Excision and Postreplication DNA Repair Capacities, Enhanced Transformation, and Survival of Syrian Hamster Embryo Cells Irradiated by Ultraviolet Light

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ABSTRACT

The frequency of ultraviolet light (UV)-induced neoplastic transformation of Syrian hamster embryo cells (HEC) is enhanced 3- to 10-fold when the cells are first treated with either X-irradiation or with methyl methanesulfonate. Maximum enhancement occurs when the interval between the two treatments is 48 hr. The relevance of UV-induced transformation to neoplasia is confirmed because the transformants produce tumors when injected into nude mice. Excision and postreplication DNA repair were studied to determine whether the enhanced transformations were associated with either of these repair mechanisms. Independent of X-ray or of methyl methanesulfonate pretreatment, approximately 25% of the pyrimidine dimers are excised within 24 hr in cells irradiated with UV with 3 J/sq m. During this period, more than 70% of the genome of cells irradiated with UV has been replicated. Postreplication repair is measured by the time required to chase pulse-labeled nascent DNA strands to parental-sized DNA. Regardless of pretreatment, 1 and 3 hr are required for pulse-labeled DNA in control and irradiated (10 J/sq m) cells, respectively, to reach parental size. Therefore, no correlation is found between a change in the rate of excision or postreplication repair and enhancement of transformation. Relative to control cloning efficiency, the survival of HEC irradiated with 3 J/sq m is higher than 70% even though HEC contain more than 105 pyrimidine dimers/genome. The level of survival is similar to the survival of human skin fibroblasts which excise pyrimidine dimers four to five times as efficiently. Moreover, postreplication repair cannot account for the ability of these cells to survive because it is three times slower than in human fibroblasts. Therefore, other repair mechanisms must be responsible for HEC survival and transformation.

INTRODUCTION

The modulation of the frequency of carcinogen-induced transformation with an in vitro model provides a valuable approach for determining the factors important for the conversion of normal cells to a neoplastic state (3). Exposure of cells sequentially to 2 agents can significantly increase the frequency of normal cells to a neoplastic state (3). Exposure of cells to UV with 3 J/sq m is higher than 70% even though HEC contain more than 105 pyrimidine dimers/genome. The level of survival is similar to the survival of human skin fibroblasts which excise pyrimidine dimers four to five times as efficiently. Moreover, postreplication repair cannot account for the ability of these cells to survive because it is three times slower than in human fibroblasts. Therefore, other repair mechanisms must be responsible for HEC survival and transformation.

MATERIALS AND METHODS

Transformation Assay and Cell Survival. For each transformation experiment, fresh HEC from fetuses 13 to 14 days in gestation (16 to 19 mm crown-rump length) are used. The tissue culture buffers and medium, the cell culture techniques, and the conditions for the transformation assay have been published (7). Random-bred hamsters are maintained in a room with a 12-hr light cycle, and the fetuses are removed from animals that have been bred at least 3 times and have had 12 to 18 viable fetuses. Primary and subsequent passage cells are grown as monolayers in plastic Petri dishes in CM at 37°C in an 11% CO2 humidified atmosphere. In all experiments, 2-day-old secondary or tertiary hamster cultures, obtained by seeding 2.5 x 106 cells/100-mm dish, are used.

For the quantitative transformation assay, 300 cells in CM with 20% serum are seeded in a 60-mm plastic Petri dish with or immediately subsequent to the addition of 6 x 104 HEC which had been irradiated (100 kVp, 3500 R) as a confluent monolayer culture using a Model T5S-433 Picker portable industrial X-ray apparatus. The latter constitute the feeder layer that facilitates the growth of the relatively low number of HEC.

