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Expression of Proteins Coincident with Inducible Radioprotection in Human Lung Epithelial Cells[1]

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ABSTRACT

Human lung epithelial cells and many other cell lines are hypersensitive to low doses of ionizing radiation (<0.2 Gy). However, above a threshold dose of 0.4–0.6 Gy, an induced radioprotective response is triggered that protects cells at higher radiation doses. At 4 h, when maximal induced radioprotection is seen in these cells after low-dose priming, the two-dimensional gel protein expression pattern in 0.5-Gy-exposed cells is subtly altered, with seven proteins being 2- to 5-fold down-regulated and one being 2-fold up-regulated. They include: (*a*) the protein kinase C inhibitor 1, or histidine triad nucleotide-binding motif (HINT) protein; (*b*) substrates for protein kinase C activity including the chloride intracellular channel protein 1; and (*c*) a cytoskeletal protein degraded during apoptosis. In addition, a lung cancer-specific protein that binds to both telomeres and nascent mRNA molecules is down-regulated, as is interleukin 1α. Therefore, at least in human lung epithelial cells, radioprotection may be the result of signaling pathway switching, which results in the removal of damaged cells and the preparation for enhanced general transcription in surviving cells during a period in which cell proliferation is repressed. This combination of events may be cell-type-specific and may have implications for the protection of normal lung tissue during unavoidable radiation exposure such as in radiotherapy.

INTRODUCTION

There is now clear evidence that cellular responses to ionizing radiation exposure can often be significantly affected by active or programmed biological intervention, resulting in induced radioresistance. For instance, when faced with excessive injury, mammalian cells can either actively promote their own death by apoptosis or up-regulate DNA damage control mechanisms, thus maintaining greater cell viability than can be provided by constitutive processes such as background DNA damage repair, replication, and housekeeping. The adaptive response to small radiation doses is a manifestation of such a process of induced resistance and has been the subject of studies for many years particularly in human lymphocytes (for a review, see Ref. 1).

This paper addresses a similar manifestation of induced radioresistance, which has implications for both radiotherapy and radioprotection. Cells in which this effect is observed exhibit hypersensitivity to very low radiation doses (<0.3 Gy), which is not predicted by back extrapolating the cell-survival response from higher doses. As the dose is increased above ~0.3 Gy, there is increased radioresistance until at doses beyond ~1 Gy, radioresistance is maximal and the cell survival follows the usual downward-bending curve with increasing dose. This effect has been recognized in nonmammalian systems for many years (for a review, see Ref. 2); however, only recently has it been possible to examine the response of mammalian cellular systems to radiation with sufficient accuracy to resolve changes in radiosensitivity at doses less than 1 Gy, at which cell survival approaches 100% (3–5). Conventional colony assays cannot reliably measure radiation-produced mammalian cell death in this low-dose region. However, improved technology permits determination of the number of cells that are at risk in a colony-forming assay either by use of a fluorescence-activated cell sorter (5) to plate an exact number of cells or by microscopic scanning to identify an exact number of cells after plating (3). Using the latter technique, Marples and Joiner (6, 7) were first to define hypersensitivity and induced radioresistance in mammalian cells (V79 hamster fibroblasts) at doses of less than 1 Gy. There is now definitive evidence for these effects in more than 15 different human cell lines (2, 5, 8) including one nontransformed lung epithelial line, L132 (9), although the underlying mechanisms have remained obscure.

To date, most mechanistic data on responses to ionizing radiation have been obtained after high-dose exposure (>8 Gy) that few cells survive, most undergoing necrotic death. We have previously shown that, with the exception of the damage-inducible gene *GADD45* (10), genes identified as responsive to such high doses are not affected by exposure of human lung cells to low doses known to induce radioresistance (11, 12). In fact, although some studies have examined molecular responses to such biologically relevant low doses (13–15), few have been carried out on cells in which the induced radioresistance effect has been well characterized. Here, we report a study of changes in protein expression after exposure of human lung L132 cells to a low dose of ionizing radiation, which we have previously shown induces radioresistance in these cells (9), to define the molecular basis of this biological effect in a target cell for both radiation carcinogenesis and morbidity.

MATERIALS AND METHODS

Cell Culture. L132 cells have a limited life span, have been verified as human bronchial epithelial in origin (16) and are wild-type for *p53* in exons 5 through 8 (17); the expression of oncogenes *c-fos* and *c-myc* in these cells is unaffected by 0.5-Gy of ionizing radiation exposure (11, 12). Cells were cultured in Eagle's MEM with 10% fetal bovine serum, supplemented with 2 mM L-glutamine, nonessential amino acids, and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin). Cells were grown in monolayer in 75-cm^2 flasks containing 12 ml of medium and incubated at 37°C with 5% CO_2.

Radiation Exposure of L132 Cells. Ionizing radiation exposure was carried out using a cobalt-60 γ source. Cells were exposed in monolayer at 70% confluence in 75-cm^2 flasks containing 12 ml of medium at a dose rate of 0.55 Gy/min. Sham irradiations were carried out concurrently, and all of the flasks were housed in Styrofoam boxes during exposure to minimize temperature change. Cells from 5 separate irradiations/sham irradiations were used for protein analysis.

Radioactive Labeling of Newly Synthesized Proteins. ^{35}S-labeled Transmix [0.5 mCi; L-methionine, L-cysteine, 43.48 TBq/mmol (ICN Ltd)] was added to 5 ml of spent medium 15 min before radiation exposure; this was inoculated into each flask of cells, which was rocked gently to mix. Both exposed and unexposed cells were incubated for an additional 4 h posttreatment, to allow incorporation of the label into proteins newly synthesized during the time of maximal expression of the induced radioprotection phenotype.

