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Similarities between human ataxia fibroblasts and murine SCID cells: high sensitivity to γ rays and high frequency of methotrexate-induced DHFR gene amplification, but normal radiosensitivity to densely ionizing α particles

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Abstract. Two γ-ray hypersensitive cell lines, human ataxia telangiectasia (AT) and murine severe combined immune deficiency (SCID) cells, proved to be very competent in amplifying their dihydrofolate reductase (DHFR) gene under methotrexate selection stress. Over a period of months, methotrexate-resistant clones were obtained which were able to grow in progressively increasing methotrexate concentrations up to 1 mM. By then methotrexate-resistant AT and SCID cells had amplified their DHFR gene 6- and 30-fold, respectively, and showed very high DHFR mRNA expression. In contrast, related cells with normal radiosensitivity (human GM637 and mouse BALB/c fibroblasts) did not show DHFR gene amplification under comparable conditions. This correlation of the capacity of DHFR gene amplification and γ -ray hypersensitivity in AT and SCID cells suggests that gene amplification may have a mechanism(s) in common with those involved in repair of γ-radiation-induced damage. No difference in cell killing could be observed following exposure to densely ionizing α particles: AT and SCID cells exhibited comparable survival rates to GM637 and BALB/c cells, respectively.

Introduction

Fibroblasts of a substrain of the BALB/c mouse with a homozygous mutation for the severe combined immune deficiency (SCID) gene show an increase in sensitivity to the killing effect of γ and X irradiation (Fulop and Phillips 1990; Hendrickson et al. 1991) similar to that found in ataxia telangiectasia disease (AT) in humans. SCID mice are severely deficient in both T- and B-cell immunity due to an inability to rearrange correctly immunoglobulin and T-cell receptor genes (Bosma et al. 1983). The SCID mutation confers radiosensitivity to the whole animal, even to tumors arising in SCID mice (Budach et al. 1992). Immune

defects have also been suggested for ataxia patients by studies showing increased frequencies of translocations in loci containing the immune system genes as well as by the finding of reduced levels of T-cell receptor α (Carbonari et al. 1990).

The phenotypic similarities of the two radiosensitive cell lines are further stressed by the finding that both cell lines show a defect in the repair of DNA double-strand breaks after γ irradiation (Coquerelle et al. 1987; Biedermann et al. 1991; Blöcher et al. 1991; Hendrickson et al. 1991). However, the kinetics of DNA synthesis following sparsely ionizing radiation is different between the two cell lines. In contrast to a radioresistant DNA synthesis of AT cells (Painter and Young 1980), DNA synthesis of SCID cells after irradiation is reduced in a similar manner to that observed with normal cells (Komatsu et al. 1993). Interestingly, cell hybrids of SCID and AT cells exhibit repair-proficient survival rates despite radioresistant DNA synthesis. This suggests that the products of at least two different genes are involved in a possible common pathway.

In the present publication I demonstrate two further similarities between SCID and AT cells: normal sensitivity to high LET (Linear Energy Transfer) α particles and a high frequency of methotrexate (MTX)-induced dihydrofolate reductase (DHFR) gene amplification indicative of increased genomic instability.

Material and methods

Cells and culture conditions

SV40-transformed normal human skin fibroblasts GM637 (kindly provided by J.E. Cleaver, San Francisco, Calif., USA) and SV40-transformed AT5BI-VA (purchased from the Genetic Mutant Cell Repository, Camden, N.J., USA) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Gibco, Eggenstein/Germany) and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin).

Murine BALB/c and SCID 3T3/w cells (kindly provided by B. Kaina, Karlsruhe, Germany) were grown in medium consisting of a mixture of 1/2 volume F12 and 1/2 volume DMEM supplemented with fetal calf serum and antibiotics as described above.

Irradiation procedures

Cells were exposed to radiation under exponential growing conditions. Gamma irradiations were performed with a cobald 60 γ source at a dose rate of 1.5 Gy/min, as determined by ferrous sulfate dosimetry; α irradiations were performed with an americium 241 α source, 85 mm in diameter, obtained from Radiochemical Center, Amersham, UK. The dishes for α irradiations each consisted of a glass ring, 45 mm in diameter, and a Melinex foil (polyethylene terephthalate) bottom of 6 μm thickness. The energy of the α particles at the cell surface was reduced to 3.3 MeV corresponding to an LET of about 120 keV/μm (Lücke-Huhle et al. 1982) with a dose rate of 0.35 Gy/min.

