DNA Damage-inducible Genes as Biomarkers for Exposures to Environmental Agents

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A biodosimetric approach to determine alpha-particle dose to the respiratory tract epithelium from known exposures to radon has been developed in the rat. Cytotoxicity assays have been used to obtain dose-conversion factors for cumulative exposures typical of those encountered by underground uranium miners. However, this approach is not sensitive enough to derive dose-conversion factors for indoor radon exposures. The expression of DNA damage-inducible genes is being investigated as a biomarker of exposure to radon progeny. Exposure of cultures of A549 cells to alpha particles resulted in an increase in the protein levels of the DNA damage-inducible genes, *p53*, *Cip1*, and *Gadd45*. These protein changes were associated with a transient arrest of cells passing through the cell cycle. This arrest was typified by an increase in the number of cells in the G_1 and G_2 phases and a decrease in the number of cells in the *S* phase. The effect of inhaled alpha particles (radon progeny) in rats was examined in the epithelial cells of the lateral wall of the anterior nasal cavity. Exposures to radon progeny resulted in a significant increase in the number of cells in the G_1 phase and a decrease in the number of cells in the *S* phase. These cell-cycle changes were concomitant with an increase in the number of cells containing DNA strand breaks. These results suggest a commonality between cell-cycle events *in vitro* and *in vivo* following exposure to ionizing radiation. In addition to ionizing radiation, A549 cells were exposed to 4-nitroquinoline-1-oxide, methyl methanesulphonate, crocidolite asbestos, and glass microfiber. These studies showed that physical and chemical agents induce different expression patterns of p53, Cip1, and Gadd153 proteins and they could be used to discriminate between toxic and nontoxic materials such as asbestos and glass microfiber. The measurement of gene expression in A549 cells may provide a means to identify a broad spectrum of physical and chemical toxicants encountered in the environment. — *Environ Health Perspect* 105(Suppl 4):913–918 (1997)

Key words: radiation, fibers, chemicals, DNA damage-inducible genes

Introduction

The decay products of radon emit alpha particles that are considered to be the most prominent environmental cause of lung cancer and the second leading cause of lung cancer (*1*). The risk of lung cancer among the general population from indoor radon progeny exposure is currently an extrapolated value from data obtained from studies of underground miners. However, exposures in the mine and in the home can differ substantially in many ways. These differences can modify exposure–dose relationships, and assumptions need to be made concerning the use of the dose-modifying factors to derive risk estimates. Some of these factors, such as particle size, breathing pattern, and bronchial architecture, accommodate differences between mine and indoor exposures. Other factors, especially those that are dose–effect modifiers, such as exposure rate, age at exposure, age at risk, gender, smoking, exposure to other carcinogens, and the presence of airway inflammation, are not addressed in current dosimetric models (*2*) and they will affect estimates of risk from indoor radon when based on risks in mines. These differences require assumptions to be used to derive dose estimates to the lung, which are used in turn to derive estimates to the general population. Because the radiation dose to the epithelium of the human respiratory tract cannot be measured directly, a biodosimetric approach to estimate dose to the respiratory tract at cumulative exposures experienced by underground miners has been developed in the rat (*3,4*). Exposure–dose relationships were established by comparing the cytotoxic effect of alpha particles in cultured cells and in cells from rats exposed to alpha particles *in vivo*. Comparing these *in vivo* end points in which the dose was not known to similar end points from *in vitro* studies in which the dose was known gave an estimate of the biologically effective dose per unit exposure to radon progeny (*5*). Unfortunately, this biodosimetric approach using cytotoxicity as an end point is not sensitive enough to detect changes at cumulative exposures experienced in the home. Our current research is focused on using the expression of DNA damage-inducible genes as a sensitive biomarker of exposure to physical and chemical agents.

