LINKING GAMMA-H2AX FOCI AND CANCER IN RAT SKIN EXPOSED TO HEAVY IONS AND ELECTRON RADIATION

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Abstract—This study uses acute doses of three test radiations, [40Ar ions (L = 125 keV μ−1), 20Ne ions (L = 25 keV μ−1) and electron radiation] to examine a potential quantitative link between radiation on skin cancer induction and gamma-H2AX foci in rat keratinocytes exposed in vitro to radiations with comparable L values. Theory provided a testable link between cancer yield and gamma-H2AX foci yields: \( Y_{Ca}(D,L) = C_{Ca}LD + B_{Ca}D^2 \) (eqn 1), where \( Y_{Ca}(D,L) \) is cancers(rat)\(^{-1}\) at 1.0 y, \( Y_{AX}(D,L) \) is in vitro gamma-H2AX foci(keratinocyte)\(^{-1}\), \( D \) is radiation dose, \( L \) is linear energy transfer, \( N \) is irradiated keratinocytes in vivo, and \( F \) is the error rate of end joining. An explicit expression for cancer yield was derived based on cancers arising in the ion track region in proportion to \( D \) and \( L \) (first term) and independently in proportion to \( D^2 \) in the delta ray region in between the ion tracks (second term): \( Y_{Ca}(D,L) = C_{Ca}LD + B_{Ca}D^2 \) (eqn 1a). Parameters quantified include: \( C_{Ca} = 0.000589 ± 0.000150 \) cancers\(\cdot\)rat\(\cdot\)keV\(\cdot\)Gy\(^{-1}\), \( B_{Ca} = 0.0088 ± 0.0035 \) cancers\(\cdot\)rat\(\cdot\)Gy\(^{-1}\), \( F = (8.18 ± 0.91) \times 10^{-10} \), \( N = (8.8 ± 1.2) \) at 1.0 y, \( Y_{AX}(D,L) \) is in vitro gamma-H2AX foci(keratinocyte)\(^{-1}\). Verification of eqns (1) and (1a) and the constancy of \( F \) support the hypothesis that end-rejoining errors play a major role in radiation carcinogenesis in rat skin. Cancer yields per rat were consistently predictable based on gamma-H2AX foci yields in keratinocytes in vitro such that 27.8 H2AX foci(keratinocyte)\(^{-1}\) predicted 1.0 cancer(rat)\(^{-1}\) at 1 y.

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Key words: cancer; exposure, radiation; genetic effects; radiation risk

INTRODUCTION

Currently little consensus exists about the mechanism by which ionizing radiation causes cancer, although key features remain single cell origin and genomic instability of constituent cells (Shuryak et al. 2011). The ability of ionizing radiation to break mammalian chromosomes by inducing DNA double strand breaks is well documented (Natarajan et al. 2008). Each break creates two broken chromosomal ends that are detectible by means of gamma-H2AX staining procedures (Li et al. 2011). Mammalian cells employ vigorous repair processes, including non-homologous end-joining (NHEJ), to reconnect broken chromosome ends, usually correctly, within a day or two after irradiation. Still the rejoining process is not perfect as has been documented cytogenetically by numerous, sometimes bizarre, chromosomal rearrangements that occur following radiation exposure (Sasaki 2009).

The hypothesis of the present study is that each carcinoma is initiated by an end joining repair error that destabilizes a single keratinocyte in a way that causes additional pro-neoplastic alterations to occur at subsequent cell divisions as the keratinocyte participates in normal turnover. After a long progression stage, a frank malignancy occurs and is counted. Although little is known about the initial destabilizing errors, they are unlikely to adversely affect the proliferative capacity of the affected keratinocytes; otherwise, malignant progression itself would also be adversely affected (Signore et al. 2011). For the purposes of the present study, the unstable cells in vivo at the single cell stage were assumed to occur in proportion to the yield of skin cancers observed at a standard time of 1 y after acute radiation exposure.

Irradiations for inducing cancers were previously performed with energetic \( ^{40} \)Ar \( [L = 125 \) (keV)\(\mu \)\(^{-1}\)] and \( ^{20} \)Ne \( [L = 25 \) (keV)\(\mu \)\(^{-1}\)] ions at the Lawrence Berkeley Bevalac (Oakland, CA) and with electrons \( [L = 0.34 \) (keV)\(\mu \)\(^{-1}\)] at the 2.5 meV van de Graaff Generator at the NYU School of Medicine (Tuxedo, NY) (Burns et al. 1989a and b, 1991, 2007). The radiations were chosen to represent three different ways that gamma-H2AX foci distribute within a
mammalian nucleus: 1) a quasi-random distribution (achieved by using electron radiation or Grenz rays), 2) a distribution consisting mostly of delta ray electrons combined with a lower density heavy ion track (achieved by using a 20Ne ion beam), and 3) a distribution consisting of a fivefold higher density ion track coupled with a lower level of delta ray electrons (achieved by using a 40Ar ion beam). Generally, broken chromosomes within an irradiated mammalian nucleus occur in association with ionization clusters on the order of 20 Å in diameter. The current analysis was simplified to exclude track structure other than L values as a basis for linking gamma-H2AX foci to cancer induction based on differing ionization distributions of the primary radiations (Singh et al. 2012).

MATERIALS AND METHODS

Skin cancer induction

A total of 478 male CD (Sprague Dawley) rats were purchased from Charles River Farms as 21-d-old weanlings. Irradiations were performed between 28 and 32 d of age during the initial resting phase of the hair growth cycle. The rats were anesthetized for positional restraint during irradiation by intraperitoneal injections of 35 mg kg⁻¹ of sodium pentobarbital. Euthanasia, when needed, was performed by intraperitoneal injections of 120 mg kg⁻¹ of sodium pentobarbital.

Radiation exposures were performed on double thickness folds of dorsal skin about 2.0 mm thick. Rats were loaded into foam-lined boxes. A dorsal skin fold was pulled upward by 1.0 cm (doubled to 2.0 cm of skin width by the fold) through a 3.0-cm-long slot in the box lid. This configuration provided a skin area of 6.0 cm² available for irradiation. Subcutaneous surgical thread stretched between 1.0-cm-high posts at both ends of the slot held the folded skin positioned, enabling simultaneous irradiations of up to three rats (6.0 mm of total skin thickness). A tantalum block shielded the bodies. The dose rate at the skin surface was monitored by a parallel plate ionization chamber connected to a dynamic capacitor electrometer (Victoreen, Cleveland, OH, USA) and was set at 1.0 Gy min⁻¹. Additional confirmatory dosimetry was routinely supplied by the beam providers.

Cancer yields were quantified based on observations made at 6-wk intervals. Cancer rates based on the number of verified new cancers interval⁻¹ rat⁻¹ were cumulated with time to provide cancer yields as a function of time since irradiation. Histological verification of each skin lesion was accomplished by assigning numbers at initial lesion detections. Drawings made from photographs taken every 6 wk documented the location and growth of each lesion. At death, each lesion was evaluated histologically, and only lesions confirmed to be malignant by persistent growth rate and local invasion were carried forward to the final analysis. Cancers were assigned to histological categories: squamous (48%), basal cell (42%), or mixed squamous and basal cell (10%). All cancer types were combined to improve statistical precision. Final comparisons were based on the 1-y accumulations. Error bars are reported as standard errors based on Poisson statistics accumulated cancer rat⁻¹ standard deviations divided by the square root of the accumulated number of cancers at 1 y.

