Profiles of Global Gene Expression in Ionizing-Radiation–Damaged Human Diploid Fibroblasts Reveal Synchronization behind the G1 Checkpoint in a G0-like State of Quiescence

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Cell cycle arrest and stereotypical transcriptional responses to DNA damage induced by ionizing radiation (IR) were quantified in telomerase-expressing human diploid fibroblasts. Analysis of cytotoxicity demonstrated that 1.5 Gy IR inactivated cell colony formation by 40–45% in three fibroblast lines; this dose was used in all subsequent analyses. Fibroblasts exhibited > 90% arrest of progression from G2 to M at 2 hr post-IR and a similarly severe arrest of progression from G1 to S at 6 and 12 hr post-IR. Normal rates of DNA synthesis and mitosis 6 and 12 hr post-IR caused the S and M compartments to empty by > 70% at 24 hr. Global gene expression was analyzed in IR-treated cells. A microarray analysis algorithm, EPIG, identified nine IR-responsive patterns of gene expression that were common to the three fibroblast lines, including a dominant p53-dependent G1 checkpoint response. Many p53 target genes, such as CDKN1A, GADD45, BTG2, and PLK3, were significantly up-regulated at 2 hr post-IR. Many genes whose expression is regulated by E2F family transcription factors, including CDK2, CCNE1, CDG6, CDG2, MCM2, were significantly down-regulated at 24 hr post-IR. Numerous genes that participate in DNA metabolism were also markedly repressed in arrested fibroblasts apparently as a result of cell synchronization behind the G1 checkpoint. However, cluster and principal component analyses of gene expression revealed a marked increase in expression up to 70% at 24 hr. Global gene expression was analyzed in IR-treated fibroblasts, indicating for the first time that the IR-induced growth arrest also included induction of quiescence-associated transcripts.

Materials and Methods

Cell lines and culture. Normal human fibroblast strains, NHFI, NHF3, and NHF10, were derived from neonatal foreskins and established in secondary culture according to established methods (Maher et al. 1981). Immortal cell lines were obtained by ectopic expression of human telomerase (hTERT), as previously described (Demin et al. 2001; Heffernan et al. 2002). Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were grown to confluence and then deprived of growth factors by replacing the medium with fresh media containing 1% FBS. After 72 hr, cells were grown to confluence and then deprived of growth factors by replacing the medium with fresh media containing 1% FBS. After 72 hr, cells were harvested and used for RNA isolation.

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2 mM l-glutamine (Invitrogen) and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were tested and shown to be free of mycoplasma contamination using a commercial kit (Gen-Probe, San Diego, CA). Cytogenetic analysis established that all three fibroblast lines were 46XY with normal chromosome numbers and structure (data not shown).

Cell irradiation. We exposed cells to IR in culture medium using a cesium-137 source (Gammarace40; Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 0.84 Gy/min. Sham-treated controls were subjected to the same movements in and out of incubators as were irradiated cells.

Clonogenic survival assay. Clonogenic survival was measured in logarithmically growing NHF1, NHF3, NHF10 fibroblasts, plated at 400–500 cells per 100-mm–diameter dish and incubated for 8 hr before exposure to IR (three dishes per dose). Cells were cultured for 2 weeks, with medium changed twice a week. Colonies were fixed and stained with a solution of 40% methanol and 0.05% crystal violet. Colonies with ≥ 50 cells were counted. The relative colony-forming efficiency of IR-treated cells was expressed as a fraction of sham-treated controls.

Cell cycle checkpoint assays. We quantified G1 checkpoint function using flow cytometry to determine the IR-induced reduction in the percentage of bromodeoxyuridine (BrdU)-labeled cells in the first half of S phase 6–8 hr after 1.5 Gy (Doherty et al. 2003). G1 checkpoint function was quantified by using flow cytometry to determine the IR-induced reduction in the percentage of phosphohistone H3–labeled mitotic cells 2 hr after 1.5 Gy (Doherty et al. 2003). To determine the percentage changes in the fractions of cells in various cell cycle compartments with time post-IR, we incubated cells with BrdU for 2 hr beginning 2, 6, 12, and 24 hr after 1.5 Gy or sham treatment. Cells were harvested and analyzed for BrdU incorporation and expression of phosphohistone H3. Summit flow cytometry analysis software (Dako Cytomation, Fort Collins, CO) was used to quantify the numbers of unlabeled cells with 2N (G0/G1) and 4N DNA content (G2), BrdU-labeled cells with 2–4N DNA content (S), and 4N cells with high phospho-histone H3 (M).