DNA damage has resulted in induction of various nuclear proteins, including p53, Cip1, Gadd45, and Gadd153 (*6,7*), and arrest of the cell cycle (*8*). However, p53 may be the most important damage response factor. Wild-type p53 also plays a pivotal role in the downregulation of genes related to transcriptional activation and cell-cycle control. *Cip1* (also known as *p21*, *Waf-1*, *Pic-1*, and *SDI-1*) and *Gadd45* appear to be downstream effector genes for *p53* involved in control of the cell cycle (*7,9,10*). *Cip1* may also be activated by a p53-independent pathway involving mitogenesis or senescence (*11,12*). Gadd45 stimulates DNA excision repair *in vitro* and inhibits entry of cells into the *S* phase (*13*). *Gadd153* is a DNA damage-inducible gene that is independent of the p53 pathway. *Gadd153* has a low basal expression such that relatively small degrees of induction can be detected. However, *Gadd153* is readily induced to high levels by many agents that cause DNA damage and/or cell-cycle arrest (*7*). The induction of *Gadd153* is transitory and is not cell-cycle regulated (*14*).

In addition to ionizing radiation, other DNA-damaging agents have been shown to increase the expression of the p53 protein. Maltzman and Czyzyk (*15*) were the first to report changes in p53 protein levels following exposure of murine cells to ultraviolet (UV) radiation and the UV mimetic

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Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; MMS, methyl methanesulfonate; 4-NQO, 4-nitroquinoline-1-oxide; UV, ultraviolet; WLM, working level months.

carcinogen 4-nitroquinoline-1-oxide (4-NQO). Fritsche et al. (*16*) exposed a murine fibroblast cell line to a variety of DNA-damaging agents and used immunohistochemistry combined with conventional fluorescence microscopy to assay p53 protein changes. Nelson and Kastan (*17*) used immunoblotting techniques to investigate the effects of various known DNA-damaging agents on human myeloid leukemia and prostatic adenocarcinoma cell lines. They showed that bleomycin, actinomycin, etoposide, and doxorubicin induced elevated levels of p53 protein, while mitomycin C and vinblastin did not alter the expression of p53 protein. The negative response with mitomycin C is in contrast with the positive response reported by Fritsche et al. (*16*). These variable responses show that the ability to induce *p53* (and therefore probably p53-regulated proteins) may be cell-type- and species-specific.

To assay a broad range of materials capable of inducing DNA damage and the subsequent expression of nuclear proteins, it is evident that proteins other than p53 need to be analyzed.

Materials and Methods

In Vitro Exposure to Alpha Particles

A549 cells, a human lung carcinoma cell line (*18*), were obtained from the American Type Culture Collection (Rochester, NY) and cultured in Ham's F12 medium supplemented with 10% heat-inactivated newborn calf serum (Sigma, St Louis, MO). The cells were grown over a 48-hr period in specially constructed 45-mm diameter culture dishes with 1.5-µm-thick mylar films serving as bottoms. Cells were exposed to alpha particles (0.6 Gy) using electroplated discs of ^{238}Pu having a activity level of approximately 2.7 Mbq. Under these conditions, cells were exposed at a dose rate of approximately 1-Gy/min (*3,4*). Nonirradiated and irradiated cells were harvested at various times after exposure, fixed in 70% methanol at –20°C, stained with either an antibody for p53 (clone DO1, Oncogene Science, Uniondale, NY), Cip1 (Ab-2, Oncogene Science), or Gadd45 (clone 4T-27, Santa Cruz Biotechnology, Santa Cruz, CA), labeled with fluorescein isothiocyanate (Vector, Burlingame, CA) and propidium iodide (Sigma), and analyzed by flow cytometry. Relative levels of the three proteins were determined by comparing the histograms from the unexposed and irradiated A549s and applying the cumulative subtraction method (*19*). The DNA content of the cells was analyzed by flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA) using MacCycle software (Phoenix Flow Systems, San Diego, CA).

