Rapid Communication

Novel Cell Imaging Techniques Show Induction of Apoptosis and Proliferation in Mesothelial Cells by Asbestos

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We developed *in situ* dual-fluorescence detection techniques for measuring apoptosis and proliferation simultaneously in single dishes of cells. The deoxyribonucleic acid (DNA)-specific labeling method, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL), first was used in conjunction with a 4′,6-diamidino-2-phenylindole (DAPI) counterstain to detect and measure morphologic characteristics of apoptotic rat pleural mesothelial (RPM) cells isolated from Fischer 344 rats and exposed to 300 μM hydrogen peroxide (H_2O_2). For this purpose, 100 TUNEL-positive nuclei were measured while being viewed with DAPI counterstaining for area, perimeter, longest diameter, and average diameter, using imaging software and an image-collection apparatus. We then exposed cells to a range of concentrations of crocidolite asbestos and putative apoptotic and mitogenic agents. Exposure to crocidolite asbestos (5 μg/cm^2) caused a striking dose-dependent apoptotic response at 24 h, 48 h, and 72 h. The nonfibrous crocidolite analogue riebeckite failed to induce apoptosis. At 24 h, tumor necrosis factor-α (TNF-α) (10 ng/ml) caused an increase in apoptotic nuclei. A second method, utilizing an antibody to 5′-bromodeoxyridine (BrdU) and oxazole yellow homodimer (YOYO), showed a dose-dependent increase in proliferation occurring in cells exposed to asbestos (5 μg/cm^2) at 48 h and 72 h. In addition, increased numbers of rat pleural mesothelial (RPM) cells exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA), TNF-α, and epidermal growth factor (EGF) exhibited incorporation of BrdU at these time points, although total numbers of cells per unit area were unchanged. Results indicate a dynamic balance between apoptosis and increased DNA synthesis after exposure of mesothelial cells to asbestos. **Goldberg, J. L., C. L. Zanella, Y. M. W. Janssen, C. R. Timblin, L. A. Jimenez, P. Vacek, D. J. Taatjes, and B. T. Mossman. 1997. Novel cell imaging techniques show induction of apoptosis and proliferation in mesothelial cells by asbestos. Am. J. Respir. Cell Mol. Biol. 17:265–271.**

Historically, carcinogenesis was thought to be a proliferative process. However, it is now known that neoplastic growth is orchestrated by an imbalance between apoptosis and proliferation (1). Apoptosis is genetically programmed cell death triggered by external stimuli, and is an important process for normal tissue development and homeostasis. Apoptosis is also observed in many pathologic conditions, such as acute lung injury (2) and pulmonary fibrosis (3). Most commonly associated with chronic cell injury, apoptosis is morphologically characterized by decreased cell and nuclear volume and condensation of chromatin during later phases of the apoptotic process (1–4). At very late stages of apoptosis, cell fragments are ultimately phagocytized by other cells or macrophages (1). Apoptosis has also been shown by biochemical methods to be a phenotypic endpoint of cell injury after exposure to oxidants such as hydrogen peroxide (H_2O_2) (5) or superoxide anion (6).

Previous studies in our laboratory (7) and others (8) have shown that increases in cell proliferation, as measured by increased numbers of cells incorporating [^{3}H]-thymidine or 5′-bromodeoxyuridine (BrdU), occur in lung after inhalation of high concentrations ($>$ 7 mg/m^3 air) of the carcinogenic and fibrogenic mineral fiber, asbestos.

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Abbreviations: 5′-bromodeoxyuridine, BrdU; calcium/magnesium-free phosphate-buffered saline, CMFPBS; 4′,6-diamidino-2-phenylindole, DAPI; epidermal growth factor, EGF; hydrogen peroxide, H_2O_2; paraformaldehyde, PFA; propidium iodide, PI; rat pleural mesothelial, RPM; room temperature, RT; terminal deoxynucleotidyl transferase, TdT; tumor necrosis factor-α, TNF-α; 12-O-tetradecanoylphorbol-13-acetate, TPA; terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling, TUNEL; oxazole yellow homodimer, YOYO.