Transformation and cell survival of HEC are determined in the same dishes. After 7 days of incubation, the colonies are fixed with methanol and stained with Giemsa. Colony morphology is determined with a stereoscopic microscope at x10 to

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: MMS, methyl methanesulfonate; HEC, Syrian hamster embryo cells; CM, Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum unless otherwise specified; CE, cloning efficiency; NTE buffer, 100 mM NaCl:50 mM Tris-HCl, pH 7.5:10 mM EDTA:BrUrd, bromodeoxyuridine; saline: EDTA, 0.8% NaCl, 0.116% NaH2PO4 and 0.02% each of KH2PO4, disodium EDTA, and KCl.

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x 40. The colonies are either light (semicontiguous) or dense (contiguous) (4). Nontransformed colonies display a regularly oriented arrangement of cells while the transformed ones exhibit a random crisscross piling up of cells not seen in the controls. Transformation frequency is calculated either per dish or per colony. The enhancement of the transformation is expressed as the ratio of the results of the double treatment to those of UV alone.

The neoplastic property of morphologically transformed colonies is confirmed by assaying for tumorigenicity. Viable colonies are identified with a phase-contrast microscope as being transformed by the same criteria as described for the Giemsa-stained colonies. Colonies are isolated by the ring technique and are placed in a 60-mm dish. At confluency, the cells are transferred to a 100-mm dish. Approximately 3 weeks from the time of isolation of the colony, 5 x 10⁶ cells are injected s.c. in nu/nu mice. The animals are then observed for progressively growing tumors (8).

The CE is determined by dividing the average number of colonies (greater than 2 mm) by the number of cells seeded per plate, and multiplying by 100. Survival is determined by comparing the CE of treated to untreated cells. When the survival of UV-irradiated normal human cells (CRL 1220 from ATC) is measured, no feeder cells are used, and 200 to 500 cells are seeded in 100-mm dishes 7 to 10 hr prior to irradiation. The cultures are incubated for 16 to 18 days; CM is renewed twice.

In some experiments, hamster mass cultures are treated with either MMS (11 μg/ml of medium) or X-irradiation (250 R). MMS is added to the cultures in CM for 1 hr. After treatment, the cells are washed with Dulbecco’s phosphate-buffered saline (without calcium or magnesium), trypsinized, and seeded for colony formation.

Cell Treatment Conditions. X-irradiation (126 R/min) is delivered from a distance of 54 cm by a Westhinghouse Quadracordex machine which has the following features: 2 tubes, above and below; 235 kVp; and 0.25 mm Cu and 0.55 mm Al filtration. Cells are irradiated with UV 24 cm from a single 15-W General Electric germicidal lamp (G15T8) at a fluence rate of 0.6 J/sq m/sec. The fluence rate measured by an International photometer has been calibrated by the potassium ferric oxalate procedure. Prior to UV irradiation, the medium is removed, and the dishes are covered with a UV-transparent Permanox top (Lux Scientific). After irradiation, fresh CM supplemented with 20% serum is added, and incubation continues. For the biochemistry experiments, CM is used.