In Vivo Exposure to Radon Progeny

Male Fischer 344/N rats (8–10 weeks old) were exposed to radon progeny and a vector aerosol in a closed-loop system (*3*). All experiments using animals were approved by an Institutional Animal Care and Use Committee in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. Radon generated by bubbling air through an acidic solution of $^{226}RaCl_2$ was piped to an aging chamber where the radon progeny remained for 30 to 50 min to increase concentration through ingrowth. Mainstream cigarette smoke (vector aerosol) generated from unfiltered, high-tar, high-nicotine research cigarettes (Type 1R3, Tobacco Health Research Institute, Lexington, KY) was mixed with the radon and radon progeny in the aging chamber. The rats were exposed for 3 to 5 hr in 27-in Hinners-type chambers using vector aerosols (10^4–10^5/ml smoke particles) either with radon progeny (radon-exposed) or without (controls). After exposure, the animal chambers were isolated from the radon source; the radon was exhausted from the chambers, which were purged with clean air, and the animals were removed.

Groups of control rats and rats exposed to graded concentrations of radon and radon progeny were killed with an ip injection of sodium pentobarbital after removal from the exposure or 2, 4, 6, 8, or 16 days after exposure. Two hours prior to the appropriate termination period, rats were ip injected with 50-µg 5-bromo-2′-deoxyuridine (BrdU; Sigma)/g bw dissolved in Dulbecco's phosphate-buffered saline to achieve a concentration of 50 mg/ml. The head of each rat was removed from the carcass and longitudinally sectioned. The lateral wall epithelium was removed and fixed in 10% buffered formalin. The cells of the lateral wall of the anterior nose were chosen because of their accessibility and the ease of isolating them for cell-cycle analysis by flow cytometry. Five micron-thick sections were indirectly immunostained to identify cells that had incorporated BrdU (*20*). In addition, sections were also stained to identify cells expressing Cip1 protein using a monoclonal antibody (Oncogene Science), and cells possessing DNA damage using terminal deoxynucleotidyl transferase to incorporate labeled nucleotides at sites of damage (Trevigen, Gaithersburg, MD). The number of labeled cells per millimeter epithelial basal lamina was determined in one section from each animal. In other groups of control and exposed rats, the cells of the nasal lateral wall were isolated as a suspension of single cells (*4*) and stained with Hoechst 3342 for DNA analysis by flow cytometry (*21*).

In Vitro Exposure to Physical and Chemical Agents

A549 cells were cultured for 48 hr and exposed during the log phase of growth. Cell cultures were exposed to either alpha particles, X-rays, UICC crocidolite asbestos, glass microfiber (JM Code 100) (both fiber types were obtained from the Medical Research Council, Toxicology Unit, Leicester, UK), 4-NQO (Sigma), or methyl methanesulphonate (MMS) (Sigma) for varying periods, after which the medium was changed and the cells harvested at set times postexposure. Cells were exposed to alpha particles (0.5 Gy) as described above. X-ray exposures (4 Gy) were performed using a Picker Vanguard console therapy unit (Picker X-Ray, Cleveland, OH) with a dose rate of 0.3 Gy/min. Cells were exposed to 2.0 µg/cm^2 crocidolite or glass microfiber for 20 hr or 4-NQO (250 ng/ml) or MMS (25 µg/ml) for 1 hr. Harvested cells were fixed in methanol at –20°C and immunostained with antibodies for p53 (clone DO1), Cip1 (Ab-2), or Gadd153 (clone R-20, Santa Cruz Biotechnology), and labeled with fluorescein isothiocyanate and propidium iodide. The relative number of cells expressing increased protein levels was determined as above.

Statistical Analysis

The statistical significance between groups was assessed by Student's t test; a value of $p < 0.05$ was chosen as the minimum level of significance.

Results

Alpha Particle Exposure of Cultured Lung Epithelial Cells

Following exposure of A549 cells to 0.6 Gy of alpha particles, there was an increase in the number of cells with elevated levels of p53, Cip1, and Gadd45 protein (Figure 1). The maximum expression of Gadd45 was seen 2 to 4 hr postexposure and the

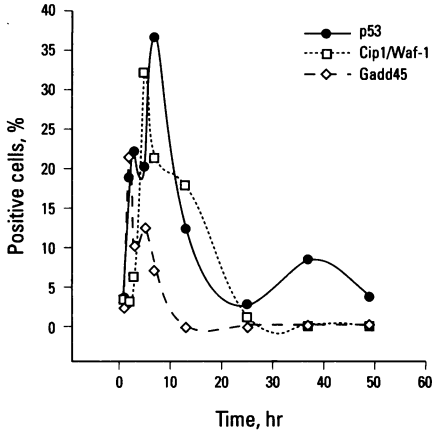

Figure 1. Protein levels of p53, Cip1, and Gadd45 in A549 cells after exposure to 0.6-Gy alpha-particle radiation. Data obtained by flow cytometric analysis of immunolabeled cells from single experiments.