The 40Ar exposure protocol included a fractionated exposure of two 1.5-Gy fractions separated by 24 h. Split dose recovery halftime for cancer induction in the rat skin model is about 4.0 h, so that by 24 h, recovery is essentially complete. Based on eqn (1a), cancer yield for a single dose of 3.0 Gy of 40Ar is $C_{Ca} \times 125 \times 3 + BCa \times 3^2$, which equals 0.3051. Two fractions of 1.5 Gy are $2 \times (C_{Ca} \times 125 \times 1.5 + BCa \times 1.5^2)$, which equals 0.2662. The fractionated cancer yield multiplied by the ratio 0.3051(0.2662)⁻¹ = 1.146 adjusted the fractionated cancer yield upward from 0.0762 cancers(rat)⁻¹ to 0.0873 cancers(rat)⁻¹. The latter is shown plotted in Figs. 4 and 8 and as per unit dose in Fig. 9.

Description of the gamma-H2AX assay

A rat cell line was established from keratinocytes obtained from newborn Sprague Dawley rat skin (Burns et al. 1991). Eventually a robust line was achieved and was passaged indefinitely in commercial keratinocyte medium (KSFM, Gibco) supplemented with 50 mg mL⁻¹ bovine pituitary extract (Gibco) and 5 ng mL⁻¹ mouse recombinant epidermal growth factor (Collaborative Research, Bedford, MA, USA). Keratinocytes were irradiated through the thin plastic bottom of culture dishes with the medium poured off, never for longer than 10 min, including setup and duration of the radiation exposures.

Keratinocytes were irradiated with L value surrogates of the cancer induction radiations as follows: (1) 150 keV μ⁻¹ 56Fe ions (Brookhaven National Laboratory, Upton, NY) as surrogate for 125 keV μ⁻¹ 40Ar ions (20% L discrepancy); (2) a 25 keV μ⁻¹ Bragg curve region of a proton beam (Loma Linda Proton Facility, Loma Linda, CA, USA) as a surrogate for 25 keV μ⁻¹ 140Ar ions (no discrepancy); and (3) 100 keV Grenz rays (NYU Grenz Ray Machine, Tuxedo, NY) as a surrogate for 0.34 keV μ⁻¹ electron radiation (little, if any, discrepancy).

For gamma-H2AX assays, rat keratinocytes were fixed in 3.7% w/v formaldehyde for 10 min, permeabilized in phosphate buffered saline (PBS) containing 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 10 min, then blocked in 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 30 min. Fixed, permeabilized cells were incubated with gamma-H2AX phosphospecific antibody at 1:400 dilution (in PBS containing 1% BSA) for 2 h, washed in PBS, incubated...
for 30 min with Alexa 488 conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) at 1:500 dilution (in PBS containing 1% BSA), followed by washing in PBS. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) (1 g L\(^{-1}\) in PBS) for 10 min. Coverslips were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA).

Fluorescent images of keratinocyte nuclei were captured by using a Zeiss Research Fluorescence Microscope equipped with digital camera. Gamma-H2AX foci were quantified by using ImageJ software in the following sequence: (1) Convert to image type 16 bit (grey); (2) activate image adjust threshold (ctrl shift T); (3) adjust threshold upward from 0 or minimal until H2AX foci mergers begin to occur (generally about 0.1% area); (4) use analyze/analyze particles [Size (pixel units\(^2\)] 5 to 100, circularity: 0.00, Show: overlay outlines] to produce Summary and Results tables of H2AX foci counts; and (5) use Edit “Inversion” and Image adjust/auto-“Save as” to create images suitable for later manual verification of H2AX foci counts.

Gamma-H2AX results for \(^{20}\text{Ne}\) ions were obtained manually by counting H2AX foci nucleus\(^{-1}\) directly on prints magnified to provide nuclear diameters of about 1 cm. Doses of \(^{20}\text{Ne}\) ions and \(^{40}\text{Ar}\) ions were lower than their respective \(D_{\text{equal}}\) values to minimize the difficulty of distinguishing central track H2AX foci from delta ray H2AX foci. In the final results, this distinction was dropped, and all H2AX foci were included in the counts, which is required to evaluate eqn (1). Foci counts keratinocyte\(^{-1}\) and cancers rat\(^{-1}\) standard errors (S.E.) were calculated as follows: standard deviations were calculated by using Excel spreadsheets, and standard errors were obtained by dividing standard deviations by the square root of the number of independent measurements.

Derivation of misjoined gamma-H2AX foci role in radiation carcinogenesis

The carcinogenic process following acute exposure of rat skin to ionizing radiation is subdivided into four stages: 1) chromosomes are broken in viable keratinocytes; 2) within days, intracellular repair systems, particularly non-homologous end-joining (NHEJ), remove nearly all broken chromosome ends by rejoining them mostly correctly; 3) a fraction, designated \(F\), of these end-joinings are defective in a way that destabilizes the keratinocyte’s genome without significantly interfering with its proliferative capability; and 4) months or years later, cancers appear and are counted to document the earlier destabilizations. The four steps give rise to four quantities: 1) initial gamma-H2AX foci yields (in vitro keratinocyte)\(^{-1}\), \(Y_{\text{AX}}(D,L)\), is a measure of broken chromosome ends; 2) end-joining yield(in vivo keratinocyte)\(^{-1}\), \(Y_{\text{EF}}(D,L)\), which nearly reverses step 1; 3) misjoining yields(in vivo keratinocyte)\(^{-1}\), \(Y_{\text{MF}}(D,L)\), which are the key causative alterations in neoplastic progression; and 4) cancer yield rat\(^{-1}\) quantified within a 6.0 cm\(^2\) dorsal skin region at an arbitrary 1 y after irradiation, \(Y_{Cd}(D,L)\), the final stage of the process.

Establishing quantitative connections among these four quantities begins by identifying the yield of misjoined chromosomes [i.e., \(Y_{\text{MF}}(D,L)\)] among the \(N\) irradiated keratinocytes. The misjoinings are assumed to be key initial events occurring in individual keratinocytes, the enumeration of which provides an estimate of cancers expected per animal; i.e., \(Y_{Cd}(D,L)(\text{rat}^{-1}) = \frac{NY_{\text{MF}}(D,L)(\text{keratinocyte})}{C_0/C_1}\). A connection is made between misjoining and end-joining by assuming that cancer-relevant misjoinings occur as a fixed fraction, \(F\), of all end-joinings in irradiated keratinocytes [i.e., \(Y_{\text{MF}}(D,L) = FY_{\text{EF}}(D,L)\)], which also establishes \(F\) as the cancer-relevant error rate of the end-joining process. Substituting \(Y_{\text{EF}}(D,L)\) into the above cancer equation produces: \(Y_{Cd}(D,L) = NFY_{\text{EF}}(D,L)\). The final step connects gamma-H2AX foci to end-joining based on the necessity that each end-joining eliminates two gamma-H2AX foci; i.e., \(Y_{\text{EF}}(D,L) = 2^{-1}Y_{\text{AX}}(D,L)\). Insertion of the latter produces a direct proportionality between cancer yield per rat and gamma-H2AX yield per keratinocyte with the proportionality constant:

\[
Y_{Cd}(D, L) = NF(2^{-1})Y_{AX}(D, L). \tag{1}
\]

Verification of eqn (1), including evaluation of NF(2\(^{-1}\)) for each of the test radiations, is a major goal of the current work. Other goals include quantifying \(N\) and \(F\) and examining the constancy of \(F\) among the three test radiations.