Pyrimidine Dimer Excision. HEC, plated at 5 x 10⁶ (to maintain log-phase growth) in a 60-mm dish, are labeled overnight in CM containing [¹⁴C]thymidine (0.1 μCi/ml; 50 μCi/mmol) or [³H]thymidine (0.25 μCi/ml; 0.25 μg/ml). The medium with radioactivity is removed 1 hr prior to UV irradiation, the cells are washed, and 4 ml of fresh CM are added. The cells irradiated with 3 J/sq m are incubated for 2, 6, or 24 hr; the medium is removed, and the cells are frozen on the dish at −40°. After being thawed, the cells are lysed by the addition of 1 ml 1% Sarkosyl in NTE buffer. Protein in the lysate is digested by incubation for 1 hr in the presence of proteinase K (50 μg/ml), the DNA solution is extracted with redistilled phenol by very gentle agitation until the liquid phase is completely emulsified. The 2 phases are separated by centrifugation at 3000 x g for 5 min, and the aqueous phase is removed. To minimize shearing, only cut-off plastic pipet tips are used for transferring the DNA. The DNA is dialyzed in collodion bags against 15 ml of NTE buffer for 9 hr, then dialyzed twice against 15 ml buffer (10 mM Tris-HCl:1 mM EDTA, pH 7.5) for 10 and 4 hr, and stored at 4°. Pyrimidine dimer content is determined by sensitivity of the DNA to Micrococcus luteus pyrimidine dimer-specific endonuclease (P-11 fraction of Riazuddin and Grossman) (15). Extracts of [¹⁴C]- and [³H]-labeled DNA are from irradiated cells that had or had not been incubated, respectively. The reaction mixture containing excess endonuclease (10 μl) and 50 μl each of 1 μM- and 14C-labeled DNA extract is incubated for 30 min at 37°. The reaction is stopped by cooling to 0°, and the mixture is layered on top of 0.3 ml of 0.5 M NaOH:10 mM EDTA over a 4.8-ml 5 to 20% (w/v) sucrose gradient (0.2 n NaOH:0.5 n NaCl:10 mM EDTA), seated on 0.1 ml of 60% (w/v) sucrose:0.5 n NaOH. The gradients are centrifuged at 45,000 rpm in a SW 50.1 rotor for about 60 min. Centrifugation is terminated when ω² t = 8.08 x 10¹¹ radians²/sec. Approximately 20 fractions are collected from each gradient by pumping the gradients through a 20-gauge cannula inserted to the bottom of the tube. The DNA in each fraction is precipitated with 1 n HCl and collected on Whatman GF/C glass fiber filters. The filters are washed once with 1 n HCl and twice with 95% ethanol. After the filters are dried, the radioactivity is counted in a toluene-based scintillation solution. The gradients were precalibrated with the following phage DNA markers: (a) T-2; (b) T-4; (c) T-7; (d) nicked φX174; and (e) λ. The data are analyzed and plotted by computer.

The Amount of DNA Replication after UV Irradiation. Cells are plated and prelabeled as for pyrimidine dimer excision. After irradiation (10 J/sq m), the cells are incubated for 6 or 24 hr in fresh CM containing BrdUrd (5 μg/ml). Subsequently, the medium is removed and the plates are frozen at −40°. After being thawed, the cells are lysed by the addition of 1% Sarkosyl in NTE buffer. RNase (50 μg/ml) is added, and the dishes are incubated for 30 min at 37°, followed by 1 hr further incubation in the presence of proteinase K (50 μg/ml). The extracted DNA is sheared by passage through a 20-gauge needle 3 times. [³H]-labeled DNA (from cells incubated without BrdUrd), [¹⁴C]-labeled DNA (from cells incubated with BrdUrd), and CsCl [60.8% (w/w) of NTE buffer] are mixed in a 1:4:2 ratio by volume. Density equilibrium is established by centrifugation for 48 hr in a SW 50.1 rotor at 33,000 rpm. The gradients are fractionated into about 50 fractions, and the radioactivity is determined. The density of Fractions 10, 20, and 30 is determined by measuring the refractive index of 2-μl samples. The data are analyzed and plotted by computer.

Postreplication Repair. HEC are plated and prelabeled overnight with [¹⁴C]thymidine as above for pyrimidine dimer excision. The CM containing radioactivity is removed, the cells are washed, and 2 ml of fresh CM are added. After a minimum of 0.5 hr, the CM is removed, and the cells are irradiated (10 J/sq m). The same medium is then replaced, the cells incubated for 25 min, and pulse-labeled for 15 min with [³H]thymidine (20 Ci/mmol, 50 μCi/ml). The pulse label is chased for 1, 2, or 3 hr with CM containing 20 μM unlabeled thymidine and incubation is terminated by washing the cells with saline:EDTA. The cells are X-irradiated (1100 R, 50 kVp using the Picker source) to facilitate the unraveling of the DNA. After a 2-ml sample of saline:EDTA is added to the dishes, the cells are detached with...
the aid of a rubber policeman, washed once by centrifugation 
(1000 × g for 4 min), and resuspended in 0.5 ml saline:EDTA. 
Between 5 × 10⁴ and 10⁵ cells are lysed on a 0.3-ml pad of 1 
M NaOH:10 mM EDTA on a 5 to 20% (w/v) sucrose gradient 
(2 M NaCl:0.33 M NaOH:10 mM EDTA) seated on 0.1 ml of 60% 
(w/v) sucrose:0.5 M NaOH. The gradients are centrifuged in a 
SW 50.1 rotor for about 130 min at 30,000 rpm and are 
terminated when ω²t = 7.7 × 10¹⁰ radians²/sec. Fractionation,
counting of radioactivity and analysis of the data are as de-
scribed above.