Colony survival assay

Survival was determined directly after irradiation of exponentially growing monolayers by measuring the ability of single cells to form colonies. Cells were

seeded into 25-cm^2 Falcon plastic flasks in numbers yielding about 30–50 colonies per flask. Prior to counting, colonies were stained with crystal violet. For each experiment 4–6 dishes per dose were evaluated.

MTX selection procedure

Cell clones were selected for increasing levels of MTX resistance by plating cells at low density (1.3×10^3 cells/cm^2) in 75-cm^2 T-flasks and cultivating the cells in the presence of MTX. MTX-resistant colonies were trypsinized and replated with 10^5 cells per 75-cm^2 T-flask in medium containing MTX concentrations progressively increasing by factors of 1.5–2. Over a period of months heterogeneous populations of cells were obtained that were able to grow in 10^{-3}, 10^{-2}, 10^{-1}, and 1 mM MTX, respectively. The starting concentration was $2.5–5 \times 10^{-5}$ mM MTX, which is the ED_{50} value of the untreated cell lines. The ED_{50} value, defined as the dose causing a 50% decrease in colony survival, had been determined in separate experiments.

Dispersed cell assay

To determine the quantitative changes in DHFR gene copy number (already at low cell numbers), the dispersed cell assay of Lavi and Etkin (1981) was applied. Single cell suspensions of 5×10^5 cells were sucked onto nitrocellulose filters (BA84 Dassel/Germany, Schleicher and Schüll). The cells trapped on the filter were lysed; at the same time their DNA was denatured by soaking the filters three times in 0.5 M NaOH/1 M NaCl. The filters were then neutralized with 0.5 M TRIS/HCl/3 M NaCl, pH 7.4, dried at 80 °C in vacuo, and filter halves (2.5×10^5 cells each) were hybridized with ^{32}P-labeled gene probes.

For quantification of the gene copy number, the β radioactivity of the filter halves was analyzed in a liquid scintillation counter (Tricarb 2660, Packard Instruments, Downers Grove, Ill. USA).

DNA isolation

High-molecular-weight DNA was isolated by phenol-chloroform extraction of sodium dodecylsulfate (SDS)- and proteinase-K-treated cell lysates and precipitated with absolute ethanol (Hinrichs 1986).

Southern DNA blotting technique

Samples of 10 µg of DNA, each digested with the restriction enzyme *Eco*RI, were subjected to electrophoresis in a 0.8% agarose gel and were afterward transferred to nitrocellulose filter paper by the method of Southern (1975). By this method DNA fragments of different sizes can be separated with high resolution. Preceding the transfer to the nitrocellulose filter, the DNA in the gel was denatured in 1 M NaOH for 40 min and neutralized in 1 M HCl/1 M TRIS for 60 min. Transfer to nitrocellulose filters was complete after 18 h.

Northern blot analysis

PolyA$^+$ mRNA was isolated using oligo (dT)-cellulose column chromatography. RNAs (5 μg each) were electrophoresed on 1% agarose gels and transferred to nitrocellulose filters (Maniatis et al. 1982).

Filter hybridization with ^{32}P-labeled DNA probes

Filters containing either DNAs or RNAs were preincubated for 6–15 h at 65 °C in sealed plastic bags containing a solution of 1 M NaCl, 10% dextran sulfate, and 1% SDS. Subsequently, a denatured ^{32}P-labeled DNA probe [labeled in vitro by nick translation (Rigby et al. 1977) with ^{32}P-dCTP to 10^8 cpm/per microgram of DNA] and 100 μg/ml of salmon sperm DNA were added. Incubation in a waterbath at 65 °C was continued for at least 15 h. Filters were then washed twice in 2 × SSC (0.3M NaCl, 0.03M Na_3 citrate, pH 7.0) at room temperature for 5 min, twice in 2 × SSC 0.1% SDS at 65 °C for 30 min, and twice more in 0.1 × SSC at room temperature for 5 min. The filters were then autoradiographed with Kodak X-Omat AR films and intensifying screens at −70 °C for various periods of time.