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Cell Harvesting and Protein Solubilization. Cell monolayers were harvested by scraping into 5 ml of PBS at 4°C and were pelleted by centrifugation at 250 × *g* for 5 min at 4°C. The supernatant was removed, and the cell pellets were drained well and then lysed by repeated pipetting in 20 μl of lysis buffer [9 M urea, 0.2% CHAPS,[3] 1% w/w DTT, 0.8% w/v Pharmalyte (pH 3–10; Pharmacia), 100 mg/ml phenylmethansulfonyl fluoride plus a few grains of bromphenol blue] and stored at −70°C. Protein concentration was determined using a modified Bradford assay (18).

Two-dimensional PAGE. IEF was performed using immobilized pH gradient strips (Pharmacia), of pH range 3–10 (linear). The solubilized protein sample was applied to the strips after gel rehydration, using the method described by Görg *et al.* (19). The samples were diluted with rehydration solution containing 8 M urea, 0.5% CHAPS, 0.2% DTT and 0.2% Pharmalyte (pH 3–10) before loading into the sample cups (Pharmacia). For analytical gels, 250 μg of total protein was loaded in a volume of 450 μl; for preparative gels, 3 mg of total protein was loaded in 450 μl. The strips were focused at 0.05 mA per immobilized pH gradient strip for 60 kVh at 20°C. After IEF, the strips were equilibrated in 1.5 M Tris-HCl (pH 6.8) containing 6 M urea, 30% glycerol, 2% SDS, and 0.01% bromphenol blue, with the addition of 1% DTT for 15 min, and then in the same buffer, with the replacement of DTT with 4.8% iodoacetamide, for an additional 15 min. SDS-PAGE was performed using 12% polyacrylamide gels without a stacking gel in the Investigator system (Genomic Solutions Ltd). The second-dimension separation was carried out overnight at 20 mA/gel at 8°C and was stopped when the bromphenol blue dye-front was approximately 1 cm from the bottom of the gels.

Protein Visualization. Analytical gels were dried under vacuum pressure at 65°C for 3 h and then were exposed to a phosphorimaging screen for 16–36 h and scanned using a Storm phosphorimager (Molecular Dynamics Ltd). Micropreparative gels were stained using 0.1% colloidal Coomassie Brilliant Blue G-250 in 2% phosphoric acid, 10% ammonium sulfate, and 20% methanol, with adequate staining being achieved after 48 h. Gels were then washed with distilled water to remove any surface-bound dye particles.

Densitometry and Computer Analysis. Gel images from the phosphorimager were analyzed using PDQuest image analysis software (Bio-Rad; Refs. 20, 21). After detection of spots, the gels were aligned, landmarked, and matched. Gels were then placed into the appropriate experimental class, and differential analysis was performed. The Mann-Whitney test was used to detect all of the spots that differed significantly between the control and exposed groups ($P < 0.05$). All of the significantly different spots were then checked manually to eliminate any artifactual differences due to gel pattern distortions and inappropriately matched or badly detected spots. For simplicity, the absorbance values for each statistically differing spot were averaged across each group (see Fig. 2).

Protein Blotting. Immediately after electrophoresis, micropreparative gels were equilibrated for 15 min in 50 mM Tris and 50 mM boric acid (pH 8.5). Semidry blotting of the gels onto PVDF membranes (FluoroTrans) was achieved using a Multiphor II Nova-Blot (Pharmacia) with a current of 0.8 mA/cm^2 for 90 min. The blots were then stained in a solution of 0.05% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid. Blots were destained by washing twice for 5 min in 50% methanol and 10% acetic acid, followed by two rinses in double-distilled water, and were stored dry.

MALDI-MS Peptide Mass Fingerprinting. Protein spots were excised from two-dimensional gels stained with colloidal Coomassie Brilliant blue G; these were cut into 1-mm cubes and destained by washing in 20-μl aliquots of 50 mM ammonium bicarbonate in 50% v/v acetonitrile for 1-h periods until colorless. The samples were then dried in a centrifugal evaporator. Modified (methylated) porcine trypsin (Promega) was prepared as a stock solution in water (0.1 μg/μl). For digestion, 4 μl of trypsin solution was added to 21 μl of 5 mM Tris-HCl, prepared fresh for each use, and the mixture was added to the gel pieces before incubation overnight at room temperature. Digestion was stopped by the addition of 15 μl of 50% acetonitrile and 0.1% trifluoroacetic acid. Tubes were then sonicated in a water bath for 10 min to extract peptides immediately before spotting onto MALDI targets. Peptide solutions were evaporated to dryness in a centrifugal evaporator.

MALDI MS (22) was performed using a Lasermat 2000 mass spectrometer (Finnigan MAT). Samples were dissolved in 2–5 μl of 20% methanol and 0.4-μl aliquots mixed with 0.4 μl of matrix (α-cyano hydroxy cinnamic acid, 10 mg/ml) on the target. After drying, data were averaged for 10–30 laser shots in positive ion mode. Peptide masses were used to search the SWISS-PROT protein database using the PeptIdent tool on Expasy[4] or MS-FIT.[5]

Searching was performed using a mass uncertainty of ± 3 Da and a molecular weight range of ± 20% of the M_r and ± 1 pI unit determined from two-dimensional electrophoresis; the output consisted of a list of proteins ranked by a statistical score.

