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Effect of Passage Number on Cellular Response to DNA-damaging Agents: Cell Survival and Gene Expression¹

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Running Title: Response to DNA-damaging Agents

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ABSTRACT

The effect of different passage numbers on plating efficiency, doubling time, cell growth, and radiation sensitivity was assessed in Syrian hamster embryo (SHE) cells. Changes in gene expression after UV or γ -ray irradiation at different passage numbers were also examined. The SHE cells were maintained in culture medium for up to 64 passages. Cells were exposed to ^{60}Co γ rays or 254-nm UV radiation. Differential display of cDNAs and Northern blots were used for the study of gene expression. With increasing passage number, SHE cells demonstrated decreased doubling time, increased plating efficiency, and a decreased yield in the number of cells per plate. Between passages 41 and 48 a "crisis" period was evident during which time cell growth in high serum (20%) was no longer optimal, and serum concentrations were reduced (to 10%) to maintain cell growth. Sensitivity to ionizing radiation was no different between early- and intermediate-passage cells. However, after UV exposure at low passages (passage 3), confluent cells were more sensitive to the killing effects of UV than were log-phase cells. At intermediate passages (passages 43, 48), confluent cells were slightly more radioresistant than were log-phase cells. By passage 64, however, both confluent and log-phase cells showed similar patterns of UV sensitivity. Expression of γ -actin, PCNA, and p53 transcripts did not change following UV exposure. p53 mRNA was induced following γ -ray exposure of the intermediate (passage 45) epithelial cells. The observed differences in radiation sensitivity associated with increasing passage number may be influenced by radiation-induced gene expression. We are conducting experiments to identify these genes.

INTRODUCTION

The functional relationship between radiation-induced cellular responses (such as apoptosis, DNA repair, etc.) and radiation-mediated changes in gene expression has been explored by a number of different laboratories in the past several years [1-15]. Genes induced after radiation exposure include those encoding cytokines [1-3], cell cycle arrest proteins [4,5], viruses [6-8], cell signal transducing agents [9,10], cytoskeletal elements [11,12], apoptosis-associated proteins [13], and a variety of unknown genes [4,14,15]. However, the precise role that this specific gene induction plays in the functional consequences of radiation exposure is still unknown.

In the experiments reported here, we set out first to explore the relationship between cell passage number and generation doubling time, plating efficiency, UV and radiosensitivity, and cell growth. Second, we designed experiments to examine relative gene expression as a consequence of radiation exposure and cell passage number. We chose the SHE³ cells for these studies because of the large body of literature examining radiation effects on these normal cells [1,9,11-13,16], our previous experience with these cells, and the well-known growth characteristics of these cells [1, 16].

The experiments reported here establish the following as a consequence of increasing cell passage number: decreased cell doubling time, decreased saturation (cell yield/plate), and increased plating efficiency. In addition, a reversion in the UV sensitivity of confluent and log-phase cells was apparent. At early passage (passage 3) the confluent cells are more sensitive to the killing effects of UV than log phase cells; at intermediate passage the log phase became more sensitive than the confluent cells and at late passage the exponentially

growing cells are equally as sensitive as the confluent cells. These changes are accompanied with changes in control, γ -ray, and UV-induced gene expression. Ionizing radiation sensitivities did not change with passage number, but changes in gene expression were also evident in control and γ -ray exposed cells.

METHODS

Cells and culture conditions. In all experiments, we examined the modulation of gene expression after exposure to UV and ionizing radiations in SHE fibroblasts; primary cultures were normal diploid cells that could be neoplastically transformed by low doses of ionizing radiation [1,16].