Derivation of cancer yield as a bivariate function of dose (D) and energy transfer (L)

An expression is needed to describe how cancer yield and gamma-H2AX foci yield depend on the radiation dose, \(D\), and the linear energy transfer, \(L\). This approach includes consideration of three types of ionizing radiations: 1) HZE (high atomic number and energy) particles, such as fully ionized \(^{20}\text{Ne}\) or \(^{40}\text{Ar}\) nuclei; 2) somewhat less energetic electrons, known as delta rays, that arise via electrical attraction forces exerted as heavy ions pass nearby; and 3) a uniformly energetic (2.0 meV) pure electron beam. Delta rays acquire sufficient energy to be ejected from their molecular or atomic orbitals and generally have a range of energies leftover to form their own ionizing tracks. By contrast, multi meV electron beams produce ionizations similarly to the way they are produced by heavy ions with the difference being that forces are repulsive and the tracks are jagged because of the light weight of the electrons.

Energetic heavy ions produce mostly straight-line tracks characterized by a dense core of ejected orbital electrons without sufficient energy to move away from the geometric path of the ion. This dense core is expected to contain
many types of biological alterations, including the chromosome breaks that generate pairs of gamma-H2AX foci. Whether the keratinocytes are in organs of animals or in culture medium, the ion track geometry and associated delta rays are expected to be identical. The ion speed and the rapidity of H2AX foci formation means that all gamma-H2AX foci within any particular intranuclear track segment are essentially simultaneous for the purpose of the present argument. Additionally the high ionization densities of the ion tracks cause the gamma-H2AX foci to be in relatively close geometric proximity. Taken together, simultaneity and close proximity establish a high likelihood that ion track gamma-H2AX foci will primarily undergo end-joining with one another rather than with gamma-H2AX foci generated by delta rays (Naruke et al. 2009). For a fixed energy nucleon \(^1\), variations of the linear energy transfer, L, are reflected in the core ionization density in a proportionate manner. For example, the Bevalac \(^{40}\)Ar ions produced an ionization density fivefold greater than that of \(^{20}\)Ne, which corresponded exactly to L increasing from 25 keV \(\mu\) \(^{-1}\) to 125 keV \(\mu\) \(^{-1}\).

For a given ion, ion energy and fluence (ions per unit area), the average keratinocyte receives a track dose \(D_{\text{ion track}}\) equal to L times the total ion track length within the average nucleus. This leaves the only available option for biological alterations distributed linearly along the ion tracks to be proportionality with total track length, which as above is identical with \(D_{\text{ion track}}\). It follows that biological alterations, such as gamma-H2AX foci and cancers in the ion track must be proportional to \(D_{\text{ion track}}\). Apparently these endpoints are capable of responding directly in proportion to ionization density in addition to responding similarly to radiation dose. To account for dual L and D dependence, an unusual bivariate expression was adopted by making cancer and gamma-H2AX foci yields in the ion track proportional to both L and D as follows: \(Y_{\text{Ca}}(D,L) = C_{\text{Ca}}D_{\text{ion track}} + B_{\text{Ca}}D_{\delta \text{ray}} \) (Fry et al. 1983; Morgan et al. 1996). Present findings confirm that gamma-H2AX foci and cancers in the ion track respond coordinate to changes in L values as expected in eqn (1a).

As noted above, delta rays have comparatively short ranges and likely follow jagged routes typical of the lesser mass of electrons. Based on comparatively low L values, it is not likely that multiple gamma-H2AX foci will occur within the short length of any particular delta ray track. Still, delta rays are numerous, so the most scenario for them is that H2AX foci end-joinings occur largely between H2AX foci originating from different ion tracks. Neither is it likely that delta ray H2AX foci would interact with ion track H2AX foci because of the latter’s unavailability, as discussed above. Origination in different tracks is sufficient to produce the randomness required for proportionality with delta ray dose-squared, as would be expected for any biological alteration arising out of an unsaturated interaction between two random events. Thus, delta ray cancer yield takes the form: \(Y_{\text{Ca}}(D,L) = B_{\text{Ca}}(D_{\delta \text{ray}})^2\) (Nikjoo and Goodhead 1991).

Combining these ion track and the delta ray terms provides an expression for cancer yield of \(Y_{\text{Ca}}(D,L) = C_{\text{Ca}}D_{\text{ion track}} + B_{\text{Ca}}(D_{\delta \text{ray}})^2\). Unfortunately, this formulation is impractical because \(D_{\text{ion track}}\) and \(D_{\delta \text{ray}}\) are not readily measurable. However, by converting to total dose \(D = D_{\text{ion track}} + D_{\delta \text{ray}}\) and defining a ratio \(G = D_{\text{ion track}}/D_{\delta \text{ray}}\) a more practical alternative is established where D is readily measurable by ionization chambers in the following alternative expression:

\[
Y_{\text{Ca}}(D,L) = C_{\text{Ca}}LD + B_{\text{Ca}}D^2, \quad \text{Cancers(rat)}^{-1}. \quad (1a)
\]

If needed, conversions back to separate ion track and delta ray doses can be accomplished by using: \(C_{\text{Ca}} = 1[G(G + 1)^{-1}C_{\text{Ca}}]^{-1}\) and \(B_{\text{Ca}} = (G + 1)^{-1}B_{\text{Ca}}\). Eqn (1a) is used throughout the current work to quantify cancer yield in units of cancers rat \(^{-1}\) at 1 y. Referring to eqn (1), three early steps precede the cancer end stage, including (1) gamma-H2AX foci(keratinocyte \(^1\)), (2) misjoinings keratinocyte \(^1\), and (3) end-joinings keratinocyte \(^1\). The validity of eqn (1) requires that intermediate steps conform to the same D and L dependencies as the cancers in eqn (1a); otherwise, eqn (1) might not be valid:

\[
Y_{\text{End}}(D,L) = C_{\text{End}}LD + B_{\text{End}}D^2, \quad \text{End-joining( keratinocyte)}^{-1}. \quad (1b)
\]

\[
Y_{\text{Mis}}(D,L) = C_{\text{Mis}}LD + B_{\text{Mis}}D^2, \quad \text{Mis-joining( keratinocyte)}^{-1}. \quad (1c)
\]

\[
Y_{\text{H2AX foci}}(D,L) = C_{\text{H2AX foci}}LD + B_{\text{H2AX foci}}D^2, \quad \text{H2AX foci( keratinocyte)}^{-1}. \quad (1d)
\]

Setting the two terms in eqn (1a) equal defines a dose \(D_{\text{equal}}\), which is important as a boundary below which linearity is the predominant feature and above which dose-squared is predominant for all of the above equations, most especially for the cancer yields:

\[
D_{\text{equal}} = C_{\text{Ca}}(B_{\text{Ca}})^{-1}L. \quad (2)
\]

### RESULTS

#### Temporal patterns of cancer yields and regression analyses

Cancer yields are shown in Figs. 1, 2, and 3 as a function of time following acute exposures for \(^{40}\)Ar ions, \(^{20}\)Ne ions or electron radiation, respectively, at various doses as indicated. These data, published in 1989, were originally fitted to time-squared functions as was customary (Burns et al. 1989). However, a reanalysis using linear functions improved the consistency within each radiation type and reduced the error bars for slopes and x-intercepts.
relative to the earlier analysis. In the current analysis, the x-intercepts averaged 134.8 ± 3.2 d, 166.0 ± 14.8 d, and 146.5 ± 24.7 days for 40Ar ions, 20Ne ions, and electrons, respectively, generally falling within the overall average range of 152.0 ± 14.2 d.