Studies *in vitro* indicate that asbestos, but not nonfibrous particles (glass beads), induces apoptosis in rat pleural mesothelial (RPM) cells at concentrations that provoke increased expression of the protooncogenes c-*fos* and c-*jun* (9). Transcriptional activation of these genes could potentially be linked to subsequent events important in carcinogenesis or pulmonary fibrosis (10). Moreover, recent work by others confirms the induction of apoptosis by silica and asbestos in cultures of human alveolar macrophages (11) and mesothelial cells (12), respectively.

An understanding of the balance between proliferation and apoptosis in cells exposed to environmental toxicants and oxidants is critical to further understanding of the mechanisms and patterns of acute lung injury and fibrogenesis. Numerous studies have used fluorescent staining techniques for deoxyribonucleic acid (DNA) and other methods to identify cells undergoing either proliferation or apoptosis (2, 7, 9, 13, 23). However, no studies have examined the development of apoptosis and proliferation simultaneously in a cell population with a single, easily quantifiable and reproducible technique. The goal of our study was to develop an *in situ* detection technique to document, in a single culture dish of cells, patterns of apoptosis and proliferation in response to environmental stimuli. In addition, we wanted to construct an identification process for apoptotic nuclei that was simple, reproducible, and subject to statistical analysis.

In experiments described here, we used traditional fluorescent staining techniques, including terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL), 4′,6-diamidino-2-phenylindole (DAPI), and labeling with BrdU and oxazole yellow homodimer (YOYO), with computer-assisted cell imaging, to obtain quantitative indices of both cell proliferation and apoptosis in RPM cells exposed to crocidolite asbestos over a 72-h time period. In comparison with other agents (i.e., 12-O-tetradecanoylphorbol-13-acetate [TPA], epidermal growth factor [EGF], and tumor necrosis factor-α [TNF-α]) reported as mitogens (14–16) or inducers of apoptosis in other cell types (17), asbestos caused striking dose-dependent increases in both the numbers of cells incorporating BrdU and those exhibiting apoptosis.

Materials and Methods

Asbestos and Chemicals

Samples of crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$] processed by the U.S. National Institute of Environmental Health Science were obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Littleton, CO). The mean fiber length of this preparation has been previously described (18). Riebeckite, a nonfibrous mineral chemically similar to crocidolite, was purchased from Wards Natural Science (Rochester, NY). TPA was purchased from Consolidated Midland (Brewster, NY), dissolved in ethanol, and stored at −20°C. TNF-α purchased from Calbiochem (San Diego, CA) and was reconstituted at 10 μg/ml in calcium/magnesium-free phosphate-buffered saline (CMFPBS) with 0.1% bovine serum albumin (BSA), filter sterilized, and stored at −80°C. A stock solution of 0.05 mg/ml EGF in Hanks' balanced salt solution (HBSS) (GIBCO BRL, Gaithersburg, MD), was purchased from Upstate Biotechnology (Lake Placid, NY). A 30% H_2O_2 solution was purchased from Sigma Chemical Co. (St. Louis, MO).

BrdU was purchased from Zymed Laboratories (San Francisco, CA) and DAPI was purchased from Sigma Chemical Co. YOYO or YOYO-1 iodide 491/509 was purchased from Molecular Probes (Eugene, OR).

Cell Culture and Addition of Test Agents

RPM cells were isolated from adult Fischer 344 rats and propagated in Dulbecco's modified Eagle's medium (DMEM) with F12 medium (DMEM/F12 medium; GIBCO BRL) containing 10% fetal bovine serum (FBS), hydrocortisone (100 ng/ml), insulin (2.5 μg/ml), transferrin (2.5 μg/ml), and selenium (2.5 μg/ml) (HITS, Sigma). Cells for use in the study experiments were grown on two or three glass coverslips in duplicate 60-mm culture dishes containing DMEM/F12 medium supplemented with 10% FBS plus HITS. When cells were confluent, the medium was changed to 0.5% serum-containing medium for 24 h prior to the addition of test agents. Cells were used between passages 5 and 10.