All cell cultures were established in Dulbecco's modified Eagle's medium containing 10% or 20% fetal calf serum (as indicated in the text), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml), called DH-10 Δ . Cells were grown to confluence; 48 h before irradiation, they were placed in medium containing 1.0% fetal calf serum to maintain the cells in plateau phase. Studies of preirradiated early passage SHE cells grown under this protocol showed them to be a mixed population of fibroblasts, with >90–95% of the cells in the G₀-G₁ phase of the cell cycle. Cells for the present experiments were from passages 3–64, as indicated throughout the text. Cells from the initial culture are called SHE-H3 cells, with the passage number indicated by a P after H3. We have cultured SHE cells from early passages [2–6] for previous experiments [1,9,11–13,16], and these cells appeared similar in morphology to the others at early passages.

Radiation treatments. For γ -ray exposures, cells were plated in 100-mm Petri plates containing 10 ml medium and irradiated with ^{60}Co γ rays at the indicated doses and dose rates. All irradiations were performed with the cells kept at 37 °C. Control cells were taken to the radiation chamber but not exposed to radiation. Plates of cells were then incubated at 37 °C for 1 or 3 h after irradiation but before harvest of the RNA.

Exposures to 254-nm UV radiation were carried out at room temperature by using a GE60TS 30-W germicidal lamp (General Electric) that was contained in a sterile hood. The irradiations were at a distance of 55.6 cm from the source. The dose rate for all exposures to UVC radiation was $2.5 \text{ J}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The media were removed from the 100-mm plates, and the plates were washed once with cold phosphate-buffered saline and irradiated without covers. The media were replaced immediately after irradiation.

Parameters of cell growth. Determination of cell doubling times, plating efficiencies, and the numbers of cells per plate was carried out as described previously [1,17]. Viable cell counts (based on >1000 cells) were determined by trypan blue dye exclusion. Cell survival experiments were determined by cell survival in 8-day colony assays [17–19].

Purification of RNA and Northern blots. RNA was prepared by isolation in 3 M guanidine isothiocyanate, extraction with phenol, and precipitation from 3 M sodium acetate, pH 6.0 [14,16,17]. Purified RNA was obtained by digestion with DNase I (37 °C for 1 h). PolyA⁺ RNA was purified and Northern blots were performed as described previously [1,9]. Total and PolyA⁺ RNA were used as indicated throughout the paper.

cDNA probes. cDNA clones were generously provided by the following: p53 by American Type Culture Collection (Bethesda, MD), γ -actin by Dr. L. Kedes (Stanford University, Palo Alto, CA), PCNA by Dr. R. Bravo (Bristol Myers), and ODC by Dr. P. Coffino (University of California, San Francisco, CA).

RESULTS

Effects of passage number on cell growth. SHE-H3 fibroblasts were harvested directly from pooled hamster embryos derived from the second pregnancy of five different mothers; cells were cultured for up to 68 passages and monitored for the various parameters of cell growth at specific passages. Figure 1 documents results from the entire experiment by depicting changes in plating efficiency, cell doubling time, and the number of confluent cells per plate as a function of cell culture passage number. These experiments established a decrease in doubling time, a decrease in the saturation density (or number of confluent cells per plate), and an increase in plating efficiency with increasing passage number. A "crisis" in the culture was apparent beginning at passage 37; at that time it was necessary to shift the cultures from 20% fetal calf serum (which was used for the initial primary cultures) to 10% fetal calf serum to allow for cell survival. This crisis period, which was evident until passage 49, was characterized by dramatic daily and weekly changes in plating efficiencies and cell growth parameters.

From these cultures, specific passages were selected for further analysis. The history of these cells and other growth characteristics are depicted in Table 1. This table documents the growth conditions and growth parameters (including half-life, plating efficiency, etc.) for

each of the passages. SHE-H3 cells that retained fibroblast characteristics were examined at passages 1, 3, 45, 48, and 64. An epidermal subline (H3CE) was obtained at passage 40 and analyzed at passage 45 to allow for a determination of possible cell subtype differences. A more detailed analysis is depicted in Fig. 2 in which these selected passages of fibroblasts (passages 3, 48, and 64) were compared for plating efficiency and doubling time. In addition, one culture showing morphological features of the epidermal (rather than fibroblastic) cells was also compared at passage 43. Doubling time and plating efficiency for that cell were similar to those observed for similar-passage (passage 48) SHE-H3 cells.