RESULTS

Cell Survival. HEC irradiated 24 hr after seeding on an X-
irradiated feeder layer are sensitive to increasing doses of UV 
irradiation, as indicated by their colony-forming ability. When 
the results are normalized by designating the CE of untreated 
controls as 100%, the dose that results in 37% survival (D₃⁷) is 
5.5 J/sq m (Chart 1). This lethality is similar to that obtained 
when normal human fibroblast cells are irradiated with UV. In 
the latter case, the UV dose corresponding to 37% survival is 
6.4 J/sq m.

Transformation Enhancement. UV irradiation also causes a 
dose-dependent increase in transformation (Table 1). The 
transformed colonies are morphologically similar to those 
which had been described previously, independent of the car-
cinogen used (4, 5). The relevance of the morphologically 
transformed colonies to neoplasia is verified by demonstrating 
their tumorigenicity. Five discrete colonies have been isolated 
and subcultured. Homozygous nude mice have been given s.c. 
injections of approximately 5 × 10⁵ cells. Progressively grow-
ing tumors result in all cases in approximately 3 to 6 weeks. 
Histopathological examination of these tumors indicates that 
they are either undifferentiated sarcomas or fibrosarcomas.

The previously published results from this laboratory that 
prior treatment with X-irradiation (250 R) enhances the UV-
induced (1.5 or 3.0 J/sq m) transformation frequency in HEC 
are confirmed. When 48 hr separate the 2 treatments, the 
enhancement is observed on both an absolute basis, number 
of transformed colonies per dish, and on a relative basis, 
number of transformed colonies per total colonies (Table 1). 
No transformation occurs with X-irradiation alone. When MMS 
(11 μg/ml medium for 1 hr) is substituted for X-irradiation, a 
similar enhancement of UV-induced transformation results. 
Data utilizing MMS pretreatment and 2 UV doses, 1.5 and 3 J/
sq m, shows the effect of increasing doses of UV (Table 1).

The variability observed between experiments is such that 
the enhanced transformation with MMS plus 1.5 J/sq m at 48 
hr ranges between 3 and 6 on a per dish basis. In these 
experiments with UV alone, the number of transformations per 
dish is more than 2-fold greater when the UV dose is increased 
from 1.5 to 3 J/sq m. The enhancement ratio for MMS plus 3 
J/sq m, however, is approximately one-half (absolute basis) 
and one-third (relative basis) that obtained with the MMS plus 
1.5 J/sq m.

As with X-irradiation plus UV (4), a temporal relationship in 
the enhancement of transformation is observed when HEC are 
treated with MMS prior to UV. The maximum enhancement 
occurs when UV exposure occurs 48 hr after MMS treatment 
(Table 2). At 72 hr, the enhancement obtained with the double 
treatment approaches that which is associated with UV alone.

The very low transformation frequency observed with MMS 
treatment alone is not considered in calculating the enhance-
ment.