H_2O_2 was diluted in CMFPBS and added to cultures at a final concentration of 300 μM. Asbestos was suspended in HBSS at 1 mg/ml, triturated eight times through a 22-gauge needle, and added to cultures at concentrations of 0.5 μg/cm^2, 1.0 μg/cm^2, and 5.0 μg/cm^2 of culture dish. Riebeckite was suspended in a manner similar to that of asbestos and added to cultures at a concentration of 5.0 μg/cm^2 of culture dish. TPA was added to medium at a final concentration of 100 ng/ml. TNF-α was added to medium at a final concentration of 10 ng/ml. EGF was diluted in HBSS and used at a final concentration of 5.0 ng/ml. Untreated control cultures were subjected to mock manipulations.

Fixation of Cells

At 24 h, 48 h, and 72 h after exposure to agents, medium was aspirated from culture dishes, and the cells were washed twice with CMFPBS. Cells then were fixed with 4% paraformaldehyde (PFA) in CMFPBS for 10 min, rinsed twice in CMFPBS for 5 min each, permeabilized for 15 min in CMFPBS plus 0.1% Triton X-100 (Sigma), and then washed three times in CMFPBS.

TUNEL Assay and DAPI Counterstain for Derivation of a Classification Function for Apoptosis in RPM Cells

To first establish criteria for measurement of apoptosis in RPM cells, we measured the nuclear dimensions of 100 apoptotic RPM cells exposed to an apoptosis-inducing concentration of H_2O_2 (300 μM) for 24 h (9), and compared the resulting numbers with the nuclear dimensions of 1,300 untreated RPM cells as visualized with the DAPI technique. For the former experiments, we used a modified version of Tornusciolo and colleagues' (19) TUNEL assay. Following fixation, cells exposed to H_2O_2 were equilibrated with TdT buffer (30 mM Tris-HCl, pH 7.2; 140 mM sodium cacodylate; 1 mM cobalt chloride). Following equilibration, the cells were incubated with TdT (25 U/100 μl TdT buffer; Boehringer Mannheim, Indianapolis, IN) and digox-

igenin-labeled deoxyuridine triphosphate (dUTP) (0.25 nmol/100 μl TdT buffer; Boehringer Mannheim, Indianapolis, IN) for 60 min at 37°C. The incubation reaction was stopped with termination buffer (300 mM sodium chloride; 30 mM sodium citrate, pH 7.0) for 15 min followed by washes with dH_2O and CMFPBS. The cells were subsequently blocked with blocking buffer (1% BSA, 0.2% powdered milk, and 0.1% Triton X-100 in CMFPBS) for 15 min. After blocking, the cells were incubated with a mouse monoclonal antidigoxigenin antibody (Boehringer Mannheim) in blocking buffer for 1 h at room temperature (RT). Cells were washed three times with blocking buffer for 15 min each and then incubated with indocarbocyanine (Cy3)-conjugated, affinity purified, donkey antimouse secondary antibody (10 μg/ml in blocking buffer; Jackson Immunoresearch Laboratories, West Grove, PA) for 45 min in the presence of DAPI (10 ng/ml). The cells were then washed twice with CMFPBS, rinsed with dH_2O, and mounted on glass slides in 90% glycerol/CMFPBS, and the mounts were sealed with nail polish.