The 40Ar results at 9.49 Gy and 12.66 Gy were not included in the analysis in deference to reduced cancer yields at these higher doses, an effect generally ascribed to inadequate keratinocyte regeneration. Neon-20 (20Ne) ion groups and electron radiation groups did not exhibit cancer yield reductions at higher doses, even at doses as high as 16.0 Gy and 26.9 Gy, respectively. Neon-20 (20Ne) ion groups at 2.8 Gy, 4.0 Gy, and 5.6 Gy were not included in the analysis because of insufficient cancers (four or fewer) in groups of 20 rats. However, one data point at 2.0 Gy was included, in spite of only two cancers in 20 rats, because of its proximity to the regression line.

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Fig. 1. Temporal onset of skin cancers induced by various single doses of 40Ar ion radiation. Rats were observed for onset of squamous and basal cell cancers at 6-wk intervals. Standard deviations were computed by Excel based on cancers on individual rats in the group, and standard errors were calculated by dividing standard deviations by the square root of the number of rats.

Fig. 2. Temporal onset of skin cancers induced by various single doses of 20Ne ion radiation. Standard deviations were computed by Excel based on cancers on individual rats in the group, and standard errors were calculated by dividing standard deviations by the square root of the number of rats.

Fig. 3. Temporal onset of skin cancers induced by various single doses of electron radiation. Rats were irradiated with single doses as indicated and were observed for onset of squamous and basal cell cancers. Standard deviations were computed by Excel based on cancers on individual rats in the group, and standard errors were calculated by dividing standard deviations by the square root of the number of rats.

Fig. 4. Regression analysis of (cancer yield)(dose)\(^{-1}\) for the purpose of evaluating \(C_{Ca}\) and \(B_{Ca}\) in the expression: \(Y_{Ca}(D,L) = C_{Ca}L + B_{Ca}D\). \(B_{Ca}\) was evaluated as the slope of the 40Ar regression line. \(C_{Ca}\) was evaluated as the y-intercept divided by \(L = 125 \text{ (keV)} \text{μm}^{-1}\). The results were: \(C_{Ca} = 0.00059\) cancer micron (rat kev Gy)\(^{-1}\) and \(B_{Ca} = 0.0088\) cancers (rat Gy)\(^{-1}\).
Fig. 4 shows cancer yield dose$^{-1}$ vs. dose for $^{40}$Ar ions and $^{20}$Ne ions. Linear regression was used to establish slopes and y-intercepts. The $^{40}$Ar data (closed circles) are shown fitted by the regression line, the slope of which defines BCa. Similarly, the y-intercept of the same line divided by L = 125 keV defines CCa for $^{40}$Ar. Note: An outlier data point at 3.16 Gy, 0.0568 cancers rat$^{-1}$ was excluded based on it and its associated error bars falling outside the 95% confidence limits, if included. A substitute for the excluded data point was generated by using the results of a two-fraction (1.5 Gy + 1.5 Gy) exposure totaling 3.0 Gy by adjusting to account for split dose sparing (see Materials and Methods for details).

Table 1. Calculation of $F = (2 \times N - 1) / C_2$ (cancers rat$^{-1}$ at 1 y/DSBs/keratinocyte) (Column F) for each radiation type and dose. $F = (8.18 \pm 0.91) \times 10^{-10}$ misjoinings/end-joining.$^a$

| Dose (Gy) | $Y_{AX}$/cell | S.E. | $Y_{Ca}$/rat | S.E. | $Y_{Ca}/Y_{AX}$ | S.E. | F       |
|----------|---------------|------|-------------|------|----------------|------|---------|
| Argon    |               |      |             |      |                |      |         |
| 1.50     | 2.06          | 0.11 | 0.130       | 0.015| 0.0631         | 0.0058| $14.35 \times 10^{-10}$ |
| 3.00     | 6.92          | 0.18 | 0.299       | 0.035| 0.0433         | 0.0065| $9.84 \times 10^{-10}$  |
| Neon     |               |      |             |      |                |      |         |
| 0.3      | 0.26          | 0.05 | 0.0052      | 0.0006| 0.0200         | 0.0131| $4.55 \times 10^{-10}$  |
| 1.0      | 0.69          | 0.14 | 0.0235      | 0.0028| 0.0341         | 0.0086| $7.74 \times 10^{-10}$  |
| Electrons|               |      |             |      |                |      |         |
| 4.5      | 2.26          | 0.13 | 0.049       | 0.006| 0.0217         | 0.0043| $4.94 \times 10^{-10}$  |
| 9.0      | 5.80          | 0.24 | 0.196       | 0.025| 0.0339         | 0.0050| $7.69 \times 10^{-10}$  |

Average 0.036 8.18 $\times 10^{-10}$

S. E. 0.006 0.91 $\times 10^{-10}$

$^a$Notes: $Ca = cancers(rat)^{-1}$; $Misjoins = Misjoinings(keratinocyte)^{-1}$; $End-Joins = End-joinings(keratinocyte)^{-1}$; $AX Foci = gamma-H2AX Foci(keratinocyte)^{-1}$; $AX Foci(Ca)^{-1} = gamma-H2AX Foci(keratinocyte)^{-1} [cancers(rat)^{-1}]^{-1}$ and $Ca(AX Foci)^{-1}$ is the reciprocal of column 5.