Amino Acid Compositional Analysis. Amino acid analysis of PVDF-bound proteins was performed as described previously (22). Single protein spots were excised from the PVDF membrane and hydrolyzed in 6 M HCl at 112°C for 18 h using a Pico-Tag workstation (Waters). Amino acid composition was determined using 9-fluorenylmethoxycarbonyl (Fmoc) pre-column derivitization on a GBC Automated Aminomate System (GBC Scientific Equipment, Dandenong, Victoria, Australia; Ref. 23), and chromatography was performed using the method of Yan *et al.* (24). Percentage amino acid composition was determined for 16 recovered amino acids by comparing the pmol yield of each amino acid to the total pmol yield of all of the amino acids. Amino acid composition and estimated M_r and pI from two-dimensional electrophoresis for each protein were used to search the SWISS-PROT protein database (Release 35) using the program AACompIdent (25, 26).[6]

Searches were made using constellation 2 (Cys, Gly, Trp not included) or constellation 4 (Cys, Lys, Trp not included) with windows of pI ± 1 unit and M_r ± 20%. Analyses were calibrated using known protein spots that had been hydrolyzed, extracted, and analyzed at the same time as the other spots.

Database Searching Using Multiident. If searches using PeptIdent or AACompIdent alone failed to identify the protein spots, peptide fingerprinting and amino acid analysis data were used in combination to search the SWISS-PROT database using Multiident[7] with the same search parameters as for the individual searches above (27).

RESULTS

More than 800 protein species, in the range pI 3–10 and M_r 10,000–150,000, could be visualized after two-dimensional gel separation of ^{35}S-labeled extracts from 0.5-Gy-irradiated or control L132 cells. A standard master gel image was created from one of the unexposed cell set using the PDQuest analysis software and is shown in Fig. 1*a*. This was used as the master pattern for a comparison of protein expression 4 h after exposure in 0.5-Gy-irradiated *versus* sham-irradiated cells. All of the matched spots were assigned a unique spot number by the computer software. To obtain statistically significant results, five gels (representing five different exposures) were analyzed in each exposure group. Eight proteins were found to be significantly regulated (Mann-Whitney test, $P < 0.05$) 4 h after exposure of L132 cells to 0.5 Gy γ rays; these, together with two nonregulated reference proteins, are indicated in Fig. 1*a*. Of the regulated proteins, seven were down-regulated (for examples see Fig. 1b, *top two panels*), with only one (spot 4712) being up-regulated (Fig. 1*b*, *bottom panel*) 4 h after exposure to the dose of ionizing radiation, which induces a radioprotective response in these cells. One down-regulated protein (8107) appeared to have several isoforms of varying pI, which formed a streak on the original gels but which was recognized by the computer software as a single spot at its region of highest density. To determine the degree of regulation of the proteins, mean protein-spot density was standardized to total gel-signal density for each of the five gels in a set. The results are shown in Fig. 2, with a 2- to 5-fold regulation of expression seen in most cases.

All of the eight 0.5-Gy-regulated proteins, plus several unregulated internal standards, were identified by amino acid compositional anal-

[3] The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; HINT, histidine triad nucleotide-binding motif; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride; MS, mass spectrometry; pI, isoelectric point.

[4] Internet address: http://www.expasy.ch/tools/peptident.html.
[5] Internet address: http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm.
[6] Internet address: http://www.expasy.ch/tools/aacomp.
[7] Internet address: http://www.expasy.ch/tools/multiident/.