The slides were viewed with an Olympus BX-50 immunofluorescent microscope (Olympus Corp., Tokyo, Japan) with a wide-band green (Cy3) and wide-band ultraviolet (DAPI) filter. Images (magnification ×400) were collected with a color video camera (DXC-960MD/LLP; Sony, Tokyo, Japan) and camera adapter (CMA-D2; Sony) coupled with an Olympus viewing screen and remote control unit (RM-930; Sony). Images were stored, manipulated, and analyzed with a Sun SPARCstation 5 computer (Sun Microsystems, Mt. View, CA), using IMIX/IMAGIST Version 8, Integrated Microanalyzer for Imaging software (Princeton Gamma-Tech, Princeton, NJ). Various modules of this software were incorporated in processing the images, including being used in the collection and feature-analysis modules. Images were first converted from real images to binary images. TUNEL-positive nuclei were observed, after which the cells containing the nuclei were observed with a filter for the DAPI stain. The area, perimeter, longest diameter, and average diameter measurements of 100 TUNEL-positive nuclei displaying evidence of condensation were made with the feature-analysis module. Additionally, measurements of approximately 1,300 DAPI-stained nuclei from untreated control dishes (20 random fields of 50 to 100 cells/field) were made. Utilizing these morphologic measurements, a statistical formula was generated to differentiate between apoptotic and normal RPM nuclei. Moreover, these characteristics enabled the computer to recognize and count apoptotic nuclei with the IMIX software. According to the formula ([0.56 × area] + [2.3 × perimeter] + [3.3 × longest diameter] − [17.4 × average diameter]), cutoff values < -24.1 were characterized as indicating apoptotic nuclei, and values > -24.1 were graded as indicating normal nuclei. Distributions for characteristics of apoptotic and normal nuclei provided very minimal overlap, with a less than 1% chance of misclassification.

Computer-assisted Detection of Apoptosis in RPM Cells

Using the formula presented earlier, which was derived from the combined TUNEL and DAPI technique, we then used DAPI staining, another technique for detecting apoptosis (20), and appropriate software to measure apoptosis in RPM cells over time. Cells were plated, grown, starved, treated with agents, and fixed at 24 h, 48 h, and 72 h as described earlier. Coverslips from duplicate dishes were incubated in DAPI (100 ng/ml in CMFPBS) for 1 h at 50°C, washed twice in CMFPBS at RT, and rinsed with dH_2O. The coverslips were mounted on glass slides in Vectashield (Vector Labs, Burlingame, CA), and were sealed with nail polish.

Slides were viewed as described earlier, with data collected from 10 random fields per coverslip in duplicate at a magnification of ×400. The feature-analysis module with nuclear classification formula was used to quantitate nuclear size and dimensions. Data are expressed as an average (mean ± SEM) percentage value.

BrdU/YOYO Dual Fluorescence Technique for Cell Proliferation

A second coverslip per dish (n = 2/time point/group) was evaluated according to two criteria for cell proliferation. The preparation, addition of test agents, and fixation of cells on coverslips was as described earlier after the addition of BrdU (10 μM) to cultures 24 h prior to all time points of examination. Following fixation of cells, DNA was denatured in 2 N HCl for 45 min at RT and neutralized in 0.1 M borate buffer (pH 8.5), with the neutralizing solution changed twice over a 10-min period. Coverslips were washed three times with CMFPBS over a 10-min period and incubated for 45 min at RT with a primary mouse anti-BrdU antibody (5 μg/ml in CMFPBS; DAKO, Carpinteria, CA). Following incubation, coverslips were washed three times with CMFPBS and incubated for 30 min in the dark at RT with a Cy3-conjugated, affinity purified, donkey antimouse secondary antibody (4 μg/ml in CMFPBS; Jackson Immunoresearch Laboratories, West Grove, PA) and nuclear counterstaining with YOYO (1:3,000 dilution in CMFPBS). Coverslips were washed once in CMFPBS at RT, rinsed with dH_2O, and mounted on glass slides in 90% glycerol/CMFPBS, and the mounts were sealed with nail polish. BrdU- and YOYO-labeled cells were viewed with wide-band green and blue filters, respectively, and images collected as described earlier. Quantitation of numbers of BrdU-positive cells per total numbers of YOYO-positive nuclei were determined as an average (mean ± SEM) percentage value. Total cell numbers per field with the YOYO technique were also evaluated.

Results

Cell Apoptosis

Two assays, the TUNEL and DAPI techniques, were used initially to detect apoptosis in RPM cells. Prior to using the DAPI counterstaining technique for determination of nuclear size, morphologic and statistical criteria for recognition of apoptotic nuclei were established with the *in situ* TUNEL labeling technique. Figure 1A illustrates an untreated control and Figure 1B shows an H_2O_2-treated (300 μM) sample. H_2O_2 was used as a positive control because it has been shown to cause apoptosis in RPM cells (9). The apoptotic nuclei are characterized by their condensed morphology and fragmented nature. Figure 1C demon-

strates TUNEL incorporation after treatment with H_2O_2 (300 μM). An untreated control coverslip produced no TUNEL-positive nuclei (image not shown).