Table 2. Calculated responses for each of the four radiation types are shown for the four transitions from gamma-H2AX foci to end-joinings to misjoinings to cancers corresponding to eqns (1a), (1b), (1c), (1d).$^b$

| Radiation | Dose(Gy) | 1. AX Foci | 2. End-joins | 3. Misjoins | 4. Cancer | 5. AX Foci(Ca)$^{-1}$ | 6. Ca(AX Foci)$^{-1}$ |
|-----------|----------|------------|--------------|-------------|-----------|-----------------------|-----------------------|
| Argon     | 0.5      | 1.069      | 0.534        | 4.29119     | $10^{-10}$ | 0.038                 | 27.8                  |
|           | 1        | 2.262      | 1.131        | 9.08239     | $10^{-10}$ | 0.080                 | 27.8                  |
|           | 5        | 16.292     | 8.146        | 6.54119     | $10^{-9}$  | 0.576                 | 27.8                  |
|           | 10       | 45.037     | 22.519       | 1.80824     | $10^{-8}$  | 1.591                 | 27.8                  |
| Neon      | 0.5      | 0.264      | 0.132        | 1.05824     | $10^{-10}$ | 0.009                 | 27.8                  |
|           | 1        | 0.652      | 0.626        | 2.61648     | $10^{-10}$ | 0.023                 | 27.8                  |
|           | 5        | 8.240      | 4.120        | 3.30824     | $10^{-9}$  | 0.291                 | 27.8                  |
|           | 10       | 28.933     | 14.466       | 1.16165     | $10^{-8}$  | 1.022                 | 27.8                  |
| Delta Rays| 0.5      | 0.226      | 0.113        | 9.075       | $10^{-11}$ | 0.008                 | 27.8                  |
|           | 1        | 0.904      | 0.452        | 3.630       | $10^{-10}$ | 0.032                 | 27.8                  |
|           | 5        | 22.603     | 11.301       | 9.075       | $10^{-9}$  | 0.799                 | 27.8                  |
|           | 10       | 90.411     | 45.205       | 3.630       | $10^{-8}$  | 3.194                 | 27.8                  |
| Electrons | 0.5      | 0.062      | 0.031        | 2.50        | $10^{-11}$ | 0.002                 | 27.8                  |
|           | 1        | 0.249      | 0.125        | 1.00        | $10^{-10}$ | 0.009                 | 27.8                  |
|           | 5        | 6.227      | 3.113        | 2.50        | $10^{-9}$  | 0.220                 | 27.8                  |
|           | 10       | 24.907     | 12.453       | 1.00        | $10^{-8}$  | 0.880                 | 27.8                  |

Notes: $Ca = cancers(rat)^{-1}$; $Misjoins = Misjoinings(keratinocyte)^{-1}$; $End-Joins = End-joinings(keratinocyte)^{-1}$; $AX Foci = gamma-H2AX Foci(keratinocyte)^{-1}$; $AX Foci(Ca)^{-1} = gamma-H2AX Foci(keratinocyte)^{-1} [cancers(rat)^{-1}]^{-1}$ and $Ca(AX Foci)^{-1}$ is the reciprocal of column 5.

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yields are direct measures of the carcinogenicity of the delta rays; i.e., the linear term is insignificant at such high doses. Also in Fig. 4 are two additional lines: 1) a regression line (dash-dot-dot) through the origin fitted to the $^{20}$Ne cancer data and 2) a putative delta ray line (dotted) with slope equal to that of $^{40}$Ar and through the origin. The “delta ray” line is well within the 95% confidence limits of the $^{20}$Ne data. The dash-dot-dot line in Fig. 4 intersecting the y-axis at 0.0135 cancers $\text{rat}^{-1} \text{Gy}^{-1}$ at 1 y is the calculated cancer yield dose $^{-1}$ for $^{20}$Ne. These results represent the best available and most likely the only available measurement of the carcinogenicity of delta rays as a separate component of heavy ion cancer yields, a result that readily justifies including the delta ray term with its own coefficient in eqn (1a).

Cancer induction $\text{rat}^{-1}$ and gamma-H2AX foci keratinocyte $^{-1}$ are the only measurable endpoints of the four being considered in these experiments, but values for misjoinings and end-joinings have been calculated as shown in Table 2 based on eqns (1b) and (1c). As summarized in Table 2, the coefficients in eqns (1b), (1c), and (1d) are calculated from the cancer coefficients, $C_{Ca}$ and $B_{Ca}$ as follows: $C_{MJ} = N^{-1}C_{Ca}$; $C_{EJ} = (NF)^{-1}C_{Ca}$; and $C_{AX} = 2(NF)^{-1}C_{Ca}$. Similarly, $B_{MJ} = N^{-1}B_{Ca}$; $B_{EJ} = (NF)^{-1}B_{Ca}$; and $B_{AX} = 2(NF)^{-1}B_{Ca}$. Values for $C_{AX}$, $B_{AX}$, $C_{Ca}$, and $B_{Ca}$ were established empirically in order to verify the link between cancers and gamma-H2AX foci implicit in eqns (1a) and (1d). The two rightmost columns in Table 2 show that for each radiation type, precisely the same ratio of gamma-H2AX foci yield to cancer yield occurs at all doses in agreement with eqn (1).

Fig. 5 is an arrangement of photos showing gamma-H2AX foci for different doses and radiation types. The gamma-H2AX data in Table 3 show no tendency in any exposure group for the H2AX foci counts to decline with time after exposure, implying that H2AX foci in these keratinocytes were not subjected to detectible end-joining in the first 4 h after irradiation. Overall, a total of 1,071 cells and 4,259 H2AX foci were examined either by ImageJ software ($^{40}$Ar ions and electrons) or by manual observation ($^{20}$Ne Ions).

**Calculation F based on cancer and gamma-H2AX foci yields**

Solving for F in eqn (1) provided an empirical approach for estimating F in the expression $F = (2 \times 10^{-5}) \left\{ [\text{Yield}_{Ca}(D,L)]/\text{Yield}_{AX}(D,L) \right\} \times 1$. The $\text{Yield}_{AX}(D,L)$ data are contained in Table 3, and the F calculation results are shown in Table 1. The $Y_{AX}$ cell $^{-1}$ column in Table 1 gives the average control-corrected gamma-H2AX foci keratinocyte $^{-1}$ values obtained from Table 3’s $H_{2}AX$ foci cell $^{-1}$ control column for each dose and radiation type. Cancer yields based on eqn (1a) shown in the $Y_{Ca}$ rat $^{-1}$ column of Table 1 were divided by the $Y_{AX}$ cell $^{-1}$ data to produce...
cancers(H2AX foci)\(^{-1}\) in the Y\(_{Ca}/Y\_(AX)\) column. Multiplying the latter column by 2 N\(^{-1}\) provided F values for each exposure group as shown in the column labeled F in Table 1. As indicated, the average was F = (8.18 ± 0.91) \times 10^{-10} misjoinings end-joining)\(^{-1}\).

Cancer induction by delta rays compared to a pure electron beam

Fig. 6 shows the cancer yield (dotted line) calculated from the delta ray term of eqn (1a) together with high dose \(^{20}\)Ne ion data (putative delta ray response) and the cancer yield for electron radiation (dash-dot, filled squares). Both cancer datasets were fitted by a least squares procedure that assumed a hypothetical exponent of 2.0. A comparison of these plots shows delta rays to be 3.63-fold more carcinogenic than pure electron radiation. There is no obvious explanation for the higher carcinogenicity of delta rays, except to note that the delta ray gamma-H2AX foci may cluster closer to their respective tracks of origin due to their energy distribution being skewed more to lower energies in comparison to the