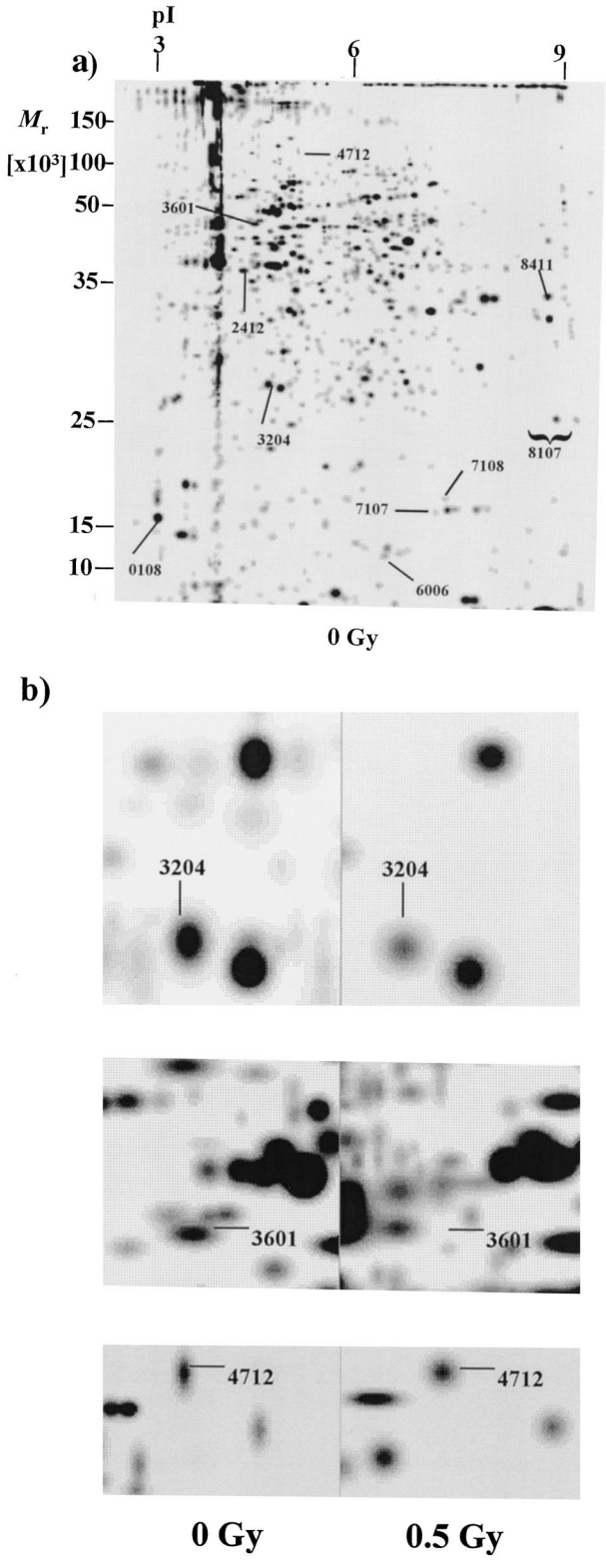

Fig. 1. *a*, two-dimensional PAGE gel image of ^{35}S-proteins from unirradiated L132 cells, generated using PDQuest analysis software. Total ^{35}S-proteins from unirradiated L132 cells were separated in the first dimension by IEF in the range pI 3–10, then by 12% SDS PAGE on 20 × 22-cm gels. Labeled proteins were visualized by phosphorimaging. This image was used as the master image, to which all of the other gels in the experiment were compared (five gels of proteins from independently 0.5 Gy-exposed cells; five from parallel mock-exposed cells). *Pointers*, eight proteins that were significantly regulated 4 h after exposure to 0.5 Gy of ionizing radiation and two control, unregulated proteins, *7107* and *7108* (Mann-Whitney test, 95% confidence limits). *Bracket*, the region in which the regulated protein 8107 appeared as a smear on the original gels. *b*, high-magnification detail of gel regions containing down-regulated proteins 3204 and 3601 (*top two panels*) and up-regulated protein 4712 (*bottom panel*) from 0-Gy- and 0.5-Gy-exposed cells.

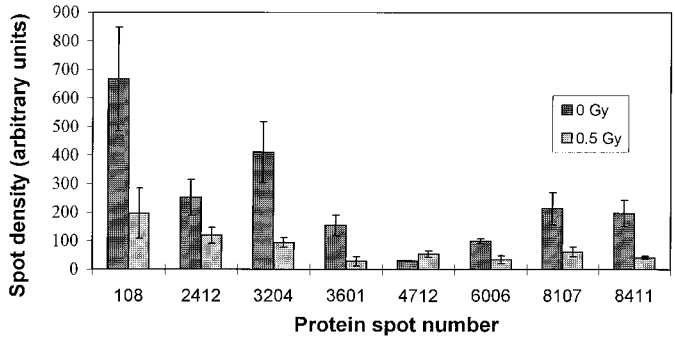

Fig. 2. Protein regulation in L132 cells 4 h after irradiation with 0.5-Gy γ rays. *Bars*, mean spot densities, standardized to total gel signal density; $n = 5$. *Bars*, ± SE. Protein identities are shown in Table 1.

ysis and MALDI MS, in conjunction with estimates of protein size and pI from the gel image. Comparison with the SWISS-PROT database resulted in the positive identification of all of the proteins analyzed. Data on the identified 0.5-Gy-regulated spots are shown in Table 1. Internal reference standards consisted of several proteins that showed no detectable regulation 4 h after 0.5-Gy exposure; these included peptidyl-prolyl *cis-trans* isomerase A (spot number 7107; pI, 7.8; M_r, 18,000), interleukin 10 (spot number 7108; pI, 8.2; M_r, 18,500) as shown in Fig. 1*a*.

The protein whose expression was up-regulated 1.8-fold by exposure to 0.5 Gy of ionizing radiation was identified as human translation initiation factor 2. The M_r and pI estimations for this protein from the gel position closely resembled those of a DNA repair protein, XPG, previously implicated in the excision of base damage of the sort caused by ionizing radiation (28). Repair of such base damage has also been shown to be induced during low dose radioprotection in human lung carcinoma cells (29). However, Western blot analysis of one-dimensional gels showed that the XPG protein migrated with an apparent M_r of 205,000 and was not up-regulated in these cells after exposure to 0.5 Gy of ionizing radiation.[8] Farnesyl PP_i synthetase, a component of the cholesterol biosynthesis and *Ras* oncogene signaling pathways (30), was down-regulated by greater than 5-fold, heterogeneous ribonucleoproteins A2/B1 and chloride intracellular channel protein 1 were down-regulated by greater than 4-fold, interleukin 1 α (a stress response cytokine) and thioredoxin peroxidase 2 (a free radical scavenger) were down-regulated by greater than 3-fold, and the contractile protein, tropomyosin, and the protein kinase inhibitor 1 (HINT) protein were down-regulated by greater than 2-fold.

DISCUSSION

Eight proteins, expressed at levels detectable in our gel system, were differentially regulated in human lung cells exposed to 0.5 Gy of ionizing radiation at the time (4 h) when maximum radioprotection is induced by this dose in these cells (9). The proteins belong to several distinct classes with members of at least two stress-responsive signaling pathways represented.

The signaling pathway predominantly affected in these cells is that of protein kinase C; the inhibitor of this enzyme, protein kinase inhibitor 1 (or HINT protein; Ref. 31), is down-regulated concomitant with the induction of radioprotection in these cells. This would result in an up-regulation of protein kinase C activity, which has been observed in many cell types and species in response to ionizing radiation (32, 33), including doses as low as 0.75 Gy (13), but has not previously been implicated in induced radioprotection. This enzyme is

[8] Unpublished observations.