Cell synchronization. NHF1, NHF3, and NHF10 fibroblasts were synchronized as previously described (Cordeiro-Stone et al. 1986). Briefly, we plated cells at a density of 1.3 × 10⁴/cm² and allowed them to grow for 8 days to confluence arrest (G0 phase). Cells were fed on days 3 and 5 postseeding. We trypsinized, reseeded, and incubated cells for 8 hr in fresh medium to allow them to reach G1 phase. Then, 1 × 10⁷ cells were harvested at each growth phase for RNA isolation. Flow cytometric analysis indicated that > 90% of cells were synchronized to G0 by confluence arrest, and 8 hr after release from confluence and reseeding in serum-containing medium, > 90% remained with 2N DNA content (G1) (Unsal-Kacmaz et al. 2005). Using this synchronization method, cells began to enter S phase 12 hr after release from confluence arrest (Cordeiro-Stone et al. 1986; Dulic et al. 1994).

Oligo DNA microarray. Logarithmically growing NHF1, NHF3, and NHF10 cells were treated with 1.5 Gy IR, or sham-treated, and harvested at 2, 6, or 24 hr after the treatment. Total RNA was isolated using an RNeasy kit (Qiagen Inc., Valencia, CA). The quality of all RNA samples was confirmed using an Agilent 2100 bioanalyzer. Microarray analysis was then performed. Briefly, 1 μg of sample RNA and global reference RNA (Stratagene, La Jolla, CA) were converted to cDNA with reverse transcriptase and then amplified using T7 RNA polymerase while labeling with either cyanine 3 deoxyuridine triphosphate (Cy3-dUTP) or Cy5-dUTP (Low RNA Input Linear Amplification Kit, Agilent Technologies). The quality of each labeled cRNA was evaluated using an Agilent 2100 bioanalyzer before hybridization; 750 ng of Cy3- and Cy5-labeled cRNA were used in the hybridization. The labeled cRNA from sham- or IR-treated samples was hybridized with the labeled global reference cRNA on an Agilent 22K human 1A array (Agilent Technologies) in a hybridization oven (model 400, 1040-60-1AG; Robbins Scientific, Sunnyvale, CA) at 60°C for 17 hr. Hybridization of sample RNA against reference RNA was done twice with dye swap. After hybridization, we scanned the arrays using the Agilent DNA Microarray Scanner with SureScan Technology; microarray images were analyzed using Agilent Feature Extraction software (version 7.1; Agilent Technologies). The gene expression level was presented as the ratio of sample intensity against reference intensity.

Microarray data analysis. The extracted intensity data from each array were preprocessed, which included array-based systematic variation normalization (Chou et al. 2005), profile-based dye-swap correction, and biological reference state alignment (Chou JW, Zhou T, Raules RS, Kaufmann WK, Bushel PR, unpublished data). In this data set, the sham-treated condition was used as a reference state. The average of the dye-swapped pair of sham-treated control arrays was aligned to log zero as a baseline, with the IR-treated samples adjusted by the same amount. Through signal-to-noise ratio evaluation applied to each correlation local cluster, EPIG extracted a set of discrete gene expression patterns. Each pattern represented a set of co-expressed genes. EPIG used the profile's signal magnitude and signal-to-noise ratio to identify biologically significant genes and categorized them within patterns according to their correlation r-values. (Chou Zhou JW, Zhou T, Raules RS, Kaufmann WK, Bushel PR, unpublished data). Two-way clustering heat maps were made by using Cluster and Treeview software (Eisen et al. 1998). Three-dimensional principal component analysis (PCA) was done using EPIG. Categories of genes that were overrepresented in a selected gene list, compared with what was represented in the microarray, were analyzed using Expression Analysis Systematic Explorer (EASE; http://david.nih.gov/david/ease.htm). Such overrepresented categories represent biological “themes” of a given list (Hawes et al. 2005).