### Table 3. Gamma-H2AX foci counts per keratinocyte for three types of radiation at two doses each.

| Dose (Gy) | Time (h) | # of Cells | # of Foci | Foci \(\text{Cell}^{-1}\) | Foci \(\text{Cell}^{-1} - \text{Control}^b\) | S.E. |
|-----------|----------|------------|-----------|----------------|--------------------------------|-----|
| \(^{40}\)Ar Ions | | | | | | |
| 1.5 Gy | 0 | 69 | 327 | 4.74 | 3.79 |
| | 1 | 142 | 189 | 1.33 | 0.38 |
| | 2 | 31 | 191 | 6.16 | 5.21 |
| | 4 | 25 | 97 | 3.88 | 2.93 |
| Total | | 267 | 804 | 3.01 | 2.06 | 0.81 |
| 3.0 Gy | 0 | 116 | 915 | 7.91 | 6.94 |
| | 1 | 59 | 440 | 7.46 | 6.51 |
| | 2 | 31 | 201 | 6.48 | 5.53 |
| | 4 | 30 | 300 | 10.00 | 9.05 |
| Total | | 236 | 1859 | 7.88 | 6.92 | 0.58 |
| \(^{90}\)Electrons | | | | | | |
| 4.5 Gy | 0 | 27 | 67 | 2.48 | 1.53 |
| | 1 | 59 | 238 | 4.03 | 3.08 |
| | 2 | 48 | 200 | 4.17 | 3.22 |
| | 4 | 49 | 83 | 1.69 | 0.74 |
| Total | | 183 | 588 | 3.21 | 2.26 | 0.47 |
| 9.0 Gy | 0 | 43 | 303 | 7.05 | 6.10 |
| | 1 | 44 | 149 | 3.39 | 2.44 |
| | 2 | 12 | 110 | 9.17 | 8.22 |
| | 4 | 17 | 221 | 13.00 | 12.05 |
| Total | | 116 | 783 | 6.75 | 5.80 | 1.58 |
| \(^{20}\)Ne Ions | | | | | | |
| 0.3 Gy | 2 | 53 | 22 | 0.42 | 0.26 |
| | 1.0 Gy | 2 | 20 | 17 | 0.85 | 0.69 | 0.14 |

\(^a\)Time (h) measured since end of irradiation.
\(^b\)Foci/cell values for \(^{20}\)Ne ions corrected by Ne-specific background = 0.13 foci cell\(^{-1}\).
\(^c\)Background correction = 0.95 Foci cell\(^{-1}\) \(^{40}\)Ar and electrons.
\(^d\)Cell = Keratinocyte.
\(^e\)S.E. = Standard Error of the mean calculated by dividing standard deviation by square root of sample number.
higher and relatively more uniform energies produced in a 2.0-meV electron beam.

Cancer yields and gamma-H2AX foci yields for 20Ne and 40Ar ions

Figs. 7 and 8 show $Y_{Ca,D,L \text{rat}}^{-1}$ (left ordinate) and $Y_{AX,D,L \text{keratinocyte}}^{-1}$ (right ordinate) for 20Ne ions [$L = 25$ (keV)$\mu^{-1}$] and 40Ar ions [$L = 125$ (keV)$\mu^{-1}$], respectively. In both plots, the left:right ordinate ratio is 0.036:1 cancer keratinocyte(rat H2AX foci)$^{-1}$. The solid lines in Figs. 7 and 8 are the calculated cancer yields based on eqn (1a) with values for $C_{Ca}$ and $B_{Ca}$ as obtained in Fig. 4 and shown in Table 4, coupled with the appropriate L values for 20Ne ions and 40Ar ions.

In Fig. 7 the cancer yield solid line is shown below the lowest actual cancer data point at 2.0 Gy. Also included are separate plots of the ion track (first) term as a straight, dashed line and the delta ray (second) term as a curved dotted line. Graphically, the intersection of the dashed line with the dotted curve calculates $D_{\text{equal}} = 1.75$ Gy, an 8% discrepancy compared to the calculated value of 1.62 Gy.

Fig. 8 indicates for 40Ar ions how the same left:right ordinate scale ratio of 0.036:1 reconciles $Y_{AX,D,L}$ on the right ordinate with $Y_{Ca,D,L}$ on the left ordinate, again as expected based on eqns (1a) and (1d). As in Fig. 7, this reconciliation is also consistent with eqn (1), where cancer yield rats$^{-1}$ equals (NF)$^{-1} = 0.036$ cancer keratinocyte(rat H2AX foci)$^{-1}$ times the gamma-H2AX foci yield (keratinocyte)$^{-1}$. Graphically, the intersection of the ion track term (shown dashed) with the dose squared delta ray term (shown dotted) gives $D_{\text{equal}} = 8.6$ Gy in comparison to calculated $D_{\text{equal}} = (C_{Ca}B_{Ca})^{-1}L = 0.067 \times 125 = 8.38$ Gy, a 3.0% discrepancy. Figs. 7 and 8 confirm the proportionality of eqn (2) with the L value of the ion, specifically $D_{\text{equal}}(^{40}\text{Ar})$. 

**A comparison of cancer yields induced by delta rays and electron radiation.** Rats were irradiated with single doses of electron radiation in the manner as described herein. The error bars are standard errors based on dividing standard deviations by the square root of total samples. The dotted line is the delta ray cancer yield projected from the delta ray (second) term in eqn (1a) at high doses of 20Ne ions. These data indicate that the delta rays and electrons are consistent with a squared dependence on dose, and delta rays are 3.63-fold more carcinogenic than electrons.
Table 4. $^{40}\text{Ar}$ cancer data per unit dose [$Y_{\text{Ca}}(D,L)/D = C_{\text{Ca}}L + B_{\text{Ca}}D$] from which the slope and y-intercept were calculated by linear regression as indicated by the circles and solid line in Fig. 4. (Courtesy of VassarStat regression program.)

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| Row # | Dose (Gy) | $Y_{\text{Ca}}$ (cancer/gy) | $(x-x_{av})^2$ | $(y-y_{av})^2$ | $(x-x_{av})(y-y_{av})$ |
| 1 | 0.51 | 0.0793 | 7.5469 | 0.000180 | 0.0369 |
| 2 | 1.62 | 0.0946 | 2.6705 | 0.000004 | -0.0032 |
| 3 | 3.15 | 0.0873 | 0.0115 | 0.000272 | 0.0018 |
| 4 | 4.76 | 0.1174 | 2.2585 | 0.000609 | 0.0371 |
| 5 | 6.34 | 0.1319 | 9.5039 | 0.001537 | 0.1209 |
| Aver. | 3.28 | 0.1021 | 21.9913 | 0.002602 | 0.1935 |

$B_{\text{Ca}}$ = slope = 0.1935(21.9913)$^{-1}$ = 0.0088 ± 0.0035 cancer (rat)$^{-1}$(gy)$^{-2}$

$C_{\text{Ca}}$ = y-intercept/125 = 0.0733(125)$^{-1}$ = 0.00059 ± 0.00015 cancer (rat keV Gy)$^{-1}$

(a) Calculated values for various parameters for $^{40}\text{Ar}$ and $^{20}\text{Ne}$ ions.

