Radiation-Induced Cell Transformation: Transformation Efficiencies of Different Types of Ionizing Radiation and Molecular Changes in Radiation Transformants and Tumor Cell Lines

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This study aims to compare the efficiencies of 5.4 keV soft X-rays, α-particles, and γ-rays in transforming C3H 10T1/2 cells and to assess the sequence of cellular and molecular changes during the process of radiation-induced transformation of Syrian hamster embryo (SHE) cells. The somewhat more densely ionizing soft X-rays are more effective than γ-rays both for cell inactivation and cell transformation. The relative biological effectiveness (RBE) appears to be independent of dose; it is approximately 1.3 for either end point. The RBE of α-particles versus γ-rays, on the other hand, increases with decreasing dose; the dose dependence is somewhat more apparent for cell transformation than for cell inactivation. SHE cells transformed by different types of ionizing radiation and related tumor cell lines isolated from nude mice tumors were found to have a distinct growth advantage compared to primary SHE cells, documented by higher plating efficiencies, shorter doubling times, and higher cloning efficiencies in semisolid medium. Most transformed and tumor cell lines that were investigated have elevated mRNA levels for the H-*ras* gene, some of them show restriction fragment length polymorphisms of the H-*ras* gene, and some exhibit a substantially amplified c-*myc* gene. In a sequence analysis of the Syrian hamster H-*ras* gene of eight tumor cell lines from radiation transformants, we have not found any mutation in codons 12, 13, 59, 61, nor in the flanking regions of these codons. The transformed and tumor cell lines tend to have lower chromosome numbers than primary SHE cells.

Introduction

The recent revision of the atomic bomb dosimetry and the subsequent reanalysis of the epidemiologic follow up of the atomic bomb survivors led to substantially increased risk estimates for radiation carcinogenesis, and this has given added urgency to the problem of extrapolating the epidemiological data to low doses. Such extrapolation needs to be supported by radiobiological data, and cell transformation studies using sparsely and densely ionizing radiation are of particular interest for this purpose. The objective of the present study is to determine transformation rates and their dependence on dose and the type of ionizing radiation.

The sparsely ionizing γ-rays are compared to somewhat more densely ionizing soft X-rays and to the far more densely ionizing α-particles. A further objective of this investigation is a mechanistic study of the sequence of steps involved in the transformation of mammalian cells by ionizing radiation.

For the study of the dose-effect relationships, C3H 10T1/2 cells were chosen because they are the system that has been subjected to the widest range of quantitative studies in different laboratories and because they are found to give the most reproducible results. The 10T1/2 cells, on the other hand, are not ideally suited for mechanistic studies because even in their untransformed state they exhibit some of the characteristics of transformed cells, such as unlimited growth potential and major chromosomal changes. Since human primary cells are not, as yet, suitable for transformation studies, we chose primary Syrian hamster embryo (SHE) cells for the study of the molecular and cytogenetic changes that accompany transformation.

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Methods

Cell Culture and Irradiation Procedures

C3H 10T1/2 mouse embryo fibroblasts (1) were used for the determination of transformation rates. The cells were cultured in Eagle's basal medium (Gibco BRL, Karlsruhe, FRG) supplemented with 10% heat-inactivated fetal bovine serum (Boehringer, Mannheim, FRG), 50 U/mL penicillin, and 50 μg/mL streptomycin (BRL) at 37°C in 95% air and 5% CO_2. Primary SHE cells isolated from 12-day-old embryos, stored in passage two to three, were cultured in IBR medium (Gibco) with 20% fetal bovine serum and antibiotics at 37°C in 90% air and 10% CO_2.

Cells were always exposed during exponential growth; γ-irradiation was performed in 25-cm² flasks (Greiner, Frickenhausen, FRG). For the exposures with soft X-rays (Cr-K_α line with energy 5.4 keV) and with α-particles, specially constructed dishes with a 2-μm foil bottom (Hostaphan, Kalle Chemie, Wiesbaden, FRG) were used. Exposures of SHE cells to 8 MeV/u carbon ions were performed at the linear accelerator UNILAC of the Gesellschaft für Schwerionenforschung (GSI, Darmstadt, FRG). The cobalt γ-ray, soft X-ray, and α-particle exposures were performed at dose rates of 0.5 Gy/min, 0.43 Gy/min, and 0.2 Gy/min, respectively. The technical procedures for the heavy ion exposures and the characteristics of the radiation sources have been described previously (2–5).