Results

Dose-dependent inactivation of clonogenic survival by IR. Treatment with IR inhibited single-cell colony formation in three diploid human fibroblast lines, NHF1, NHF3, and NHF10, with similar dose kinetics (Figure 1). A shoulder on survival curves was apparent below the 1.5-Gy dose. The slope of the curves between the 1.5- and 4.5-Gy doses approximated a D0 (lethel dose) dose of about 1.5 Gy similar to D0 (mean lethal dose) values previously recorded for IR-treated normal human fibroblasts (Arlett et al. 1988). The 1.5-Gy dose reduced colony formation in fibroblasts by 40–45% relative to sham-treated controls and was selected for further analysis of cell cycle checkpoint responses and changes in gene expression.

IR-induced cell cycle checkpoint responses synchronize cell division. We quantified the IR-induced G1 checkpoint by measuring the incorporation of bromodeoxyuridine 6–8 hr after 1.5-Gy or sham treatment (Figure 2A, left). IR-treated fibroblasts displayed a severe reduction in the fraction of BrdU-labeled S-phase cells with 2–3N DNA content (first half of S) due to ATM- and p53-dependent G1 arrest (Kaufmann et al. 2003). The three
fibroblast lines exhibited > 93% G1 arrest (Table 1). The IR-induced G2 checkpoint was quantified by measuring mitosis-specific phosphohistone H3 immunostaining 2 hr post-IR or sham treatment (Figure 2A, right). IR-treated fibroblasts displayed a severe reduction in the fraction of mitotic cells because of ATM-dependent G2 arrest. The three fibroblast lines exhibited > 94% G1 arrest (Table 1).

Dynamic changes in DNA content, DNA synthesis, and mitosis were charted 2–24 hr post-IR (Figure 2B). The three cell lines showed very similar responses to IR. The percentage of cells with 2N DNA that did not incorporate BrdU (G1) showed a small decline at 2 hr post-IR and then rose to a plateau 10% above control by 12 hr. S-phase cells with 2–4N DNA content and labeled with BrdU declined between 6 and 12 hr post-IR to a nadir at < 5% of control, then recovered by 24 hr to 10–30% of the sham-treated control. There was a rapid post-IR accumulation of 4N cells with no BrdU incorporation (predominantly G2), which peaked at 6 hr and then declined to near control levels by 24 hr. Mitosis was severely inhibited 2 hr post-IR and then recovered to control levels at 6 and 12 hr before falling again at 24 hr to < 25% of control. The recovery of mitotic cells 6 and 12 hr post-IR and the decline in G2 cells 6–24 hr post-IR indicate that the G2 checkpoint response to IR was a transient arrest that was largely reversed by 6 hr. The severe (70–90%) reduction in S- and M-phase cells 24 hr post-IR is consistent with synchronization of fibroblasts behind a persistent G1 checkpoint response.

Profiles of gene expression in response to IR in normal human fibroblasts. We used Agilent human 1A arrays (22K) to monitor gene expression post-IR in the three different fibroblast lines. Fibroblasts were harvested 2, 6, and 24 hr after 1.5 Gy, which are times of maximal initial G2 arrest with minimal reduction in S phase, maximal G1 arrest with recovery of mitosis, and sustained G1 arrest with depletion of S phase and mitosis, respectively. Controls were harvested 6 hr after sham treatment. Gene expression profiles extracted from microarray data included 24 arrays. Each cell line (NHF1, NHF3, and NHF10) had four treatment states (sham and 1.5 Gy IR) and dye-swapped pairs for each treatment. EPIG extracted nine patterns of change in gene expression and identified a total of 1,811 genes as significantly altered in response to IR (Figure 3). The numbers of genes in each pattern varied from several to several hundreds, and about one-third of the 1,811 selected genes were expressed sequence tags (ESTs). Pattern 1 included 18 genes that were highly induced at 2 hr, then declined modestly through 24 hr. This pattern included prototypical p53-target genes that mainly contribute to initiation and maintenance of G1 arrest through inhibition of cyclin-dependent kinases. Pattern 2 included 24 genes that were progressively induced from 2 to 24 hr. These genes also are known to be induced by p53-dependent signaling. CCNG1 may contribute to recovery of DNA synthesis through attenuation of p53 signaling (Ohtsuka et al. 2004). Pattern 3 included 15 genes that were induced only at 2 hr, including immediate early-response genes IER3 and IER5 and a p16 (INK4A) antagonist, SEL1. Pattern 4 included 18 genes that were repressed only at 2 hr. Repression of MYC and the early growth-response gene EGR1 in this group may further negatively regulate E2F1 and its target gene expression. Pattern 5 included 6 genes that were induced modestly at 6 hr but repressed at 24 hr. Pattern 6 included 9 genes that were induced at 2 and 24 hr but not at 6 hr. Pattern 7 included 14 genes that were highly repressed at 6 and 24 hr, of which CCNE1 (cyclin E) repression is an important indicator of G1 arrest starting at 6 hr. Several DNA repair genes, such as MSH2, FANC E, and UNG, were in this group. Pattern 8 was composed of 901 genes that were repressed moderately at 6 hr but highly repressed at 24 hr, including many genes whose products participate in various DNA metabolic events during the cell division cycle, such as ASK, CCNA2, CCNB1, CCNB2, CDK2, CD2, CDC6, CDC45L, CDC7L, MCMs, RFC subunits, and TOP2A (Mendez and Stillman 2000; Stillman 1996). Several DNA repair genes, MSH2, RAD18, RAD51, RAD54, XRCC1, XRCC4, and XRCC5, and two apoptosis inducers, CASP3 and HCS, were also in this category. Pattern 9 included 806 genes that were induced at 24 hr, including CCNDBPI (a negative regulator of E2F1), the stress-response genes GSTM3 and SOD3.

