ethanol. After TUNEL and annexin V assays, cells were counterstained with Hoechst 33258 and examined as described.

BrdU labeling and identification of S phase cells

For BrdU incorporation, cells were incubated with 30 μM BrdU for 1 h before fixation with ice-cold 70% ethanol for 10 min. After DNA denaturation in 2 N HCl for 20 min, cells were incubated with FITC-conjugated anti-BrdU antibody (Dako) in the dilution as recommended by the supplier, cells were counterstained with Hoechst 33258 and examined as described.

Senescence-associated β-galactosidase assay

MRC-5 cells were grown to passage 40 and subjected to senescence-associated β-galactosidase (SA β-gal) assay, as described by Dimri et al. (1995). Briefly, cells were fixed in 3% formaldehyde for 5 min and incubated with SA β-gal solution (40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM potassium ferro cyanide, 5 mM potassium ferric cyanide, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/ml X-Gal) for up to 12 h, and examined under light microscope.

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