Table 1 *Protein identities and their regulation 4 h after exposure in 0.5-Gy-irradiated L132 cells*

The M_r and pI of proteins statistically significantly regulated by 0.5-Gy X-rays were deduced from their positions in the two-dimensional gels by comparison to internal and external standards. A consensus identity for each regulated protein was then determined by the methods indicated.

Spot number	M_r (from gel)	pI (from gel)	Consensus identity[a]	SWISS-PROT accession code	M_r (from database)	pI (from database)	Analysis methods[b]	Regulation relative to control[c]
0108	16	3	Interleukin 1 α	P01583	18	5.3	A, M	down 3.4
2412	35	4.5	Tropomyosin isoform TM3	P09494	32.8	4.7	A, M	down 2.2
3204	28	4.8	Chloride intracellular channel protein 1	O00299	26.9	5.0	A, M	down 4.4
3601	48	4.6	Farnesyl pyrophosphate synthetase	P14324	40.5	5.0	A, M	down 5.3
4712	110	5.2	Translation initiation factor 2	O60841	139	5.4	A	up 1.8
6006	13	6.5	Protein kinase C inhibitor 1 (HINT protein)	P49773	13.6	6.5	A, M	down 2.9
8107	25	8–9	Thioredoxin peroxidase 2	Q06830	23.1	7.6	A, M	down 3.4
8411	34	9.0	Heterogeneous ribonucleo-proteins A2/B1	P22626	37.4	9.0	M	down 4.6

[a] Amino acid composition and peptide masses were used to search the SWISS-PROT databases AACompIdent, PeptIdent, and MS-FIT, the combined results of which were used to assign identities to the proteins.
[b] A, amino acid compositional analysis; M, MALDI mass spectrometry.
[c] Regulation and degree (exposed value as a proportion of unexposed value; data derived from Fig. 2).

known to phosphorylate—and, thus, to regulate the activity of—many downstream proteins in signaling cascades (34). These protein kinase C substrates include the chloride intracellular channel protein 1 (35), which has also been detected here as a down-regulated protein coincident with expression of the induced radioprotection phenotype. Both protein kinase C activation and elevation of intracellular Ca^{2+} cause closure of chloride ion channels (36). An additional protein kinase C substrate is the translation initiation factor 2, the only protein seen here to be up-regulated as part of the radioprotective response. This protein acts as a general translation initiation factor promoting binding of methionyl tRNA to ribosomes (37); its up-regulation may enhance expression of many genes, including those that encode housekeeping enzymes and proteins such as repair and cell cycle enzymes, which are not normally deemed to be stress-inducible. Protein kinase C is also known to phosphorylate p53 *in vitro*, though its role *in vivo* is equivocal (38, 39). This could implicate both cell cycle regulation and apoptosis as possible mediators of the induced radioprotective effect in these human lung cells. Interestingly, protein kinase C inhibitor 1 has been found to colocalize with the ATDC protein, a downstream component of the ATM radiation-activated signaling pathway (40) and with vimentin, an intermediate filament protein that is degraded during radiation-induced, Ca^{2+}-mediated apoptosis (41) along with tropomyosin, an accessory contractile protein also found to be down-regulated in this study coincident with expression of the induced radioprotection phenotype.

The stress response pathways characterized by activation of the transcription factors NFκB and AP-1 may not be components of induced radioprotection in these cells. This is suggested by the down-regulation of the inflammatory and stress-responsive cytokine, interleukin 1 α, after exposure to 0.5 Gy of ionizing radiation. This membrane-associated cytokine is, however, normally induced in a cooperative fashion with tumor necrosis factor in many cell types and species in response to ionizing radiation (42, 43) and seems to be critical for protection of mouse tissues against high doses (>8 Gy; Ref. 43). It activates the transcription factors NFκB and AP-1 via Traf6, Tak1, and the mitogen-activated protein kinase pathway (44) to produce classical stress/inflammatory responses. AP-1 has been shown to be high-dose radiation-inducible in some cells, including human fibroblasts and keratinocytes (45); however, we have previously shown that its component, *c-fos*, is not induced by 0.5-Gy exposure in L132 cells (11, 12). The induction and radioprotective action of interleukin 1 α is both tissue- and species-specific (46), and its response to low-dose radiation in normal human lung epithelial cells has not previously been reported. The M_r 18,000 interleukin 1 α protein is the cleavage product of a M_r 31,000 precursor that is irreversibly myristoylated during translation on the NH_2-terminal portion that ultimately forms the active cytokine (47). This modification targets the protein to the membrane and may explain the apparently aberrant pI seen in our gel system. Interleukin 1 α also up-regulates a number of cellular mechanisms, such as the radical scavenger, glutathione, and the manganese-dependent superoxide dismutase enzyme, which protect against metabolic and radiation-induced free radicals. Thioredoxin peroxidase 2 is an additional member of this class of cellular free-radical defense molecules (48) and, consistent with the down-regulation of interleukin 1, is also down-regulated during the induced radioprotective effect analyzed here. We speculate that perhaps there is insufficient free-radical damage to cellular components to act as a trigger during induced radioprotection or that the cell may deliberately accumulate enough damage to constitute a signal.

An additional activity of interleukin 1 α is seen during liver carcinogenesis in which it acts as a copromoter of epithelial tumor progression (49). Its down-regulation at the time of maximal induced radioprotection in these lung epithelial cells may result in repressed proliferation of cells damaged during the initial challenge with the 0.5-Gy radiation dose. Farnesyl PP_i synthetase, another protein found to be down-regulated in L132 cells exposed to 0.5 Gy of ionizing radiation, is a component of the cholesterol biosynthetic pathway (30); cholesterol synthesis is elevated in proliferating normal tissues and in tumors, the farnesyl moiety also being necessary for the activation of G-proteins and the ras oncoprotein, p21 (30). The down-regulation of farnesyl PP_i synthetase may, therefore, also contribute to growth repression of damaged cells.