Table 1. DNA damage G1 and G2 checkpoint responses to 1.5 Gy IR (mean ± SD, n = 4–6).

| Cell line   | G1 arrest (%) | G2 arrest (%) |
|-------------|---------------|---------------|
| NHF1-hTERT  | 95 ± 2        | 97 ± 1        |
| NHF3-hTERT  | 93 ± 2        | 95 ± 1        |
| NHF10-hTERT | 94 ± 1        | 94 ± 2        |

*G1 arrest was quantified by determining the percentage reduction in the fraction of cells in the first half of the S phase (Figure 2A). G2 arrest was quantified by determining the percentage reduction in the fraction of cells in mitosis (Figure 2A).
and p53-dependent genes (TP53BP1 and TP53TG1). A G0-specific gene GAS1 was highly induced at 24 hr. Gene Ontology (http://david.niaid.nih.gov/david/ease.htm) analysis of the 1,811 genes using EASE revealed that more than 30 biological processes were overrepresented in response to IR-induced DNA damage (Table 2). Categories in which the proportion of IR-responsive genes exceeded expectation based on chance included cell cycle, cell proliferation, DNA and RNA metabolism, M phase of mitotic cell cycle, S phase of mitotic cell cycle, response to DNA damage stimulus, DNA repair, and cell-cycle checkpoint (Table 2). When EASE analysis was focused on each pattern, genes in pattern 1 indicated negative regulation of cell proliferation and cell cycle arrest (Table 3), genes in pattern 8 showed similar categories as analyzed in the entire 1,811 gene list, and genes in pattern 9 showed only several categories that were not obviously related to DNA damage response, such as extracellular matrix, calcium ion binding, and

Figure 3. Patterns of gene expression in the three fibroblast lines NHF1, NHF3, and NHF10 in response to IR-induced DNA damage. Nine different DNA-damage–responsive patterns of gene expression were extracted using EPIC, each pattern representing a group of genes that were expressed in the same way upon DNA damage. In each panel (patterns 1–9) the numbers of genes in each pattern are shown in parentheses. For each cell line, the log2 ratios of sample RNA against reference RNA are indicated at each time point (IR 2 hr, IR 6 hr, IR 24 hr) with the sham-treated controls adjusted to zero and with both dye-flip replicates shown. The thick lines show the average response to IR of genes in the pattern for each of the fibroblast lines, and the thin lines show the responses of the selected genes listed to the right. For the genes listed, the coefficient of correlation with the average pattern, the magnitude of change in gene expression (log2), and the signal-to-noise ratio (SNR) are given.
antigen processing. Patterns 2–7 did not display overrepresentation of specific gene categories.

Global gene expression patterns in fibroblasts reveal individual genetic background, previous IR treatment, and IR-induced G0 quiescence. Many genes display cell cycle–dependent changes in expression, and it has been demonstrated that IR treatment can induce a senescence-like permanent G1 arrest in fibroblasts, even after a low dose of 1 Gy (Di Leonardo et al. 1994). We were interested in determining whether the IR-induced changes in gene expression reflected a pattern of G1 arrest. Cells were synchronized to G0 by growth to confluence and cells were synchronized to G1 by replating G0 quiescent cells at lower cell density in serum-containing medium with harvest at 8 hr after replating. Previous studies using this method indicated that G0-synchronized cells expressed low levels of cyclins A and D1 and hypophosphorylated retinoblastoma (RB), whereas G1-synchronized cells expressed increasing levels of cyclins A and D1 with hyperphosphorylated RB (Dulic et al. 1994).