After the establishment of criteria for recognizing apoptosis, RPM cells were cultured, treated with agents, and harvested at 24 h, 48 h, and 72 h as described in MATERIALS AND METHODS. Trend analysis revealed that when compared with untreated controls, exposure to crocidolite asbestos (5.0 μg/cm^2) caused a striking dose-dependent ($P < 0.05$) increase in apoptosis at 24 h, 48 h, and 72 h (Figure 2A). In addition, the cytokine TNF-α (10 ng/ml) caused significant increases in apoptosis at 24 h. In contrast, exposure to the tumor promoter TPA and to EGF produced no statistically significant increase in numbers of apoptotic cells at any point. Exposure to riebeckite, the negative control, also caused no apoptotic response.

Indices of Proliferation

Dual labeling, using BrdU and YOYO, is a highly sensitive technique for detecting DNA synthesis and DNA, re-

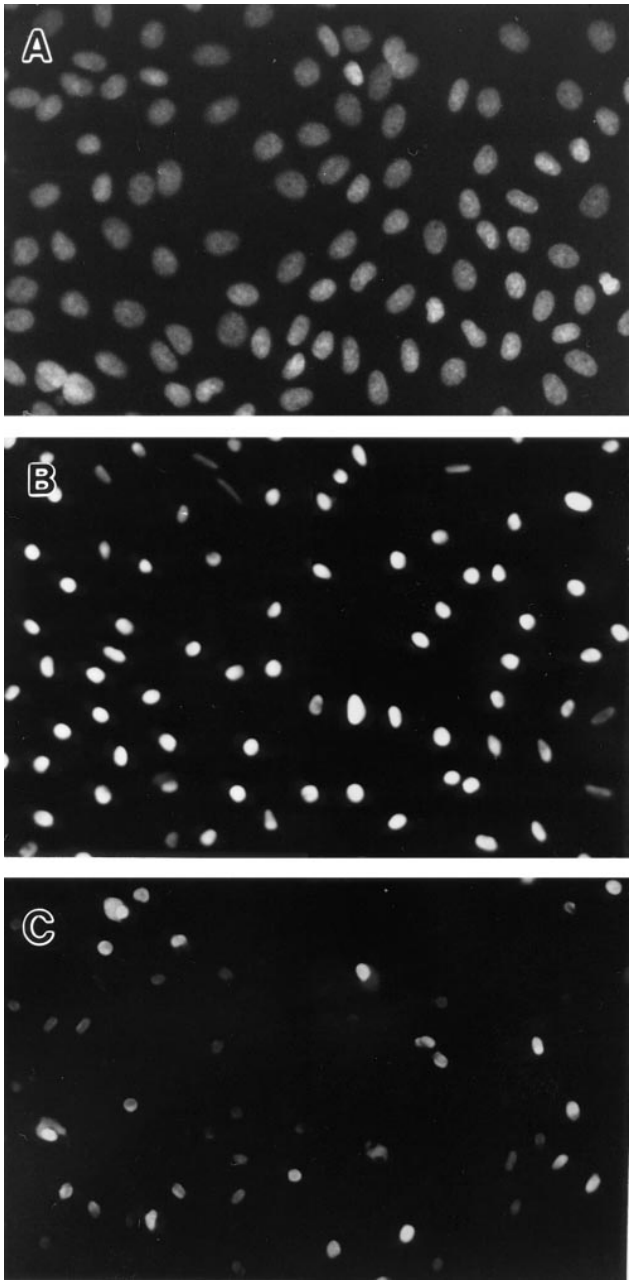

Figure 1. Intercalation of DAPI by RPM cells untreated (*A*) and exposed to H_2O_2 (300 μM) (*B*) for 1 h. Apoptotic nuclei were identified with the TUNEL method after treatment with H_2O_2, (300 μM) (*C*) for 1 h. Original magnification: ×400.