Survival and Transformation Assays

After irradiation, C3H 10T1/2 cells were trypsinized, counted, and then replated in 25-cm² flasks at cell densities of about 80 or 250 to 300 viable cells per flask for the survival or transformation assays, respectively. Surviving fractions were determined from the number of colonies after 10 days of incubation. Transformation frequencies were estimated from the number of foci of type 2 and 3 (1) per surviving cell, as described previously (4).

After irradiation, SHE cells were repeatedly subcultured with 5 × 10⁵ cells per 75-cm² flask. The primary cells aged within a few passages, and immortalized or transformed cells were then readily recognized as they overgrew the nonproliferating aged cells. The cells of transformed phenotype were then tested for their ability to grow in semisolid medium and for tumor induction in athymic nude mice. These animal tumors were then used to establish tumor cell lines.

Determination of Chromosome Numbers

Cells were grown for 24 hr; 0.5 μg/mL colcemide was added for 3 hr; and mitotic cells were harvested, fixed, spread on microscope slides, and stained with 10% Giemsa. Chromosome numbers per cell were counted in at least 50 metaphase cells.

Expression of H-*ras* Oncogene

RNA was isolated from the different cell lines (6). H-*ras* expression was determined by Northern analysis. Hybridizations were performed with the ³²P-nick-translated human H-*ras* proto-oncogene (Amersham, Buchler, Braunshweig, FRG). The relative amount of ras mRNA was estimated by densitometer scanning of the H-*ras* signals; α-actin probes were used as reference.

Gene Amplification and Restriction Fragment Length Polymorphism

DNA from the different cell lines was cut with the restriction endonucleases EcoRI, BamHI, and HindIII and was then fractionated by agarose gel electrophoresis. Subsequently DNA was blotted on nylon filters, and hybridized either with a 1.2 kb c-DNA fragment of the Syrian hamster H-*ras* gene (provided by R. Ebert, Institute for Toxicology, University of Würzburg) or a XbaI-BamHI-fragment of pSVc-*myc*-1 (7).

Determination of Specific Point Mutations in the H-*ras* Gene

The H-*ras* gene was amplified by polymerase chain reaction (PCR) (8). The primers R14 (homologous to a 5' untranslated region) and R17 (homologous to intron 2 sequences of the hamster H-*ras* gene) were provided by R. Ebert. Codons 12, 13, 59, 61, and flanking regions were then directly sequenced according to the technique of Sanger et al. (9) with a modified T7 DNA polymerase (10).

Results and Discussion

Inactivation and Transformation of C3H 10T1/2 Cells

The survival curves of C3H 10T1/2 cells after exposure to graded doses of γ-rays and 5.4 keV Cr-K_α characteristic X-rays had pronounced shoulders, whereas the experiments with α-particles led to purely exponential survival curves, with a D_{37} of 0.61 Gy (Fig. 1). The mean inactivation doses, D, were 2.99 Gy and 2.27 Gy for the γ-rays and the soft X-rays. The relative biological effectiveness (RBE) of soft X-rays versus γ-rays showed no recognizable dependence on dose; equating the RBE to the ratio of D yielded the value 1.3. For α-particles, on the other hand, the RBE varied with dose; we obtained a value versus γ-rays of about 10 at low doses and a value of 3.7 at an α-particle dose of 2 Gy.