| Name of Parameter | Value | S.E. | Units |
|-------------------|-------|------|-------|
| $B_{\text{Ca}}$ ($^{40}\text{Ar}$ Slope) | 0.0088 | 0.0035 | cancer (rat)$^{-1}$(gy)$^{-2}$ |
| $^{40}\text{Ar}$ Y-Intercept | 0.0733 | 0.0148 | cancer (rat)$^{-1}$(gy)$^{-1}$ |
| $C_{\text{Ca}}$ (Y-Intercept/125) | 0.00059 | 0.00015 | cancer (rat keV Gy)$^{-1}$ |
| $^{20}\text{Ne}$ Y-Intercept | 0.0147 | 0.0030 | cancer (rat keV Gy)$^{-1}$ |
| Ratio $C_{\text{Ca}}/B_{\text{Ca}}$ | 0.067 |
| $^{40}\text{Ar}$ $D_{\text{equal}}$ | 8.33 Gy |
| $^{20}\text{Ne}$ $D_{\text{equal}}$ | 1.67 Gy |
| P-Value (1-tail) | 0.01 |

(b) Cancer yields calculated from $Y_{\text{Ca}}(D,L) = C_{\text{Ca}}L + B_{\text{Ca}}D^2$ at doses where H2AX foci were quantified empirically.

| $^{40}\text{Ar}$ Ions | Dose (Gy) | $Y_{\text{Ca}}(D,L)$ (cancers/rat at 1 y) |
|-------------------|-------|--------------------------------|
| 1.5 | 0.131 |
| 3.0 | 0.302 |

| $^{20}\text{Ne}$ Ions | Dose (Gy) | $Y_{\text{Ca}}(D,L)$ (cancers/rat at 1 y) |
|-------------------|-------|--------------------------------|
| 0.3 | 0.0052 |
| 1.0 | 0.0238 |

| Electrons | Dose (Gy) | $Y_{\text{Ca}}(D,L)$ (cancers/rat at 1 y) |
|-----------|-------|--------------------------------|
| 4.5 | 0.050 |
| 9.0 | 0.201 |

$[D_{\text{equal}}(^{20}\text{Ne})]^{-1} = L(^{40}\text{Ar})L(^{20}\text{Ne})^{-1} = 125(5)^{-1} = 5$. Table 4 contains the following derived quantities: $^{40}$Ne y-intercept = 0.0142 Gy and the $C_{\text{Ca}}/B_{\text{Ca}} = 0.067$ and $D_{\text{equal}}$ values.

The gamma-H2AX foci data [$Y_{\text{AX}}(D,L)$] for $^{40}$Ar ions in Fig. 8 was obtained at doses below the $D_{\text{equal}}$ value, which emphasizes the importance of L in the first term of eqn (1a) and justifies its insertion as a second variable in the linear term (eqn 1a). The results in Figs. 7 and 8 establish that eqn (1a) for cancers and eqn (1d) for gamma-H2AX foci differ only by the coefficient (NF)$^{-2}$ = 0.036 cancer keratinocyte(rat H2AX foci)$^{-1}$.

Cancer yield(unit dose)$^{-1}$ vs. gamma-H2AX foci yield(unit dose)$^{-1}$

Conversion to the (unit dose)$^{-1}$ format permits all current findings to be included in a single graph as shown in Fig. 9. The left:right ordinate ratio remains at 0.036:1 cancers keratinocyte(rat (H2AX foci)$^{-1}$ as in Figs. 7 and 8. The results in Fig. 9 confirm the validity of eqn (1) and evaluate (NF)$^{-2}$ for both $^{20}$Ne and $^{40}$Ar ions as $Y_{\text{Ca}}(D,L)$ D$^{-1} = (NF)^{2-1}Y_{\text{AX}}(D,L)D^{1} = 0.036 Y_{\text{AX}}(D,L)D^{3}$. Dividing eqns (1a) and (1d) by dose gives: $Y_{\text{Ca}}(D,L)$ D$^{-1} = C_{\text{Ca}}L + B_{\text{Ca}}D = (NF)^{2-1} (C_{\text{AX}}L + B_{\text{AX}}D)$, from which evaluation of $C_{\text{AX}}$ and $B_{\text{AX}}$ becomes $C_{\text{AX}} = 27.8$ and $B_{\text{AX}} = 27.8$. This analysis demonstrates how $Y_{\text{AX}}(D,L)D^{3}$ and $Y_{\text{Ca}}(D,L)D^{1}$ share analytic forms differing only in coefficients and how gamma-H2AX foci yield (keratinocyte)$^{-1}$ converts directly to cancer yield rat$^{-1}$ as demonstrated by the superimposition of gamma-H2AX foci data near the fitted lines of the cancer data for each of the three radiation types. Also these data provide a rule of thumb conversion that 1.0 cancer rat$^{-1}$ at 1 y...
DISCUSSION

The premise of the current study is that a specific type of cytogenetic damage, a genomically destabilizing misjoining between two gamma-H2AX foci is a key initial molecular lesion leading to cancer causation by acute doses of ionizing radiation. The equation, $Y_{Ca}(D,L) = (N) F^{-1} Y_{AX}(D,L) D^{-1} \text{ keratinocyte}^{-1}$ (eqn 1), was developed to make use of the high sensitivity of the gamma-H2AX foci technique as a way to evaluate a possible numerical link between cancer yield and gamma-H2AX foci (Golfier et al. 2009; Ozasa et al. 2012). In spite of variability in the gamma-H2AX foci and cancer data, the following features of the model were confirmed for the three test radiations: 1) the constancy of NF(2$^{-1}$); 2) the proportionality of the bivariate linear term with D and L for heavy ions in eqns (1a) and (1d) for cancers and gamma-H2AX foci, respectively; 3) the consistency with dose to the power of 2 of delta rays and high energy electrons for cancer induction and gamma-H2AX foci formation; and 4) a constant value of F (S.E. = 11.1%) as a measure of misjoinings end-joining$^{-1}$ among the three test radiations. The constancy of F is critical for connecting gamma-H2AX foci yield to cancer yield as expressed in eqn (1). For the three test radiations, gamma-H2AX foci predicted cancer yields independent of the presumed differing physical distributions of gamma-H2AX foci among the test radiations. Cancer induction for all three radiations was completely explained by the gamma-H2AX foci yields.

Another important implication of these results is that the dose dependence of gamma-H2AX foci mimics the dose dependence of the cancers; otherwise, Equation I would be not be consistent. This point is emphasized by showing how gamma-H2AX coefficients are independently related to the cancer induction coefficients. Equating eqns (1a) and (1) gives $Y_{Ca}(D,L) = C_{Ca}LD + B_{Ca}D^2 = (\{NF(2^{-1})C_{AX}LD + \{NF(2^{-1})B_{AX}D^2\}$. Equating equivalent coefficients on the two sides of the second equal sign produces $C_{AX} = 2(\text{NF})^{-1} C_{Ca}$ and $B_{AX} = 2(\text{NF})^{-1} B_{Ca}$. Inserting 2 (NF)$^{-1} = 27.8$ rat H2AX foci (cancer keratinocyte)$^{-1}$, $C_{Ca} = 0.00059$ cancer (micron) [rat(kev)Gy]$^{-1}$ and $B_{Ca} = 0.0088$ cancers (ratGy)$^{-1}$, $C_{AX}$ and $B_{AX}$ are calculated to be 0.0164 H2AX-focus (micron) [rat(kev)Gy]$^{-1}$ and 0.245 H2AX-focus (ratGy)$^{-2}$, respectively.