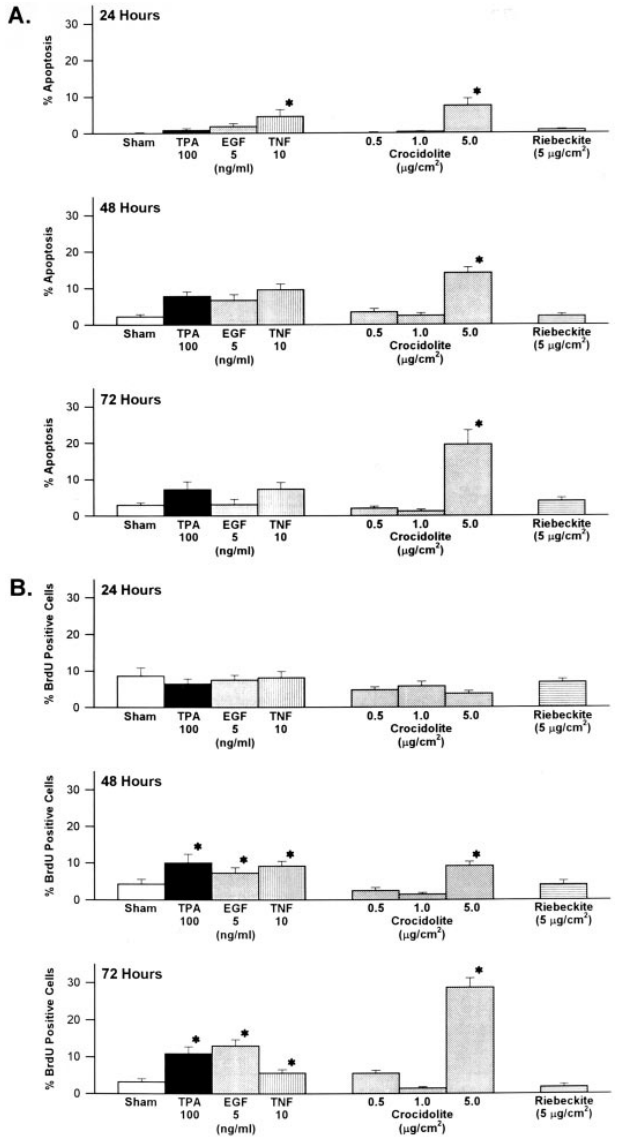

Figure 2. (*A*) Quantitation of apoptosis in RPM cells exposed to crocidolite asbestos (5.0 μg/cm^2), riebeckite (5.0 μg/cm^2), TPA (100 ng/ml), EGF (5 ng/ml), and TNF-α (10 ng/ml). (*B*) Quantitation of BrdU incorporation into RPM cells exposed to agents. Cells were prepared as described in MATERIALS AND METHODS and exposed to test agents for 24 h, 48 h, and 72 h. Experiments were performed in duplicate and results are shown as mean percentage ± SEM. *$P < 0.05$ in comparison with untreated controls at each time point.

spectively. Incorporation of BrdU and YOYO is illustrated in the right- and left-hand columns of Figure 3, respectively. Using both techniques, it was possible to count the total number of cells undergoing DNA synthesis (BrdU-positive) per total numbers of cells (YOYO-positive) in a field from a single culture dish. An untreated sample at 48 h (Figures 3A and 3B), a crocidolite (5.0 μg/cm^2)-treated sample at 48 h (Figures 3C and 3D), and a riebeckite (5.0 μg/cm^2)-treated sample at 72 h (Figures 3E and 3F) are shown. Note that in Figures 3C and 3D, most nuclei are condensed and/or fragmented. Trend analysis revealed that as compared with untreated controls, cells treated with crocidolite asbestos (5.0 μg/cm^2), TPA (100 ng/ml), EGF (5 ng/ml), and TNF-α (10 ng/ml) showed significant ($P < 0.05$) increases in BrdU incorporation at 48 h and 72 h (Figure 2B). In contrast, cells exposed to riebeckite showed no significant response at any time point. Assessment of total cell numbers with the YOYO technique