The last protein component of the response that we have begun to dissect here seems to be specific to lung epithelial cells. Along with interleukin 1 α, the heterogeneous ribonucleoproteins A2/B1 are known to be expressed during proliferation of initiated lung cells to form a carcinoma (50). In fact, they have been used as a tumor marker in this tissue. Interestingly, these proteins bind not only to nascent RNA but also to telomeres (51), which are known to be stabilized during oncogenesis. The A2/B1 protein is substantially down-regulated at the time of maximal expression of induced radioprotection in these cells, which suggests a role in the suppression of growth of potentially initiated cells.

These proteins that we have characterized are likely to comprise just part of an array of molecules that possibly contribute to the radioprotective effect that we have shown to be induced maximally at 4 h after 0.5-Gy exposure of these cells; the rest may be expressed at a level below our limits of reliable detection during two-dimensional gel analysis. Indeed, parallel studies in our laboratory of differential gene expression in response to 0.5 Gy of ionizing radiation in the cells used here (11, 12) have identified a further down-regulated gene (whose mRNA is of a low-abundance class) recently characterized as a member of the immunophilin family of heat-shock related genes

(52). Such genes have a role in general stress response and cell cycle control, but the low abundance of their protein products may preclude accurate quantitation in our gel system.

Possible drawbacks of previous studies of the molecular basis of radiation response include: (*a*) the use of high radiation doses not relevant to the induced radioprotection response; (*b*) the use of cells that may not show induced radioprotection; or (*c*) the use of individual candidate genes (53, 54) deemed to be potential mediators of the effect. The array of proteins expressed here show little overlap with those expressed in human melanoma cells in response to X-ray doses of 3–4.5 Gy (14), although spots 3601 (farnesyl PP_i synthetase) and 4712 (translation initiation factor 2) have pI and M_r values close to those of the M_r-47,000 and M_r-126,000 proteins reported in that study and are similarly regulated at 0.5 Gy. The discrete array of proteins that we have found to be regulated coincident with the well-characterized 0.5-Gy-induced radioprotective response in human lung L132 cells, at the approximate time of maximal expression of this response, suggest the selective up-regulation of protein kinase C-regulated signaling responses, culminating in enhanced general transcription, with the concurrent down-regulation of the specific inflammatory and stress-responsive pathways involving NFκB and AP-1. In this particular cell type, there is evidence for the initiation of additional cellular defense mechanisms including cytoskeletal protein breakdown (indicative of limited apoptosis of damaged cells) and for the inhibition of cell proliferation of potentially initiated cells via interleukin 1 α, heterogeneous ribonucleoprotein A2/B1, and farnesyl PP_i synthetase down-regulation.

The responses characterized here are consistent with the overall biological effect, in which low-dose-irradiated lung epithelial cells are protected from subsequent high-dose lethal, or perhaps carcinogenic, effects of ionizing radiation by the selective removal of a few damaged cells, with the growth regulation of surviving cells and preparation for enhanced expression of key genes necessary for the maintenance of cellular and genetic integrity. The results of this study will permit further detailed characterization of the time course and dose response of the eight individual protein species and will both confirm their role in the radioprotective response and assess their value in the modification of normal tissue morbidity during radiotherapy of lung cancer.