![Chart 1. UV survival curves of HEC and normal human fibroblasts. HEC (•), 
300 cells/60-mm dish (12 dishes/dose), are seeded with x-irradiated feeder 
cells; human cells (x), 200 to 500 cells/100-mm dish (5 dishes/dose), are 
seeded without feeder cells. Within 24 hr incubation, the medium is removed for 
irradiation. HEC are incubated for 7 days and human cells are incubated for 16 
days before staining. Results are normalized to the CE of nonirradiated HEC 
(25%) and human cells (30%).](cancerres.aacrjournals.org)
Excision of Pyrimidine Dimers under Enhancement Conditions. The rate of pyrimidine dimer excision is measured under enhancement and control conditions with the endonuclease-sensitive site assay. HEC that have been X-irradiated (250 R), or incubated with MMS (11 μg/ml) for 1 hr in CM, or not treated are trypsinized and plated. The cells are labeled over night with [14C]thymidine, irradiated with UV (3.0 J/sq m) 48 or 72 hr postplating, and further incubated for 2, 6, and 24 hr. DNA is extracted and subjected to pyrimidine dimer-specific endonuclease digestion. The size of the DNA digestion products is analyzed by alkaline sucrose sedimentation. The rate of loss of UV-induced endonuclease-sensitive sites in pretreated and control cells is the same. Independent of pretreatment conditions, approximately 25% of the pyrimidine dimers are removed within 24 hr post-UV (Chart 2; Table 3). By 72 hr post-UV, no more than 35% of the pyrimidine dimers are excised in any of the above cases (data not shown). Therefore, the enhancement of transformation is not the result of a change in the rate of pyrimidine dimer excision.

Postreplication Repair under Enhancement Conditions. In this study, the rate of postreplication repair is functionally defined as the time required for small pulse-labeled DNA to be chased into parental-sized DNA in cells irradiated with UV. This rate is measured in HEC, pretreated 48 or 72 hr with 250 R of X-ray (conditions which enhance transformation), and compared to the rate in HEC treated with UV (10 J/sq m) alone. When the size of pulse-labeled DNA nascent strands is determined by alkaline sucrose sedimentation, their rate of chase into parental-length DNA is the same whether or not the cells had been treated with X-ray (48 or 72 hr) prior to UV (Chart 3). The size of pulse-labeled nascent strands is smaller in UV-irradiated than in non-UV-irradiated cells under all pretreatment conditions (compare Chart 3, A and B). Furthermore, for nascent strands to be chased into parental size in cells irradiated with or without UV, 1 and more than 3 hr, respectively, are required (Chart 3, B and F). Results similar to those in Chart 3 are obtained with cells X-irradiated 24 hr prior to UV. Therefore, the enhancement of transformation by X-ray does not result from a change in the rate of postreplication repair.

The Amount of DNA Replication in HEC Irradiated with UV. The percentage of DNA replicated in a given period can be measured by determining the fraction of DNA that has a hybrid density (heavy-light) in cells grown with BrdUrd in the medium. By 24 hr post-UV (3.0 J/sq m), more than 70% of the dimers still remain in the DNA (Table 3). The BrdUrd density shift technique demonstrates that UV (10 J/sq m) slows the rate of DNA replication (Chart 4) relative to the controls. By 6 hr after UV, only 10% of the DNA in irradiated cells is replicated, compared to 50% of the DNA in nonirradiated cells (Chart 4A); while by 24 hr post-UV, 70% of the DNA has replicated in irradiated cells and virtually all of the prelabeled DNA in non-irradiated cells is heavy-light by 24 hr (Chart 4B). Evidence for further rounds of replication cannot be observed because fully BrdUrd-substituted DNA contains no label. At this UV dose, there are approximately 15 dimers/replicon and 8 X 10⁵ dimers/genome, assuming that the replicon size and the average amount of DNA per chromosome is the same as in the Chinese hamster (10, 11) whose diploid number is one-half that of the Syrian hamster. Therefore, DNA replication...
animal studies had not been performed to confirm their tumor
same types of tumors when injected s.c. into nude mice.

chemical classes had been used (5). Finally, the transformation
induced by chemical carcinogen treatment and produce the
consistent with a previous study of X-ray and MMS enhance
ment in which a variety of carcinogens belonging to diverse
irradiation plus UV. Moreover, the enhancement resulting from
X-irradiation is greater than that attributable to MMS. This
is consistent with a previous study of X-ray and MMS enhance-
ment in which a variety of carcinogens belonging to diverse
chemical classes had been used (5). Finally, the transformation
frequency is dependent on the UV dose in both enhanced and
nonenhanced conditions (Table 1). Similar results occur when
HEC are transformed by benzo(a)pyrene (7). Thus, transforma-
tion is a random event induced by the carcinogen treatment.