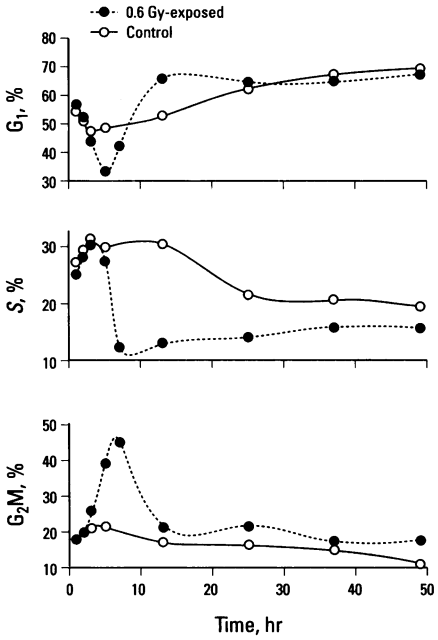

Figure 2. Number of cells in the G_1, *S*, or G_2M phase of the cell cycle in untreated cells and cells exposed to 0.6-Gy alpha-particle radiation. Data obtained from single experiments.

maximum expression of p53 and Cip1 occurred 8 to 12 hr postexposure. Following exposure to alpha particles there were marked changes in the distribution of cells among the phases of the cell cycle (Figure 2). At 6 hr postexposure, the fraction of cells in *S* phase was markedly reduced while the fraction of cells in G_2/M phase increased.

In Vivo Radon Exposure

The number of cells in the nasal lateral wall labeled with BrdU showed a significant decrease 2, 4, and 8 days after exposure (Figure 3); the decrease in cell numbers was most marked 2 days postexposure. The number of cells labeled with BrdU in rats sacrificed immediately after exposure and 16 days postexposure showed no significant difference. In rats exposed to graded concentrations of radon progeny, the number of cells labeled 2 days postexposure showed a dose-dependent decrease (data not shown). Cell-cycle analysis of cells isolated from exposed rats showed a significant increase in the number of cells in the G_1 phase 4 days postexposure (Figure 4). The number of cells with DNA strand breaks was significantly increased over control values in rats exposed to 500 working level months (WLM) and sacrificed 24 hr after exposure (Figure 5). There were no differences in number of cells with DNA strand breaks between exposed and control rats rat 48 hr after exposure. The number of cells showing unequivocal nuclear staining for Cip1 protein was not different between

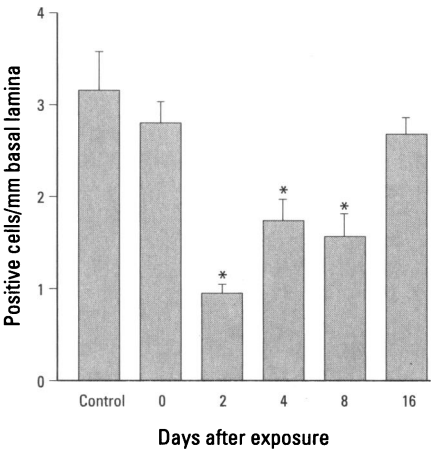

Figure 3. Number of cells labeled with BrdU in control rats and rats exposed to 500 WLM ($n = 4$). Error bars ± SD. *Significantly different from control.

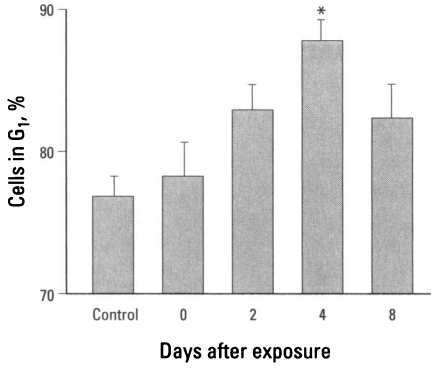

Figure 4. Fraction of cells in G_1 phase of the cell cycle at various times after exposure to 1000 WLM ($n = 5$). Error bars ± SD. *Significantly different from control.