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REFERENCES

1. Wolff, S. Aspects of the adaptive response to very low doses of radiation and other agents. Mutat. Res., *358:* 135–142, 1996.
2. Joiner, M. C., Lambin, P., Malaise, E. P., Robson, T., Arrand, J. E., Skov, K. A., and Marples, B. Hypersensitivity to very-low single radiation doses: its relationship to the adaptive response and induced radioresistance. Mutat. Res., *358:* 171–183, 1996.
3. Spadinger, I., and Palcic, B. Cell survival measurements at low doses using an automated image cytometry device. Int. J. Radiat. Biol., *63:* 183–189, 1993.
4. Palcic, B., and Jaggi, B. The use of solid-state image sensor technology to detect and characterize live mammalian cells growing in tissue culture. Int. J. Radiat. Biol., *50:* 345–352, 1986.
5. Wouters, B. G., Sy, A. M., and Skarsgard, L. D. Low dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. Radiat. Res., *146:* 399–413, 1996.
6. Marples, B., and Joiner, M. C. The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. Radiat. Res., *133:* 41–51, 1993.
7. Marples, B., and Joiner, M. C. The elimination of low-dose hypersensitivity in Chinese hamster V79–379A cells by pretreatment with X-rays or hydrogen peroxide. Radiat. Res., *141:* 160–169, 1995.
8. Lambin, P., Malaise, E. P., and Joiner, M. C. Might intrinsic radioresistance of human tumour cells be induced by radiation? Int. J. Radiat. Biol., *69:* 279–290, 1996.
9. Singh, B., Arrand, J. E., and Joiner, M. C. Hypersensitive response of normal human lung epithelial cells at low radiation doses. Int. J. Radiat. Biol., *65:* 457–464, 1994.
10. Papathanasiou, M. A., Kerr, N. C., Robbins, J. H., McBride, O. W., Alamo, I. J., Barrett, S. F., Hickson, I. D., and Fornace, A. J. Induction by ionizing radiation of the *gadd45* gene in cultured human cells: lack of mediation by protein kinase C. Mol. Cell. Biol., *11:* 1009–1016, 1991.
11. Arrand, J. E., Singh, B., Robson, T., Gamble, S. C., and Joiner, M. C. Molecular dissection of X-ray induced responses in human cells. Radioprotection, *32:* C1-311–314, 1997.
12. Robson, T., Lohrer, H., Bailie, J. R., Hirst, D. G., Joiner, M. C., and Arrand, J. E. Gene regulation by low-dose ionizing radiation in a normal human lung epithelial cell line. Biochem. Soc. Trans., *25:* 335–341, 1997.
13. Woloschak, G. E., Chang-Liu, C-M., and Shearin-Jones, P. Regulation of protein kinase C by ionizing radiation. Cancer Res., *50:* 3963–3967, 1990.
14. Boothman, D. A., Bouvard, I., and Hughes, E. N. Identification and characterization of X-ray- induced proteins in human cells. Cancer Res., *49:* 2871–2878, 1989.
15. Boothman, D. A., Meyers, M., Fukunaga, N., and Lee, S. W. Isolation of x-ray-inducible transcripts from radioresistant human melanoma cells. Proc. Natl. Acad. Sci. USA, *90:* 7200–7204, 1993.
16. Arrand, J. E., and Murray, A. M. Benzpyrene groups bind preferentially to the DNA of active chromatin in human lung cells. Nucleic Acids Res., *10:* 1547–1555, 1982.
17. Gamble, S. C., Cook, M. C., Riches, A. C., Herceg, Z., Bryant, P. E., and Arrand, J. E. *p53* mutations in tumors derived from irradiated human thyroid epithelial cells. Mutat. Res., *425:* 231–238, 1999.
18. Ramagli, L. S., and Rodriguez, L. V. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis, *6:* 559–563, 1985.
19. Görg, A., Postel, W., and Günter, S. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis, *9:* 531–546, 1988.
20. Krauss, M. R., Collins, P. J., and Blose, S. H. A two-dimensional acrylamide gel electrophoresis/computer software approach to decoding the human genome. Biotechniques, *8:* 218–223, 1990.
21. Corbett, J. M., Wheeler, C. H., Baker, C. S., Yacoub, M. H., and Dunn, M. J. The human myocardial two-dimensional gel protein database: update 1994. Electrophoresis, *15:* 1459–1465, 1994.
22. Wheeler, C. H., Berry, S. L., Wilkins, M. R., Corbett, J. M., Ou, K., Gooley, A. A., Humphery-Smith, I., Williams, K. L., and Dunn, M. J. Characterisation of proteins from two-dimensional electrophoresis gels by matrix-assisted laser desorption mass spectrometry and amino acid compositional analysis. Electrophoresis, *17:* 580–587, 1996.
23. Haynes, P. A., Sheumack, D., Greig, L. G., Kibby, J., and Redmond, J. W. Applications of automated amino acid analysis using 9-fluorenylmethyl chloroformate. J. Chromatogr., *588:* 107–114, 1991.
24. Yan, J. X., Wilkins, M. R., Ou, K., Gooley, A. A., Williams, K. L., Sanchez, J. C., Golaz, O., Pasquali, C., and Hochstrasser, D. F. J. Large scale amino-acid analysis from proteome studies. J. Chromatogr. A, *736:* 291–302, 1996.
25. Wilkins, M. R., Ou, K., Appel, R. D., Sanchez, J. C., Yan, J. X., Golaz, O., Farnsworth, V., Cartier, P., Hochstrasser, D. F., Williams, K. L., and Gooley, A. A. Rapid protein identification using N-terminal "sequence tag" and amino acid analysis. Biochem. Biophys. Res. Commun., *221:* 609–613, 1996.
26. Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golaz, O., Sanchez, J. C., Yan, J. X., Gooley, A. A., Hughes, G., Humphery, S. I., Williams, K. L., and Hochstrasser, D. F. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnology, *14:* 61–65, 1996.
27. Wilkins, M. R., Gasteiger, E., Wheeler, C., Lindskog, I., Sanchez, J-C, Bairoch, A., Dunn, M. J., and Hochstrasser, D. F. Multiple parameter cross-species protein identification using MultiIdent—a world-wide web accessible tool. Electrophoresis, *19:* 3199–3206, 1998.
28. Klungland, A., Hoss, M., Gunz, D., Constantinou, A., Clarkson, S. G., Doetsch, P. W., Bolton, P. H., Wood, R. D., and Lindahl, T. Base excision repair of oxidative DNA damage activated by XPG protein. Molecular Cell, *3:* 33–42, 1999.
29. Le, X. C., Xing, J. Z., Lee, J., Leadon, S. A., and Weinfeld, M. Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage. Science (Washington DC), *280:* 1066–1069, 1998.
30. Rao, K. N. The significance of the cholesterol biosynthetic pathway in cell growth and carcinogenesis. Anticancer Res., *15:* 309–314, 1995.
31. Lima, C. D., Klein, M. G., Weinstein, I. B., and Hendrickson, W. A. Structure-based analysis of catalysis and substrate definition in the HIT protein family. Proc. Natl. Acad. Sci. USA, *93:* 5357–5362, 1996.
32. Kim, C. Y., Giaccia, A. J., Strulovici, B., and Brown, J. M. Differential expression of protein kinase C ϵ protein in lung cancer cell lines by ionising radiation. Br. J. Cancer, *66:* 844–849, 1992.
33. Hasan, N. M., Parker, P. J., and Adams, G. E. Induction and phosphorylation of protein kinase C-α and mitogen-activated protein kinase by hypoxia and by radiation in Chinese hamster V79 cells. Radiat. Res., *145:* 128–133, 1996.
34. Hallahan, D. E., Sukhatme, V. P., Sherman, M. L., Virudachalam, S., Kufe, D., and Weichselbaum, R. R. Protein kinase C mediates x-ray inducibility of nuclear signal transducers EGR1 and JUN. Proc. Natl. Acad. Sci. USA, *88:* 2156–2160, 1991.
35. Valenzuela, S. M., Martin, D. K., Por, S. B., Robbins, J. M., Warton, K., Bootcov, M. R., Schofield, P. R., Campbell, T. J., and Breit, S. N. Molecular cloning and expression of a chloride ion channel of cell nuclei. J. Biol. Chem., *272:* 12575–12582, 1997.
36. Duan, D., Cowley, S., Horowitz, B., and Hume, J. R. A serine residue in CIC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. J. Gen. Physiol., *113:* 57–70, 1999.