Unsupervised hierarchical cluster analysis of all genes in the arrays, totaling 16,390 single clones, was performed using data from sham-irradiated control cells, cells treated with 1.5-Gy IR, 1-Gy IR, or 24-hr post-IR cells and cells synchronized to G0 clones, was performed using data from sham-irradiated control cells, cells treated with 1.5-Gy IR, 1-Gy IR, or 24-hr post-IR cells and cells synchronized to G0, G1, or G2 as indicated by EPIG as showing significant response to IR-induced DNA damage. As found in the cluster analysis with all 16,390 genes, the pattern of gene expression using the smaller set of 1,811 IR-responsive genes revealed similarity between 24-hr post-IR and G0 cells, and differences with G1 cells (Figure 4B). One group of genes induced by IR at 24 hr was expressed at the highest level in G0 cells compared with G1, S, G2, and M-synchronized cell populations (Zhao et al., unpublished observations) and asynchronous cell populations. Genes in this group included GAPI (growth-arrest-specific 1) that was first recognized as induced in G0 cells (Del Sal et al. 1992) and several p53 target genes, FDXR, DDB2, BTG2, CCNG1, PA26, and SNK. A second group of genes that was more highly expressed in G1 than in G0 and 24-hr post-IR cells included G0S2, MYC, CCND1, CNK, ID1, and ID3. The marked differences in gene expression patterns between G1 cells and G0 or 24-hr post-IR cells were largely contributed by these two groups of genes. Most of the cell cycle–regulated genes, functioning in the G1/S and G2/M transitions, were expressed at similar low levels in 24-hr post-IR, G0, and G1 groups compared with other asynchronous groups. The same set of 1,811 genes was used for three-dimensional PCA. The results with both asynchronous and synchronized cell populations showed that 24-hr post-IR components were very close to but did not overlap with the G0 components, and both 24-hr post-IR and G0 components were far from G1 components (Figure 4C).

Discussion

Cell cycle checkpoint functions and gene regulation in response to IR. Although each cell displayed a unique pattern of gene expression (Cheung et al. 2003), changes in gene expression related to cell cycle control, DNA metabolism, and apoptosis were very similar in the three lines in response to IR treatment.

When DNA damage occurred in G2 cells (~10% of the total cell population), a stringent G2 arrest was observed 2 hr post-IR, as indicated by ≥94% reduction in mitotic cells. We did not expect the 1.5-Gy dose of IR to impede progress through and completion of mitosis. As a consequence of the IR-induced G2 arrest due to ATM- and ATR-dependent signaling (Deng et al. 2001; Kaufmann et al. 2003), the mitotic compartment emptied nearly fully. The expression of genes that controls the G2/M transition, particularly Plk1, Cdc25C, CDC2, and cyclin B1, was not changed at 2 hr post-IR, demonstrating the importance for this rapid G2 arrest of posttranscriptional modification of the above proteins. The ATM-CHK1 pathway responsible for changes in the phosphorylation status of CDC2 and Polo-like kinases has been well studied in activation of the immediate G2 checkpoint response post-IR (for a review, see Kaufmann et al. 2002). The G2 arrest induced by 1.5 Gy IR did not last long, however, because by 6 hr post-IR the frequency of mitotic cells in IR-treated cultures equaled or exceeded control values.

Synchronization of cells behind the G1 checkpoint can be expected to produce significant changes in gene expression. Given the approximately 4-fold reduction in S- and M-phase cells 24 hr post-IR as cells were

Table 2. EASE analysis of all genes selected by EPIG based on the Gene Ontology biological process.