Effects of passage number on radiosensitivity. The next series of experiments were performed to determine the effects of cell passage number on sensitivity to ionizing and UV radiations. Table 2 presents the results from experiments in which the survival rate was examined after exposure of cells to 96 cGy of ^{60}Co γ rays. There was no difference in the survival rate of early- (passage 1) or medium-passage (passage 45) SHE-H3 fibroblasts after γ -ray exposure. The epidermal cells (SHE-H3CE) did show increased radiosensitivity, which may be attributed to cell type differences.

Studies of the effects of cell passage number on UV sensitivity are depicted in Table 3 which compares cultures of SHE-H3 cells from passages 3, 48, and 64. These results showed a change in the D_0 value with passage number. Early- and late-passage cells showed similar D_0 values, but the intermediate-passage (passage 48) cells had markedly lower D_0 values than either early or late passage cells. This was not related to cell type since the epidermal type SHE cells (SHE-H3CE) from passage 43 also expressed low D_0 values.

Comparisons of survival curves for each of these cells from selected passage numbers after UV exposure of the cultures either in log phase or when confluent are evident in Fig. 3. In Figure 3A, it is apparent that passage 3 cells had increased UV-resistance when in log phase. Figure 3B demonstrates that in intermediate-passage (passage 48) cells, the confluent cultures are more resistant than the log-phase cultures over most of the survival curve. However, in late-passage (passage 64) cultures, the log-phase and confluent cultures were similar with respect to survival curve. The intermediate-passage (passage 43) epidermal cells (Fig. 3D) showed a more dramatic resistance of confluent cells even than the intermediate-passage fibroblasts. In addition, the confluent cells appear to have a resistant tail, suggesting the presence of a resistant cell subpopulation in the cultures or the possible shielding of a subset of cells in the Petri plate from UV radiation (perhaps those around the perimeter).

Effects of passage number on gene expression. Initially, we set out to examine the expression of several known genes as a function of passage number and radiation exposure. Untreated and irradiated (96 cGy) confluent fibroblasts and epidermal SHE cells from various passages (1, 40, 45) were harvested 1 h post-radiation exposure. RNA was prepared and Northern blots were performed. Figure 4 presents results from several such experiments. γ -actin mRNA is expressed similarly regardless of passage, cell type (H3 fibroblasts vs. H3CE epidermal cells), RNA preparation (total or Poly A+) or γ -ray exposure. PCNA mRNA expression was enhanced in later passages regardless of radiation exposure or cell type. mRNA specific for p53, however, was detectable only in γ -ray exposed H3CE epidermal cells. This γ -ray induction of p53 mRNA was not detected in any other passages or in the intermediate passage H3 fibroblasts. These transcripts (PCNA and p53) could not be

adequately detected in total RNA preparations (data not shown). mRNA for ornithine decarboxylase (ODC) was poorly detected in all of the preparations and quantitation was not possible (data not shown).

Effects of UV exposure ($5-75 \text{ Jm}^{-2}$) administered at a dose-rate of $2.5 \text{ Jm}^{-2}/\text{sec}$) on SHE cells from the same passages described above (for γ -ray exposures) similarly revealed that changes in γ -actin and PCNA RNA were passage-related and not affected by UV exposure. p53 was not induced following UV exposure of cells from any passage, including the H3CE epithelial cells which showed p53 mRNA induction following γ -ray exposure above (data not shown). This suggests that p53 induction in epidermal cells may be specific for γ -rays.