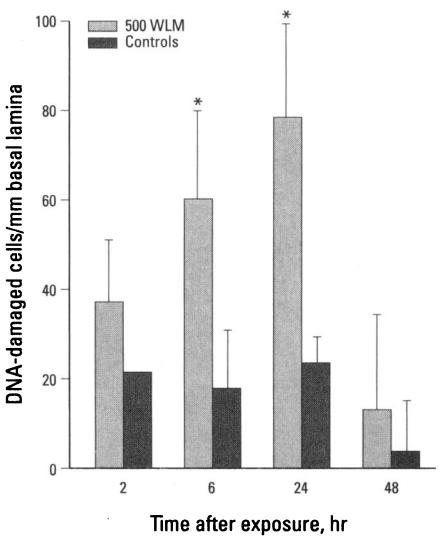

Figure 5. Number of cells with DNA strand breaks in control rats and rats exposed to 500 WLM ($n = 3$). Error bars ± SD. *Significantly different from control.

controls and exposed animals except at 6 hr postirradiation, where there were significantly more stained cells in rats exposed to radon progeny (data not shown).

Exposure of Cultured Lung Cells to Physical and Chemical Agents

The initial experiments were conducted with confluence-arrested cells that could only be induced to express limited amounts of p53, Cip1, and Gadd153 proteins after exposure to 4-NQO or MMS (Figure 6). In contrast, cells in log-phase growth had elevated levels of p53, Cip1, and Gadd153 proteins (Figure 7). All subsequent studies were conducted with cells in log-phase growth. The pattern of protein expression was different for 4-NQO and MMS; 4-NQO a sustained expression of all three proteins, while MMS exposure resulted in a sustained expression of only Gadd153 protein (Figure 7). A marked difference in protein expression was also seen between crocidolite asbestos and glass microfiber-treated cells. Crocidolite induced marked levels of expression, while in glass microfiber-treated cells the expression was at background levels (Figure 8). The differing patterns of protein expression among the physical and chemical agents was most clearly observed 18 hr postexposure (Figure 9).

Discussion

The alpha-particle response in A549 cells showed this established cell line to have a functional *p53* gene and *p53*-associated

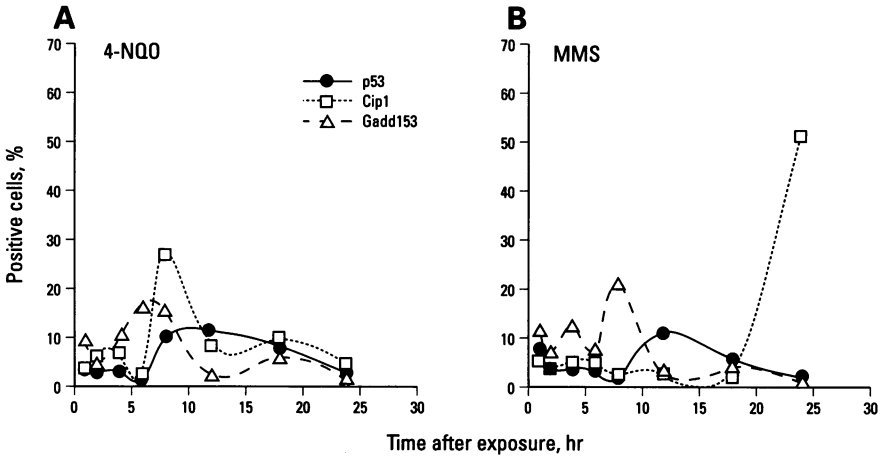

Figure 6. Protein levels of p53, Cip1, and Gadd153 in confluence-arrested A549 cells after exposure to (*A*) 4-NQO (250 ng/ml for 1 hr) or (*B*) MMS (25 µg/ml for 1 hr). Data obtained by flow cytometric analysis of immunolabeled cells from single experiments.