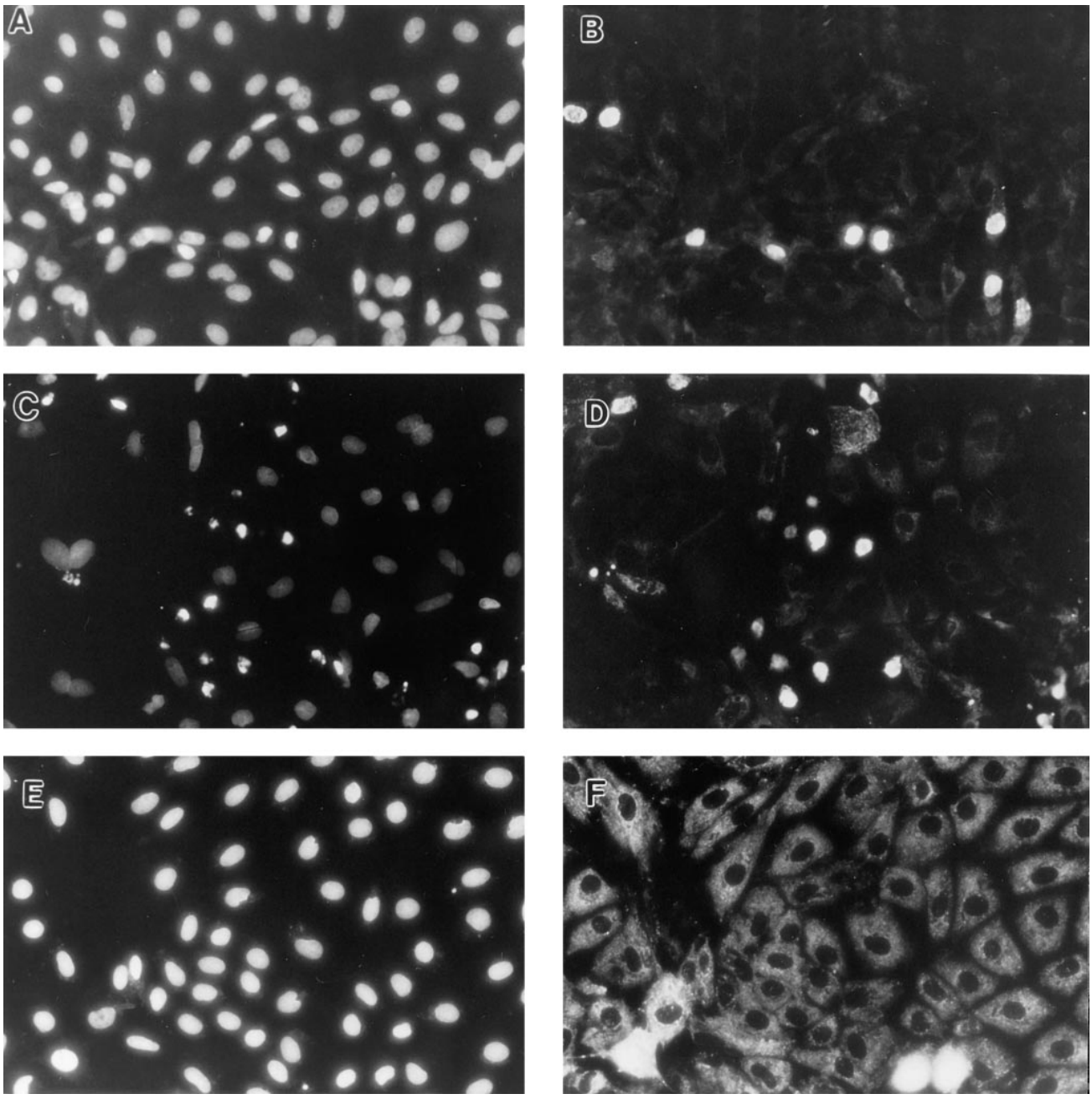

Figure 3. Incorporation of YOYO (*A*) and BrdU (*B*) by untreated RPM cells and RPM cells exposed to 5.0 μg/cm^2 crocidolite (YOYO [*C*], BrdU [*D*]) and 5.0 μg/cm^2 riebeckite (YOYO [*E*], BrdU [*F*]) for 48 h, 48 h, and 72 h, respectively. Original magnification: ×400.

showed no statistical changes in groups exposed to various agents in comparison with untreated controls during each time period (data not shown).