| Gene category | EASE score | Bonferroni |
|---------------|------------|------------|
| Mitotic cell cycle | 3.19 × 10^-13 | 7.87 × 10^-30 |
| Cell cycle | 4.33 × 10^-28 | 1.07 × 10^-43 |
| DNA metabolism | 5.35 × 10^-23 | 1.32 × 10^-19 |
| DNA replication and chromosome cycle | 1.59 × 10^-21 | 3.93 × 10^-18 |
| M phase | 6.16 × 10^-20 | 1.52 × 10^-16 |
| Mitosis | 2.92 × 10^-16 | 7.20 × 10^-13 |
| M phase of mitotic cell cycle | 7.48 × 10^-16 | 1.84 × 10^-12 |
| Nuclear division | 7.64 × 10^-16 | 1.88 × 10^-12 |
| S phase of mitotic cell cycle | 1.38 × 10^-15 | 3.40 × 10^-12 |
| DNA replication | 1.38 × 10^-15 | 3.40 × 10^-12 |
| RNA metabolism | 1.47 × 10^-13 | 3.61 × 10^-10 |
| RNA processing | 6.96 × 10^-13 | 1.62 × 10^-10 |
| Regulation of cell cycle | 8.67 × 10^-13 | 1.68 × 10^-9 |
| Response to endogenous stimulus | 1.49 × 10^-12 | 3.68 × 10^-9 |
| Response to DNA damage stimulus | 2.42 × 10^-12 | 5.96 × 10^-9 |

*P-Value adjusted for multiple comparison.

Table 3. EASE analysis of overrepresented genes in pattern 1.

| Gene category | EASE score | Bonferroni |
|---------------|------------|------------|
| Negative regulation of cell proliferation | 7.93 × 10^-16 | 1.07 × 10^-31 |
| Regulation of cell proliferation | 1.09 × 10^-04 | 1.48 × 10^-02 |
| Regulation of cellular process | 2.83 × 10^-04 | 3.82 × 10^-02 |
| Regulation of biologic process | 2.94 × 10^-04 | 3.98 × 10^-02 |
| Cell proliferation | 4.47 × 10^-04 | 6.04 × 10^-02 |
| Regulation of cell cycle | 6.12 × 10^-04 | 8.26 × 10^-02 |
| Cell cycle arrest | 1.42 × 10^-03 | 1.91 × 10^-01 |
arrested in G1, one would expect that the levels of expression of genes that are normally expressed in S, G2, and M phases would decline to a similar extent. More than 20 genes involved in cell cycle control and DNA metabolism were down-regulated by 4-fold or more 24 hr post-IR, implying that there was a common response to IR-induced DNA damage affecting large numbers of genes. The down-regulation of genes required for passage through S, G2, and M phases will be a necessary secondary consequence of prolonged accumulation of cells behind the G1 checkpoint. It is notable that the kinetic pattern of change in gene expression in diploid human fibroblasts given 1.5 Gy IR is quite different from that observed in HeLa cells given 10 Gy IR, where cells experience a prolonged G2 delay (Crawford and Ptwnica-Worms 2001).

Genes that play important roles in the G1/S transition and initiation of DNA replication, including CCNE, CDK2, CDC6, CDC45, CDC7, RFC subunits, and MCM family members, were down-regulated at 6 and 24 hr post-IR as an expected consequence of the p53-dependent induction of CDKN1A (p21Waf1) to enforce the G1 checkpoint. However, the p53/p21Waf1 pathway may not be the only one contributing to G1 arrest. For example, ARF (alternative reading frame) induced biphasic (G1 and G2) arrest in a p21-independent pathway (Modestou et al. 2001), and expression of c-Abl tyrosine kinase down-regulated the activity of CDK2 and induced G1 arrest in p21−/− but not p53−/− cells (Yuan et al. 1996). Many other p53 target genes for early induced genes, see patterns 1 and 2 in Figure 3) may also play roles in p53-dependent cell cycle arrest. BTG2 induces accumulation of unphosphorylated RB by inhibiting cyclin D1 (Guardavaccaro et al. 2000). GADD45 stabilizes phosphorylation of serine-15 of p53 providing a positive feedback signal in activation of the p53 pathway (Jin et al. 2003), Plk3 phosphorylates p53 on serine-20 and enhances p53 stabilization (Xie et al. 2001), and p53-dependent activation of Plk2 prevents mitotic catastrophe after spindle damage (Burns et al. 2003). PPMD1, a member of the PP2C family of Ser/Thr protein phosphatases that is induced in a p53-dependent manner in response to various environmental stresses, reduces the phosphorylation of p53 through negative regulation of p38 MAP kinase, and in turn suppresses p53-mediated transcription and apoptosis (Bulavin et al. 2004). The p53-dependent G1 checkpoint orchestrates a complex signaling network to regulate the G1/S transition. Other p53-target genes seen in patterns 1 and 2, including FDXR, PLAB, PA26, SES2, and TRF4, may function in cell cycle regulation, although their correspondent functions have not been determined. However, at least one gene in pattern 2, CCNG1, has been reported to contribute to recovery of DNA synthesis after DNA damage through negative feedback regulation of p53 signaling (Ohtsuka et al. 2003). Several genes in pattern 1 have not been reported as p53-target genes (Bunz et al. 2002; Hoh et al. 2002; Sax and El-Deiry 2003; Yu et al. 1999).