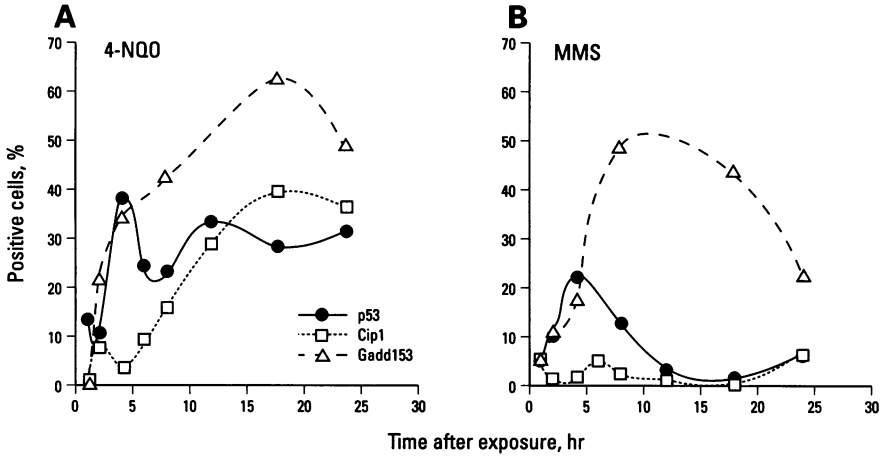

Figure 7. Protein levels of p53, Cip1, and Gadd153 in A549 cells in log phase growth after exposure to (*A*) 4-NQO (250 ng/ml) or (*B*) MMS (25 µg/ml) for 1 hr. Data obtained by flow cytometric analysis of immunolabeled cells from single experiments.

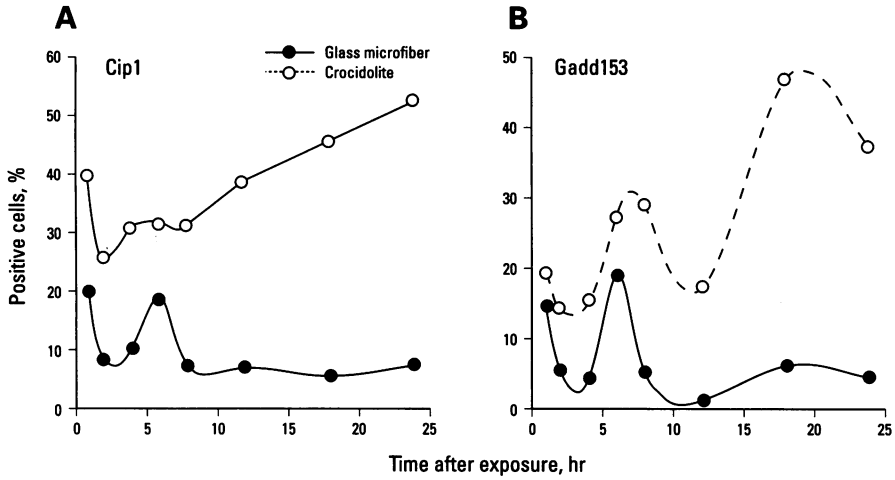

Figure 8. Protein levels of (*A*) Cip1 and (*B*) Gadd153 in A549 cells in log phase growth after exposure to crocidolite asbestos (2.0 µg/cm^2) or glass microfiber (2.0 µg/cm^2) for 20 hr. Data obtained by flow cytometric analysis of immunolabeled cells from single experiments.