In a comparison with data obtained by other authors with soft X-rays of different energies between 0.3 keV and 18.5 keV for the inactivation of a variety of mammalian cells (11–16), our RBE value for 5.4 keV X-rays fits well into the trend of RBE values which increase with decreasing X-ray energy. The increasing values of
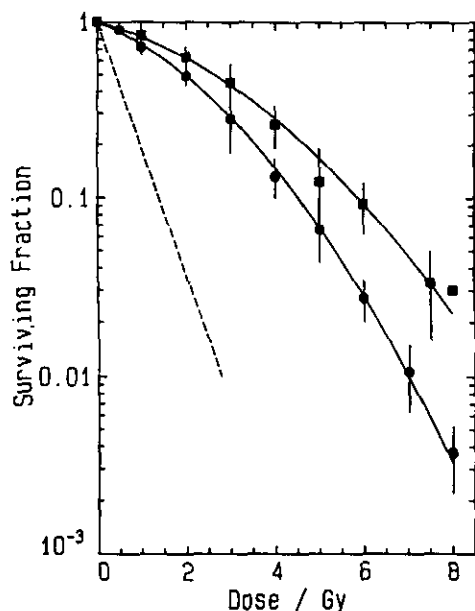
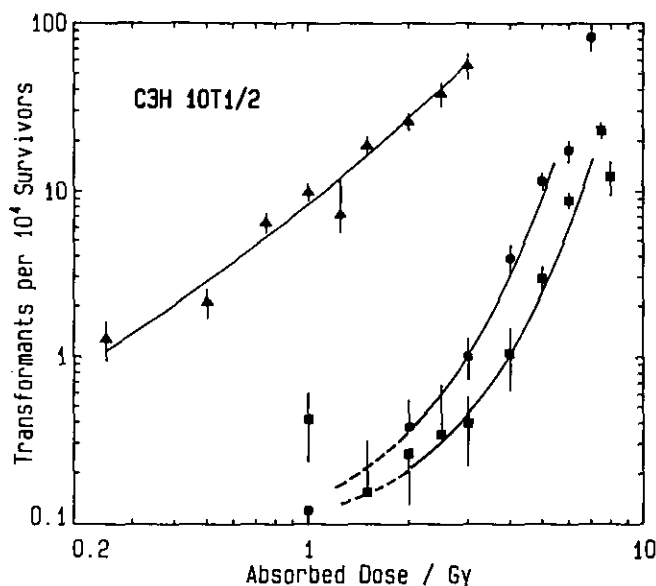FIGURE 1. Inactivation of C3H 10T1/2 cells by Co-γ-rays (■), Cr-K$_α$ soft X-rays (●), and α-particles (— —).

FIGURE 2. Transformation frequencies per surviving cell after exposure to Co-γ-rays (■), Cr-K$_α$ soft X-rays (●), and α-particles (▲). The bars indicate standard errors.

RBE reflect the increased ionization density of electrons at lower energies.

In contrast to the situation with inactivation studies, there have been, up to now, no data for the transformation efficiency of soft X-rays. In our study we find that the relative biological effectiveness of the soft X-rays for transformation and for cell inactivation are similarly enhanced; as seen in Figure 2, one obtains the same value of RBE of about 1.3. The data suggest that the RBE for cell transformation by soft X-rays versus

γ-rays is independent of dose at least in this dose range. Similar values of RBE for cell inactivation and cell transformation are also found for α-particles. As seen in Figure 2, the RBE versus γ-rays is about 6 at 1 Gy for transformation, whereas the corresponding value for cell inactivation is about 5. Although the overall picture is similar for cell inactivation and cell transformation, there is an indication that the changes of RBE are somewhat larger for transformation than for cell inactivation.

The essential result of our soft X-ray study is that low energy electrons with ranges less than 1 μm are substantially more effective than fast electrons with regard to cell transformation. This observation is an important addition to the earlier findings that were limited to cell inactivation studies, preliminary data on micronuclei induction, induction of DNA double strand breaks (17), and the production of chromosome aberrations (18,19).

Transformation Studies with SHE Cells

Growth Behavior. Syrian hamster embryo fibroblasts were transformed by different doses of γ-rays, α-particles, and 8 MeV/u carbon ions. All of the transformed cell lines induced tumors in athymic nude mice, 5 to 6 weeks after SC injection of 2×10^6 cells. All isolated cell lines (now at least 30 cell lines) are, therefore, neoplastically transformed and tumorigenic. The transformants and the tumor cell lines have a pronounced growth advantage both on solid surfaces and in semisolid medium. Their plating efficiencies are increased by a factor of about 2, the population doubling times are reduced by about 30%, and the cloning efficiencies in semisolid medium are increased by factors between 10 and 10^4 relative to the primary cells.

Expression of the c-H-ras Gene. Eight radiation-transformed SHE cell lines and eight related tumor cell lines have been analyzed by Northern blotting with regard to the expression of the H-*ras* gene. As shown in Figure 3 and summarized in Table 1, all transformed cell lines and 6 out of 8 tumor cell lines have levels of H-*ras* transcripts that are enhanced by a factor of 2 to 3. Only 2 tumors expressed this gene at the same level as primary SHE cells. The enhancement of the H-*ras* mRNA levels is observed even in cell lines that have just overcome the growth crisis and one may, therefore, surmise that it is a relatively early event in the transformation process. The assumption is supported by the observation of increased H-*ras* expression in early passages of SHE cells that have immortalized spontaneously and are not yet tumorigenic in these early passages (unpublished results obtained in this laboratory).