We found that several genes of unknown function, L_1000314, OSBP3L, FLJ12484, and I_931541, were regulated in a p53-dependent way upon DNA damage. These genes and most of the genes in patterns 1 and 2 were not induced by IR in fibroblasts expressing the HPV16E6 oncoprotein or a dominant-negative mutant p53 allele to inactivate p53 function (Zhou et al., unpublished observations). IR synchronized fibroblasts to a G0-like state. An important finding in the present study was that 1.5 Gy IR induced a pattern of gene expression 24 hr post-IR that was more similar to G0 than to G1. Moreover, a G0-specific gene, GAS1, was markedly induced at 24 hr, indicating induction of a G0-like state of quiescence. Quantification of colony formation post-IR indicated that 40–45% of cells were permanently arrested and unable to form colonies. Although 55–60% of irradiated cells re-entered the cell cycle and successfully produced colonies, this resumption of DNA synthesis was not evident 24 hr post-IR. Di Leonardo et al. (1994) have charted the kinetics of DNA replication in irradiated fibroblasts and shown

**Figure 4.** Comparison of gene expression patterns between G0 and G1-synchronized cells, and IR- or sham-treated cells. (A) Unsupervised hierarchical cluster analysis was done by using the 16,330 genes listed in the Agilent human 1A array. Before clustering, the mean center of genes was repeated 5 times, and an uncentered correlation was used for the similarity metric calculation. (B) Supervised hierarchical cluster analysis was done using the 1,811 genes significantly changed in response to IR. To the right of the cluster are listed selected genes that show similar expression patterns in G0 and 24-hr post-IR samples but a different pattern in G1 cells. (C) PCA analysis using 1,811 significant genes showed that 24-hr post-IR patterns were very close but did not overlap with G0 patterns, and 24-hr post-IR and G0 patterns were far from G1 patterns.
that a portion of cells arrested behind the G1 checkpoint resume DNA synthesis 24–72 hr post-IR. DNA double-strand breaks are thought to be the IR-induced lethal damage, and a small portion (2–5%) of DNA double-strand breaks is not rejoined by DNA repair (Dikomey et al. 2003). These unrepaired or misrepaired double-strand breaks likely account for an unremitting signal to arrest growth. Most of the observed 40–45% reduction of colony formation was considered previously to be a permanent G1 arrest (Di Leonardo et al. 1994; Walworth 2000). The present analysis of more than 16,000 genes showed that cells arrested behind the G1 checkpoint displayed a pattern of gene expression more reflective of G0 than of G1. This suggests that during prolonged IR-induced G1 arrest, additional reprogramming of gene expression occurs to bring cells into a G0-like state. To test for induction of replicative senescence, the senescence-associated marker pH 6.0 β-galactosidase (Dimri et al. 1999) was determined in a senescent fibroblast line (positive control) and in sham-treated or 1.5 Gy IR–treated cells from 1 to 7 days after the treatment. The percentage of β-galactosidase–positive cells was much higher in the senescent cells than in the sham- or IR-treated young cells, and no increased β-galactosidase staining was observed in IR-treated cells compared with sham-treated cells (data not shown). This result further supports the conclusion that IR induced a G0-like growth arrest instead of a senescence-like G1 arrest.

In summary, low-dose (1.5 Gy) IR induced marked G1 and G2 delays in normal human fibroblasts and caused stochastic changes in the patterns of gene expression associated with these cell cycle delays. Early transcriptional activation of CDKN1A (p21Waf1) and many other p53-target genes as the effectors of G1 checkpoint response may account for the great majority of changes in gene expression recognized in irradiated fibroblasts. However, the sustained G1 arrest associated with induction of p21Waf1 in IR-damaged fibroblasts resembled a G0-like state of quiescence. In addition, direct transcriptional repression of important cell cycle–regulated genes through p53-dependent signaling may maintain the growth arrest. This aspect of DNA damage response will be addressed in a subsequent report.

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