genes, which was evident from the induction of p53, Cip1, and Gadd45 proteins following irradiation and the associated cell arrest. The results of *in vivo* exposure to alpha particles in the form of radon progeny showed that inhalation of radon progeny by rats causes cell-cycle arrest. This arrest was evidenced by an increased number of cells in the G_1 phase of the cell cycle and a significant decrease in the number of cells synthesizing DNA following exposure to 500 WLM. In cultured cells exposed to alpha particles a G_1 block was evidenced by the reduced number of cells entering *S* phase and the subsequent drop in number of cells in *S* phase. At an exposure level of 500 WLM, a significant difference in the number of DNA strand breaks was detected compared to the control exposures. The number of breaks was greatest at 24 hr post-exposure; it is unknown whether these breaks were due directly to the radiation or to an apoptotic response; however, there was no morphological evidence of apoptosis. An exposure to 500 WLM is equivalent to 1.2 Gy of alpha radiation, assuming a dose conversion factor of 2.4 mGy/WLM, and is associated with a significant reduction in the colony-forming ability of cultured nasal epithelial cells (*5*). These results show that epithelial cells exposed *in vivo* behave in a fashion similar to cells in culture with respect to radiation-induced cell-cycle arrest. Further work is being conducted to determine the pattern of radiation-induced gene expression; DNA damage *in vivo* is similar to that observed in cultured lung epithelial cells, thus allowing a biodosimetric determination of radon dose to airway cells at low cumulative exposures.

p53 Response kinetics vary widely for different cell types and exposure agents. In different human cell lines with wild-type *p53*, there can be a 6-fold difference in the levels of gamma-ray-induced p53 protein (*22*). In addition, the expression of Gadd45 can vary 20-fold in cells exposed to gamma radiation (*9*), with the most marked elevation in protein levels found in cells of hematogenous origin. Both Kastan et al. (*6*) and Lu and Lane (*23*) have reported fairly rapid and transient elevations in p53 levels in mouse prostate and human hematopoietic cells, respectively, exposed to X-rays, while Khanna and Lavin (*24*) observed a sustained response in Epstein–Barr virus-transformed human lymphoblastoid cells. Nonlethal doses of X-rays to human hematopoietic cells and human fibroblasts increase the levels of p53 protein, which is temporally correlated with cell-cycle arrest

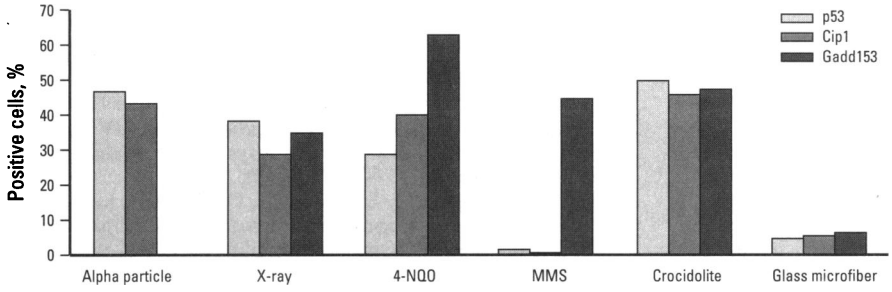

Figure 9. Protein expression for p53 Cip1, and Gadd153 18-hr posttreatment for alpha particle (0.6 Gy), X-ray (4 Gy), 4-NQO (250 ng/ml), MMS (25 μg/ml), crocidolite asbestos (2 μg/cm^2) and glass microfiber (2 μg/cm^2).

in the G_1 phase of the cell cycle (*25*). DNA strand breaks may be the critical trigger common to the many DNA-damaging agents capable of inducing elevated levels of p53 protein (*17,23*). These authors found that the peak expression of the p53 protein occurs 3 to 5 hr postirradiation and that the duration of G_1 arrest is 12 to 24 hr. In contrast, exposure to UV radiation (*23,24*) or to mitomycin C (*16*) produces prolonged increases in a variety of cell types; the time required to reach maximal p53 levels is also prolonged compared to that seen following X-ray treatment. In Hickman et al. (*19*), exposures to alpha particles and X-rays have produced differences in time of peak expression and dose–response relationships in rodent lung epithelial cells. The number of p53-positive cells reached a maximum value by 8 hr following exposure to 1 Gy of X-rays; this time was prolonged to about 20 hr following 28-cGy alpha-particle exposure. Delmolino et al. (*27*) have reported that the p53 protein in unexposed mammary epithelial cells is more stable (protein half-life of > 3 hr) than that in mammary fibroblasts (protein half-life < 15 min). Thus, both cell type and kind of DNA damage appear to influence the kinetics of the p53 protein response.