DISCUSSION

This work explored the effect of cell passage number on doubling time, plating efficiency, cell growth, and sensitivity to radiation. The results of these experiments indicated decreased doubling time and cell yield per plate but increasing plating efficiency with increasing passage number (Table 4). In addition, while sensitivity to ionizing radiation was not affected, UV sensitivity, especially with regard to cell cycle effects, was markedly dependent on passage number. In early-passage cells, confluent cells were more sensitive than log-phase cells; a tendency in the reverse was apparent with intermediate-passage cells. By late passage (passage 64), log-phase and confluent cultures demonstrated similar patterns of sensitivity to UV. In addition, changes in gene expression were found to accompany these alterations in radiosensitivity. This is probably not surprising in light of the fact that with

each passage there is a selection for faster growing, better plating cells. Many investigators [17–19] note that cells progress toward transformation with increasing passage number. The fact that doubling time decreases (a feature characteristic of many transformed relative to non-transformed cell lines) is not surprising. This may be causally related to the cell cycle differences in UV radiosensitivity found associated with increasing passage number.

The appearance of a "crisis" period in long-term cultures is well documented in the literature [20–22]. It has been hypothesized that this is related to the achievement of growth factor independence in the culture, another step associated with increasing transformation. The fact that this crisis is associated with lower serum requirements confirms a possible growth factor influence. This is further supported by the finding that fewer cells per plate and decreased cell doubling time also occur with increasing passage number. Autocrine growth factors may replace exogenous factors during the crisis period.

Studies of changes in expression of known genes with radiation exposure and cell passage number revealed few differences. γ -actin mRNA was unaffected by exposure to γ -rays, or irradiation, a result which is consistent with previous work from our group [11,12]. PCNA expression also did not change following exposure to UV or γ -rays, although the early passage (passage 1) fibroblasts expressed lower levels than the intermediate passage cells. This is not surprising in light of the fact that PCNA is a cell cycle-specific transcript [23–25] and that later passage cells have an increased doubling time relative to the early passage cells (Fig. 2). p53-mRNA was undetected in all untreated fibroblast cell cultures. Epidermal type H3CE cells isolated at passage 45 expressed p53 mRNA only following exposure to γ rays (but not following exposure to UV). This is interesting in light of the fact that most studies

have reported p53 induction at the protein but not at the mRNA level [26,27]. It is not clear whether this γ -ray induction of p53 in the H3CE cells is due to their epidermal characteristics or whether the cells are partially transformed (based on growth characteristics, plating efficiency, etc. described throughout this manuscript) or perhaps have acquired a p53 mutation/alteration. These experiments do suggest, however, that γ -ray induction of p53 can occur. Its induction is not related to cell passage alone since intermediate passage H3 cells do not induce p53 following γ -ray exposure alone. p53 induction was not observed following UV exposure, suggesting that p53 induction in these cells is γ -ray-specific.

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Table 1. Cell history^a

Cell line/ Passage No.	No. of initial inoculated cells/ plate	No. of cells harvested/ plate	Growth period (days)	Harvested cells/In- oculated cells	Plat- ing effic- iency (%)	Doubling time (h)
H3/P3	3.5×10^5	3.5×10^6	3	10	1	22
H3/P3	3.5×10^5	4.0×10^6	6	11	5	-
H3/P48	5.0×10^4	1.8×10^6	3	36	25	14
H3/P48	5.0×10^4	3.5×10^6	6	70	6	-
H3/P64	2.0×10^5	9.0×10^6	3	45	28	13
H3/P64	2.0×10^5	7.0×10^6	6	35	49	-
H3/P1	3.5×10^5	3.5×10^6	4	10	1	28
H3/P1	3.5×10^5	5.0×10^6	4	14	-	25
H3/P45	5.0×10^4	2.7×10^6	4	54	9	17
H3CE/P40	5.0×10^4	1.7×10^6	4	34	13	19
H3CE/P45	5.0×10^4	1.0×10^6	3	20	30	17
H3CE/P45	5.0×10^4	1.6×10^6	6	32	6	-

^aAll cells grown in medium DH-10Δ.