Restriction Fragment Length Polymorphism. Only a few radiation-transformed and tumor cell lines have, up to now, been analyzed in respect to restriction fragment length polymorphism (RFLP) of the H-*ras* gene (Fig. 4). One (A40 I-1) out of three transformed cell lines shows an extra 4.7 kb fragment in the HindIII-cut DNA. The related tumor line (T 2802) has the same additional fragment. Two other tumor lines, derived

Table 1. Cytogenetic and molecular effects in radiation-transformed and tumor cells.

Cell line[a]	Radiation	c-H-ras expression[b]	RFLP[c]	Sequence of codon[d]				c-myc Amplification[e]	Chromosome number/cell
				12	13	59	61		
82-9	Primary	1	−	GGA	GGC	GCA	CAA	−	44
37IIc1	γ-Rays	2	ND					ND	ND
T 2742		2	ND	***	***	***	***	+	ND
37IIc3	γ-Rays	3	ND					−	43
T 2662		2	−	***	***	***	***	ND	38
38IIa2	α-Particles	2	ND					ND	43
T 2746		3	ND	***	***	***	***	ND	43
38IIb1	α-Particles	2	ND					ND	65
T 2739		2	ND	***	***	***	***	ND	76
37IIIc	α-Particles	3	ND					ND	41
T 2653		1	ND	***	***	***	***	ND	41
401-1	C ions	2	+					−	42
T 2802		2	+	***	***	***	***	+	ND
40II-1	C ions	2	−					−	46
T 2800		2	+	***	***	***	***	+ + +	41
40III-1	C ions	2	−					−	40
T 2799		1	+	***	***	***	***	+ + +	ND

[a] Cell lines beginning with numbers are transformed lines; cell lines beginning with T are tumor cell lines.
[b] Numbers correspond to the factors of enhanced mRNA levels.
[c] (+) Extra bands; (−) no extra band; ND, not done.
[d] (***) Codons identical to those in the primary cells.
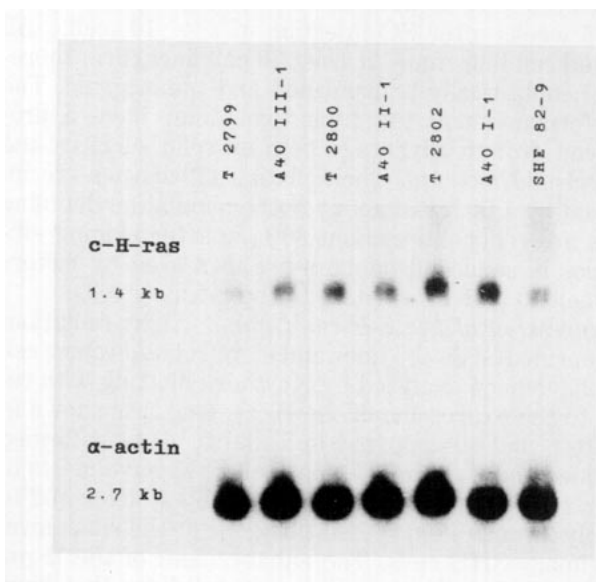[e] (+) Weakly amplified, (+ + +) substantially amplified, or (−) not amplified c-myc gene.

FIGURE 3. H-ras gene expression in various transformed and tumor cell lines. Cells were transformed by 8 MeV/u carbon ions. For data from further cell lines see Table 1.

FIGURE 4. RFLP of the H-ras gene of carbon ion-transformed and the related tumor cell lines. DNA was cut with HindIII.

from carbon ion-transformed cell lines (T 2799 and T 2800), showed two extra fragments with 4.7 kb and 5.1 kb. EcoRI digestion also led to additional fragments in the same cell lines (data not shown).