Cytotoxicity in a regenerative epithelial tissue like epidermis often competes with carcinogenesis at high radiation doses, where proliferative regeneration cannot fully compensate for keratinocytes eliminated by cell death (Mothersill et al. 2000). In vitro studies have established that ionizing radiation reproductively inactivates ~50% of keratinocytes when exposed to low L radiations at doses in the range of 2 or 3 Gy, yet rat skin tolerates doses 10- to 15-fold higher without evidence of a reversal of the upward trend of cancer induction with dose. The lack of reversal for electron doses as high as 28.9 Gy implies that destabilized keratinocytes are participating in regenerative replacement at about the same rate as normal keratinocytes; otherwise, reversal would occur at lower doses. The implication is that keratinocytes containing misjoined gamma-H2AX foci retain a proliferative capacity comparable to that of normal keratinocytes, which is a key requirement for these altered cells to participate in cancer progression, indirectly supporting the idea that genomic instability permits cancer-progression relevant proliferative activity up to 16 Gy for $^{20}$Ne ions and up to 28.9 Gy for electron radiation.

Current results establish that 27.8 gamma-H2AX foci (keratinocyte)$^{-1}$ predicts an average cancer yield of 1.0 cancer $\text{rat}^{-1}$ at 1 y after irradiation. However, if only stem cell rather than all keratinocytes are at risk and if, as an arbitrary example, stem cells are 1(20)$^{-1}$ of total keratinocytes, N would be adjusted to N = (8.8 $\pm$ 1.2 $\times$ 10$^7$) (20$^{-1}$) = 4.4 $\pm$ 0.6 $\times$ 10$^6$, and (NF)2$^{-1}$ would be adjusted downward to 0.00166 misjoinings end-joining$^{-1}$, but F would be unaffected. The population of stem cells would need to be quantified independently as a fraction of total keratinocytes.

The occurrence of misjoined chromosome ends is made clear by the many types of chromosomal rearrangements,
such as dicentrics, reciprocal translocations, etc., associated with exposure to ionizing radiation even at very low doses as in biodosimetric screening (Hagmar et al. 1998; Terzoudi and Pantelias 2006). Such chromosomal alterations have been used as cytogenetic biomarkers to survey cancer and other radiation risks in the work environment. Nordic and Italian cohorts of subjects examined with cytogenetic tests have shown a positive trend between chromosome aberration frequency and increased cancer risk. Based on translocations and deletions as hallmarks of human leukemias, several reports implicated gamma-H2AX foci and their repair by non-homologous end-joining (NHEJ) in the generation of some key translocations in myeloid and lymphoid leukemias (Elliott and Jasin 2002). DNA sequence analyses of the translocation breakpoint junctions in some of these leukemias revealed the presence of sequence homologies, suggesting that NHEJ had been responsible for gamma-H2AX foci ligations. NHEJ is much faster than homologous recombination and seems to represent the primary gamma-H2AX foci repair pathway in mammalian cells (Lees-Miller and Meek 2003).

The gradual diminution of the gamma-H2AX fluorescence signal as DNA repair progresses has been a useful predictor of gamma-H2AX foci end-joining kinetics (Cucinotta et al. 2008). The question arises: at which time point following irradiation should gamma-H2AX foci be counted for purposes of estimating cancer risk? The present study indicates that gamma-H2AX foci counts obtained immediately after irradiation (about 5 min) gave essentially the same results as counts made up to 4 h later. By restricting the gamma-H2AX foci counts to be prior to significant gamma-H2AX foci repair, the results maximize counts of initial levels of gamma-H2AX foci, which should provide the most informative results for correlative studies and for establishing calibrations relevant to cancer risk estimation.

The technique of using gamma-H2AX foci for detecting initial levels of DNA damage, as was applied here, may turn out to be more sensitive than scoring dicentrics or reciprocal translocations. Based on a direct comparison of both types of biological assays, scoring dicentrics and quantifying gamma-H2AX foci formation in human lymphocytes allowed the detection of biological damage after low dose CT scans (Golfier et al. 2009).

Epidemiological studies have documented associations between genomic instability, aberrant chromosomes, and leukemias (Ballarini and Ottolenghi 2004). Instability has been shown to occur in connection with disjunctural errors during later anaphases (Holmberg et al. 1993; Kadhim et al. 1995; Rydberg et al. 2005), but the destabilizing aberration(s) with relevance to cancer causation have not yet been identified (Naruke et al. 2009).

Transgenic mice deficient in DNA-PKcs(−/−) provide an instructive example of how cells may become progressively unstable as cellular natural selection accumulates pro-neoplastic alterations (Rothkamm et al. 2001). NHEJ, a key repair system consisting of DNA protein kinase subunits Ku70 and Ku80, Artemis, XRCC4 and DNA ligase IV, is largely responsible for rejoining gamma-H2AX foci in mammalian cells (Jeggo and Lobrich 2005). DNA-PKcs sense and repair gamma-H2AX foci as demonstrated by the high susceptibility of DNA-PKcs(−/−) mice to cytolytic doses of ionizing radiation (Iliakis et al. 2004). DNA-PKcs(−/−) cells are suitable genetic targets for molecular therapeutics, including siRNA, antisense, and novel inhibitions by small molecules (Stracker et al. 2009; Iliakis et al. 2012). Interventions such as these applied soon after irradiation give hope that initial alterations may be targeted once destabilizing structures are identified.

Eqn (1a) allows cancer yields to be calculated for acute exposures to any type of radiation for which an average LET value is available and might even be useful for mixtures of different types of radiation with differing L values. The finding that identical functional forms fit gamma-H2AX foci yields (eqn 1d) and cancer yields [eqn (1a)] represents support for gamma-H2AX foci misjoinings as an entry point for understanding radiation carcinogenesis more completely. Still none of the above contradicts the idea that the cancer-relevant misjoinings are embedded in the DNA at the single cell stage. Depending on the radiation dose, a large proportion of irradiated keratinocytes are expected to exhibit gamma-H2AX foci and end-joinings, but only $F = 8.18 \pm 0.91 \times 10^{-10}$ misjoinings are expected for each end-joining. This low frequency of misjoinings (and cancers) in comparison to the high frequency and ease of detection of the gamma-H2AX foci is a basis for optimism that the gamma-H2AX foci will prove useful as biomarkers of radiation carcinogenesis.

Whether the formulation described here applies to human cancers cannot be answered at present. However, radiation-induced chromosome breakage and rejoining has been found to be somewhat similar among mammalian species (Kligerman et al. 1992). The difference between radiation carcinogenesis in humans versus experimental animals resides primarily in the very much longer progression stage in humans (Shore 2001). It is currently unknown how ratios, such as $C_{C_0B_{C_0}}$, might differ among organs in the same species or among different mammalian species.

The present results support the hypothesis that gamma-H2AX foci are a component of cancer causation by ionizing radiation in the rat skin model, but the constancy of the (cancer keratinocyte)/(rat H2AXfoci) $^{-1}$ ratio and the constant $F$ among the three test radiations in the present study supports rejoining errors as a likely source of cancer-relevant alterations. The results imply that cancer yields in rat skin are quantitatively linked to gamma-H2AX foci in irradiated keratinocytes in a manner that permits prediction of...
cancer risks. Similar analyses will be needed on in vivo keratinocytes and in other organs and species in order to evaluate the possible generality of these findings.

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