Table 2. Survival assay of SHE cells exposed to ^{60}Co γ rays^a

Cell line/ passage no.	Growth period (days)/Cell cycle	Plating efficiency (%) ^b	Survival rate (%)
H3/P1	4/log cells (Asynchronous)	1	91
H3/P45	4/log cells (Asynchronous)	9	97
H3/CEP40	4/log cells (Asynchronous)	13	69

^aDose rate = 14 cGy/min, dose = 96 cGy.

^bIn medium DH-10 Δ .

Table 3. Survival assay of SHE cells exposed to UV radiation^a

Cell Line/ passage no.	Growth period (days)/Cell cycle	Plating efficiency (%) ^b	D ₀
H3/P3	3/log cells (Asynchronous)	1	6.5
H3/P3	6/confluent cells (Synchronous)	1	4.5
H3/P48	3/log cells	25	1.0
H3/P48	6/confluent cells	6	1.0
H3/P64	3/log cells	28	5.5
H3/P64	6/confluent cells	49	6.0
H3CE/P43	3/log cells	30	1.0
H3CE/P43	6/confluent cells	6	2.0

^aDose rate = 2.5 J·m⁻²·sec⁻¹.^bIn medium DH-10A.

Table 4. Summary of parameters affected by cell passage number

Passage no.	Doubling time (h)	Plating efficiency (%)	UV sensitivity (cell cycle)	No. of cells/plate
3	22	5	confluent > log ^a	1.5×10^7
43	17	6	log > confluent	4.0×10^6
48	14	6	log $\geq$ confluent	4.0×10^6
64	13	49	log $\equiv$ confluent	6.0×10^6

^alog phase = exponentially growing cells

FIGURE LEGENDS

- Figure 1. Plot of plating efficiency (x), growth doubling time ($\cdot$), and the number of confluent cells per 100-mm plate (Δ) as a function of cell culture passage number. Cell growth doubling times and plating efficiencies (% control) use the left y-axis, while the numbers of confluent cells per plate use the right y-axis. Arrows indicate the passages at which concentrations of fetal calf serum (FCS) were changed from 20% to 10% in the culture medium.
- Figure 2. Plot of the cell number per plate as a function of the growth period per day. The doubling time (T^2) is indicated for each of four different cell passages. H3 cells are SHE cell fibroblasts at various different passages (passage 64 = x; passage 3 = $\cdot$; passage 48 = Δ). H3CE cells (at passage 43) show features of epidermal cells. The percentages indicate the plating efficiencies of the cells at the indicated points.
- Figure 3. Survival curves as a function of UV dose with a dose rate of $2.5 \text{ J}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for SHE-H3 cells at A, passage 3; B, passage 48; and C, passage 64. D, curves for the epidermal-like cell line in passage 43. Cells were irradiated in log phase (Δ) or when confluent ($\cdot$).

Figure 4. SHE cells from various passages (p1-p45) were grown to confluence. Fibroblast (H3) and epidermal (H3CE) cells were used. Untreated (0) and γ -ray (γ) exposed (96 cGy) cells were harvested 1 h following completion of the exposure. RNA was purified and analyzed by Northern blot hybridization to (A) γ -actin, (B) PCNA, and (C) p53 probes. Fig. 4A is a phosphorimager print; all others are x-ray films. Total RNA loaded was 20 μ g; poly A+ RNA loaded was 5 μ g in each well.

Figure 1

Figure 2

H3P3

Figure 3A

H3P48 Cells

Figure 3B

H3P64 Cells

Figure 3C

H3CEP43

Figure 3D

Figure 4A

H_3CEP_{45}	H_3P_{40}	H_3P_1
└─┬─┘	└─┬─┘	└─┬─┘
γ 0	γ 0	γ 0

PCNA
96 cGy-1 h
SHE cells

Figure 4B

H ₃ CEP ₄₀	H ₃ P ₄₅	H ₃ P ₁
└─┘	└─┘	└─┘
γ 0	γ 0	γ 0

p53
SHE cells

Figure 4C