A549 cells also respond to the presence of chemical and physical agents other than ionizing radiation. The difference in protein response with 4NQO and MMS suggest that this expression pattern may be reflective of the mode of action of these materials. Agents that have been shown to induce elevated levels of p53 protein possess different putative modes of action. Bleomycin and sparsely ionizing radiation directly cause DNA strand incision, and mitomycin C is an alkylating agent that creates DNA strand cross links. MMS induces DNA base modifications, actinomycin D is a DNA intercalating agent that binds to topisomerase II, and cisplatin is a metal complex that induces DNA strand breaks. Methotrexate inhibits purine biosynthesis, thus depriving the cell of purines for DNA repair, and 4-NQO induces single-strand breaks through metabolism to several bulky adducts in addition to cross-linking DNA adducts.

An increased level of p53 protein is not the only response to DNA damage. Gadd45 and Gadd153 can be readily induced by MMS independently of the p53 status of the cell (*28*). Gadd153 mRNA is only weakly induced by ionizing radiation, which readily induces high levels of Gadd45 and p53 mRNA (*29*). 4-NQO induces bulky DNA adducts that are repaired slowly compared to the base alkylation caused by MMS, which is repaired relatively more quickly (*30*). mRNA levels of Gadd153 increase after exposure to a wide variety of DNA-damaging chemicals (*31*). However, after exposure to ionizing radiation, Gadd153 mRNA levels do not increase significantly (*31*). Our results demonstrate that in A549 cells protein levels increase after exposure to ionizing radiation. Gadd153 protein levels may increase either through transcription, as in the case of chemical exposure, or through posttranscriptional modification, as appears to be the case with ionizing radiation. Increased levels of p53 detected after irradiation result from posttranslational stabilization of the protein. This modification of the p53 protein may result from changes in the redox status of the cell. Hainaut and Milner (*32*) have shown that oxidation disrupts wild-type protein conformation and that reduction favors folding into the wild-type form. Ionizing radiation and chemical exposures can readily produce oxygen radicals that would alter the redox state of the cell (*33*).

A surprising result of these studies was the clear difference in response of A549 cells to crocidolite asbestos and glass microfiber. *In vitro* assays such as the colony-forming efficiency assay are generally poor at discriminating between fibers that are toxic or nontoxic by inhalation (*34*). Crocidolite is a known human and animal carcinogen, whereas glass microfiber does not induce excess lung tumors in rats exposed by inhalation (*35*). Human exposure to glass fiber has not been associated with an excess risk of lung cancer (*35*). If the differential expression of DNA damage-inducible genes demonstrated by this study holds true for other toxic and nontoxic fibers, this approach may be a useful screen for new fibrous materials. It is unclear how DNA damage-inducible genes may be able to discern between crocidolite and glass microfiber since fiber length is a major determinant of toxicity *in vitro* and both these fibers have similar size distributions and number of fibers per milligram of dust (*34*). Recent studies have shown that asbestos fibers longer than 20 μm are associated with the induction of lung cancer in rats (*36*). However, glass wool aerosols that contain fibers longer than 20 μm are not associated with excess lung cancer in rats (*37*). This difference in biological activity of fibers longer than 20 μm may be due to their biopersistence. Glass wool fibers are cleared more rapidly than shorter fibers due to their solubility in lung tissue; glass wool fibers typically have a half-life of 11 to 54 days (*38*).

Microbial assays are insensitive to inorganic fibrous materials such as asbestos and glass fibers (*39,40*). Microbial and mammalian cells have been genetically engineered to express individual stress genes (*41,42*), which necessitates multiple exposures to analyze a number of stress genes. The use of established cell lines such as A549 that have functional *p53* and *p53*-associated genes enables the analysis of multiple DNA damage-inducible proteins in one cell system following exposure to a wide range of materials. Measurement of proteins associated with DNA damage-inducible genes may provide a method to delineate the adverse biological effects of chemical and physical agents including inorganic fibers.

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