37. Lee, J. H., Choi, S. K., Roll-Mecak, A., Burley, S. K., and Dever, T. E. Universal conservation in translation initiation revealed by human and archaeal homologs of bacterial translation initiation factor IF2. Proc. Natl. Acad. Sci. USA, *96:* 4342–4347, 1999.
38. Milne, D. M., McKendrick, L., Jardine, L. J., Deacon, E., Lord, J. M., and Meek, D. W. Murine p53 is phosphorylated within the Pab421 epitope by protein kinase C *in vitro* but not *in vivo*, even after stimulation with the phorbol ester *O*-tetradecanoylphorbol 13-acetate. Oncogene, *13:* 205–211, 1996.
39. Baudier, J., Delphin, C., Grunwald, D., Khochbin, S., and Lawrence, J. J. Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein. Proc. Natl. Acad. Sci. USA, *89:* 11627–11631, 1992.
40. Brzoska, P. M., Chen, H., Zhu, Y., Levin, N. A., Disatnik, M-H., Mochly-Rosen, D., Murnane, J. P., and Christman, M. F. The product of the ataxia-telangiectasia group D complementing gene, *ATDC*, interacts with a protein kinase C substrate and inhibitor. Proc. Natl. Acad. Sci. USA, *92:* 7824–7828, 1995.
41. Prasad, S. C., Soldatenkov, V. A., Kuettel, M. R., Thraves, P. J., Zou, X., and Dritschilo, A. Protein changes associated with ionizing radiation-induced apoptosis in human prostate epithelial tumor cells. Electrophoresis, *20:* 1065–1074, 1999.
42. Woloschak, G. E., Chang-Liu, C-M, Shearin Jones, P., and Jones, C. A. Modulation of gene expression in Syrian hamster embryo cells following ionizing radiation. Cancer Res., *50:* 339–344, 1990.
43. Neta, R., Oppenheim, J. J., Schreiber, R. D., Chizzonite, R., Ledney, G. D., and MacVittie, T. J. Role of cytokines (interleukin 1, tumor necrosis factor and transforming growth factor β) in natural and lipopolysaccharide-enhanced radioresistance. J. Exp. Med., *173:* 1177–1182, 1991.
44. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J-I., Cao, Z., and Matsumoto, K. The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature (Lond.), *398:* 252–256, 1999.
45. Martin, M., Vozenin, M. C., Gault, N., Crechet, F., Pfarr, C. M., and Lefaix, J. L. Coactivation of AP-1 activity and *TGF-β1* gene expression in the stress response of normal skin cells to ionizing radiation. Oncogene, *15:* 981–989, 1997.
46. Ross, H. J., Antoniono, R. J., Buckmeier, J. A., and Redpath, J. L. Variable expression of IL-1β has minimal effect on the radiation sensitivity of three human glioma cell lines. Int. J. Radiat. Biol., *66:* 785–791, 1994.
47. Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M., and Lovett, D. H. The 31-kDa precursor of interleukin 1α is myristoylated on specific lysines within the 16-kDa N- terminal propiece. Proc. Natl. Acad. Sci. USA, *90:* 7245–7249, 1993.
48. Prosperi, M. T., Ferbus, D, Karczinski, I., and Goubin, G. A human cDNA corresponding to a gene overexpressed during cell proliferation encodes a product sharing homology with amoebic and bacterial proteins. J. Biol. Chem., *268:* 11050–11056, 1993.
49. Oberyszyn, T. M., Sabourin, C. L., Bijur, G. N., Oberyszyn, A. S., Boros, L. G., and Robertson, F. M. Interleukin-1α gene expression and localization of interleukin-1α protein during tumor promotion. Mol. Carcinogenesis, *7:* 238–248, 1993.
50. Sueoka, E., Goto, Y., Sueoko, N., Kai, Y., Kozu, T., and Fujiki, H. Heterogeneous nuclear ribonucleoprotein B1 as a new marker of early detection for human lung cancers. Cancer Res., *59:* 1404–1407, 1999.
51. McKay, S. J., and Cooke, H. hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat $TTAGGG_n$. Nucleic Acids Res., *20:* 6461–6464, 1992.
52. Robson, T., Joiner, M. C., Wilson, G. D., McCullough, W., Price, M. E., Logan, I., Jones, H., McKeown, S. R., and Hirst, D. G. A novel human stress response-related gene with a potential role in induced radioresistance. Radiat. Res., *152:* 451–461, 1999.
53. Sherman, M. L., Datta, R., Hallahan, D. E., Weichselbaum, R. R., and Kufe, D. W. Ionizing radiation regulates expression of the *c-jun* protooncogene. Proc. Natl. Acad. Sci. USA, *87:* 5663–5666, 1990.
54. Kasid, U., Suy, S., Dent, P., Ray, S., Whiteside, T., and Sturgill, T. W. Activation of Raf by ionizing radiation. Nature (Lond.), *382:* 813–816, 1996.