Discussion

Recognition of apoptotic cells is possible through a number of different techniques including the TUNEL assay, DAPI and propidium iodine (PI) staining, flow cytometry, electrophoretic DNA laddering, and DNA denaturation using acridine orange (21–23). DAPI, PI, and acridine orange are relatively inexpensive DNA-intercalating agents that permit morphologic analysis. However, these techniques applied alone are questionable and potentially variable when used in large experiments. Flow cytometry, albeit specific for identifying stages of the cell cycle, presents problems when handling samples that have been treated with mineral fibers or particulate matter, since cells associated with long fibers or agglomerates of particles may be excluded upon filtering for single-cell preparations. Quantitation of apoptosis with any statistical accuracy using electrophoretic DNA laddering is difficult. In contrast, numerous studies have used TUNEL, an extremely sensitive technique that labels 3′-OH ends in DNA fragments with fluorescein-conjugated dUTP, to detect early stages of apoptosis (22–24). Even though the specificity of this technique is highly regarded, TUNEL proves to be cost-ineffective and is not easily applied to large-scale studies. In the present study, we developed a technique that takes advantage of the specificity of the TUNEL technique, is inexpensive, and is applicable to large screening experiments. Using TUNEL to develop criteria for identifying apoptosis in RPM cells allows subsequent use of the DAPI technique alone with a precise formula to measure apoptosis.

In previous experiments, we have shown that RPM cells exposed to crocidolite asbestos (5 $\mu g/cm^2$) exhibit apoptosis when examined with flow cytometry, the DAPI, and the TUNEL techniques individually (9). Our results presented here are consistent with these observations. Exposure of RPM cells to crocidolite asbestos (5 $\mu g/cm^2$) caused a dose-dependent apoptotic response when compared with untreated controls at 24 h, 48 h, and 72 h. Moreover, when RPM cells were exposed to TNF-α (10 ng/ml), an apoptotic response was observed at 24 h but not at later time points. Proliferation was observed in cells exposed to TPA (100 ng/ml), EGF (5 ng/ml), and TNF-α (10 ng/ml) at 48 h and 72 h. We also observed that a unique response to asbestos was a dose-dependent increase in BrdU incorporation that increased in magnitude over time. As with TNF-α, increases in DNA synthesis followed increases in apoptosis at 24 h.

These results suggest that cell death by apoptosis may cause a compensatory hyperplasia, as has been observed in tracheal epithelium in organ culture after exposure to crocidolite asbestos or long chrysotile fibers (25). Our results here with riebeckite support the concept that the fibrous geometry of asbestos is important in the induction of cell death and increased DNA synthesis.

In the studies described here, RPM cells were exposed to agents of interest in a contiguous monolayer, as they occur *in situ* in the pleura. The study data indicate a dynamic balance between increased DNA synthesis, a condition favoring aberrant DNA repair and susceptibility to additional genetic errors, and cell death by apoptosis.

The novelty of our work lies not only in the generation of a statistically reproducible and inexpensive technique for measuring apoptosis, but also in demonstrating the ability of the dual-fluorescence techniques used here to provide information on numbers of cells undergoing DNA synthesis and apoptosis in the same culture dish. The YOYO method alone measures total cell nuclei per area, thus verifying whether increases in total cell numbers also occur. These nonradioactive, contemporaneous approaches allow quantification of proliferating and apoptotic cells in a single culture dish, and are especially valuable with the limited cell numbers afforded by primary isolates of alveolar Type II cells and other cell types from lung and pleura.

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