In the past, this laboratory has referred to UV-induced trans-
formations of HEC as morphological transformations because
animal studies had not been performed to confirm their tumor-
igenicity. UV transformants are morphologically similar to those
induced by chemical carcinogen treatment and produce the
same types of tumors when injected s.c. into nude mice.

Pretreatment of HEC with agents which increase their sus-
ceptibility to UV light-induced transformation (Chart 1; Tables
1 and 2) affects neither the ability of cells to excise pyrimidine
dimers (Chart 2; Table 3) nor the rate of postreplication repair
(Chart 3). The endonuclease-sensitive site assay used to meas-
ure the removal of pyrimidine dimers from DNA is independent
of the type of excision mechanism. The rate of excision has
been measured up to 3 days post-UV which is 5 days post-
plating. Any changes in excision repair occurring after this time
would not be expected to affect the observed transformation
frequency because those experiments are terminated on Day
7. The rate of postreplication repair can be measured only
during the first several hr post-UV because, by 6 hr post-UV,
the size of pulse-labeled nascent strands increases to that of
those in nonirradiated cells and postreplication repair is no
longer needed. Neither excision of pyrimidine dimers nor post-
replication repair can be responsible for the enhancement of
transformation frequency.

Numerous studies have shown that excision and postrepli-
cation repair are the 2 most important mechanisms used by
prokaryotic organisms to protect themselves from potentially
lethal damages in their DNA. Postreplication repair in prokar-
yotic organisms involves the filling of gaps in daughter strands
which have been replicated on damaged templates (16). Most
investigations of DNA repair in mammalian cells have, there-
fore, been predicated on the assumption that these are also
the 2 most important mechanisms for mammalian cell survival.
These results, however, suggest that these 2 repair mechani-
isms have a minimal effect on HEC survival when the cells are
irradiated with 3 J/sq m, a "physiological dose" that results in
only about 30% lethality. With respect to excision repair, at
least 70% of the DNA is replicated within 24 hr even though
only 25% of the pyrimidine dimers are removed (Chart 4; Table
3). A dose of 3 J/sq m yields 2.4 × 10^6 pyrimidine dimers/
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genome and 4.5 pyrimidine dimers/replicon. Since the excision and replication measurements are from cells irradiated with 3 and 10 J/sq m, respectively, it is likely that even more than 70% of the DNA would have been replicated within the first 24 hr if HEC had received the lower dose. With respect to postreplication repair, the size of pulse-labeled nascent strands in cells irradiated with 10 J/sq m recovers to that of nonirradiated cells by 6 hr post-UV, as has been observed in other mammalian cells (1, 13). Moreover, by 6 hr post-UV, only 10% of the DNA has been replicated (Chart 4). Therefore, only a small percentage of the genome is subject to postreplication repair. HEC excise pyrimidine dimers at one-fourth to one-fifth the rate of human cells (Table 3; Ref. 7) and require 3 times longer to chase pulse-labeled nascent strands to parental size (Chart 2). Although the repair rates for excision and postreplication differ significantly, the survival curves for UV-treated human fibroblasts and HEC are similar (Chart 1). Therefore, repair mechanisms other than excision of pyrimidine dimers and postreplication repair must be responsible for the survival of HEC irradiated with UV.

Other repair systems, such as the recovery of nascent strands to normal size, may be important to cell survival and to the mechanism of transformation and its enhancement.

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