Possible Activation of the H-ras Gene by Point Mutation. The Syrian hamster c-H-ras gene has recently been cloned and sequenced be Ebert et al. (20), and a sequence analysis has, therefore, become possible. Certain codons in the ras gene family may be responsible for the activation because they are frequently found to
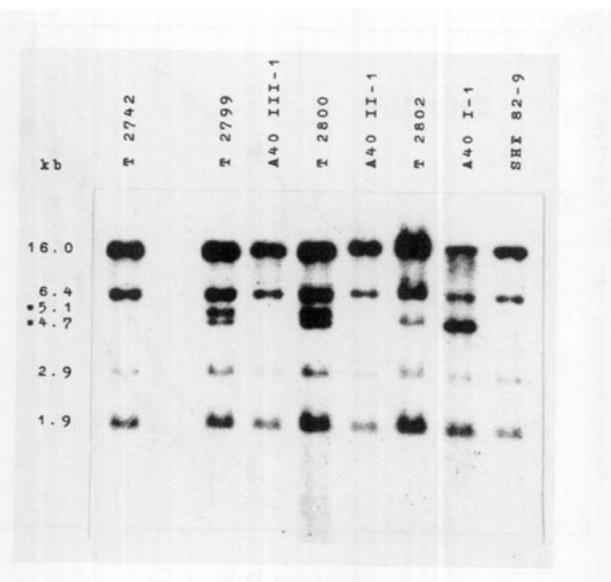
be mutated in tumor cells (21). We have sequenced the H-ras gene (from codon 1 to 23 and from codon 52 to 83) of 8 tumor-cell lines and from the parental SHE cells. No mutations in codon 12 (GGA/gly), 13 (GGC/gly), 59 GCA/ala), or in codon 61 (CAA/gln) were found (Table 1); all of the tumor lines were free of mutations even in the flanking regions of these codons. The absence of point mutations in the specific codons would seem to weaken the assumption that radiation-induced transformations are co-determined by H-ras gene activation through point mutations.
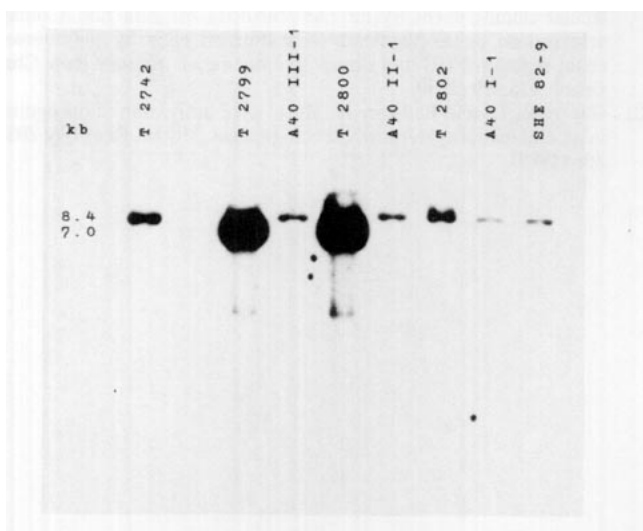
FIGURE 5. Southern blot analysis for the amplification of the c-*myc* gene of the same cell lines as in Figure 4. DNA was cut with HindIII.

Amplification of the c-myc Gene. In the same tumor cell lines where RFLPs of the H-*ras* gene were found, an amplified c-*myc* gene was identified by Southern blot analysis (Fig. 5). The tumor cell lines T 2799 and T 2800 showed substantially increased c-*myc* signals; the lines T 2742 and T 2802 showed a smaller, but still a significant, increase. On the other hand, the transformed cell lines that have been investigated have normal c-*myc* signals as found in primary SHE cells. These observations suggest that the restriction fragment length polymorphisms of the H-*ras* gene and the amplification of the c-*myc* gene are late changes that occur predominantly during growth of the transformed cells in the animal. This finding contrasts with the conclusion mentioned above that the enhanced expression of the H-*ras* gene is an early change in the process of radiation-induced cell transformation.

In the analysis of chromosome numbers per cell in transformed and tumor cell lines, it was found that about 75% of the cell lines have lower and about 25% have higher chromosome numbers than primary SHE cells (44 chromosomes). The possibility that the loss of one or more chromosomes in a transformed cell might correspond to the loss of certain suppressor genes suggested a search for genes that are expressed in normal but not in radiation-transformed cells or in tumor cells. Therefore, we have constructed λgt11 c-DNA libraries of normal, transformed, and tumor cells. In initial plaque hybridization tests with the library for normal cells we have, up to now, found five λ-clones that did not hybridize with c-DNA from a transformed cell line and the corresponding tumor cell line. The extension of these experiments and the identification of the sequences remains a major challenge for future work.

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