The gene probes used for the hybridization assay were mouse α-actin c-DNA (Minty et al. 1981) and mouse DHFR c-DNA (Gasser et al. 1982).

Results and discussion

Figure 1 demonstrates SCID 3T3/w and AT5BI-VA cells to be more sensitive to the killing effect of γ rays (open symbols) than their normal counterparts, the unmutated mouse BALB/c fibroblasts and the normal human GM637 cells, respectively. Radiosensitivity differs by factors of 2.9 and 2.1 for SCID and AT cells, respectively, as calculated at 10% survival level (listed in Table 1). A high sensitivity of SCID cells to γ rays and bleomycin has been described earlier by Biedermann et al. (1991) and Itoh et al. (1993). New is the finding that no difference can be observed in cell killing following exposure to densely ionizing americium 241 α particles. Here SCID cells show the same sensitivity as BALB/c cells, which is comparable to the result obtained with AT5BI-VA and GM637 cells (closed symbols in Fig. 1). All survival parameters are compiled in Table 1. As expected, relative biological effectiveness (RBE) values calculated at 10% survival level are much lower for the radiosensitive SCID and AT5BI-VA cell lines compared with BALB/c or GM637 cells, respectively.

Alpha-particles are supposed to kill a cell mainly via the production of DNA double-strand breaks. The finding, however, that no difference in radiosensitivity with respect to cell killing exists, indicates that α-particle-induced DNA strand breaks may be equally well repaired (or not repaired) in AT5BI-VA and GM637 cells or, correspondingly in SCID 3T3/w and BALB/c cells.

For γ-ray-induced DNA double-strand breaks, Biedermann et al. (1991) concluded from rejoining measurements by pulse field gel electrophoresis that a reduced level of double-strand-break repair is responsible for the high killing rate in γ irradiated SCID cells. Correspondingly, impaired double-strand-break rejoining or high fractions of nonrejoined double-strand breaks were found in γ irradiated AT cells (Coquerelle et al. 1987; Blöcher et al. 1991). In contrast

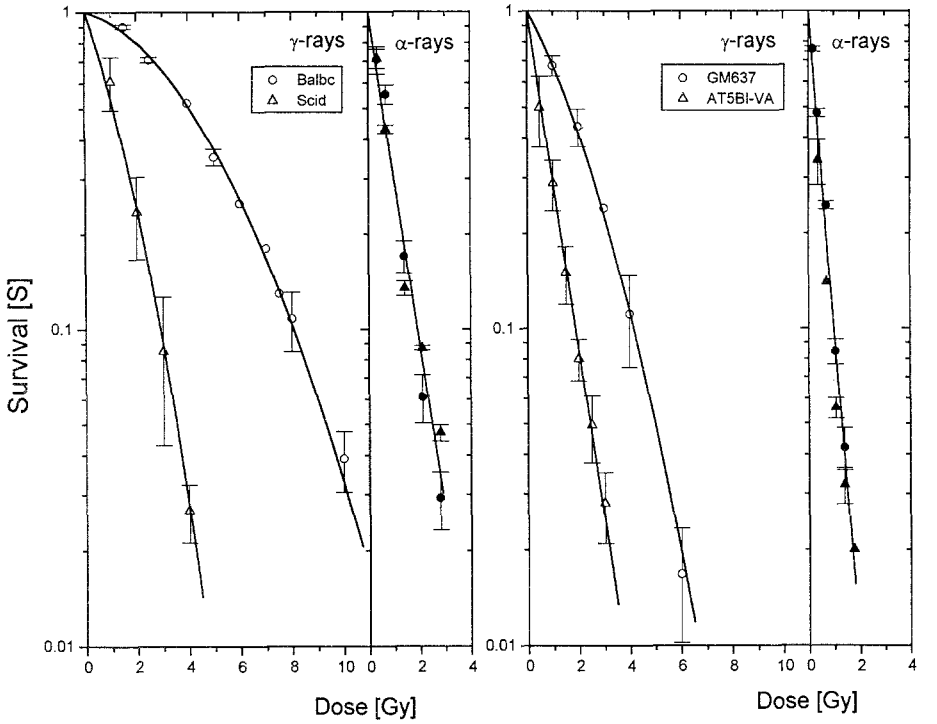

Fig. 1. Survival curves of murine BALB/c and severe combined immune deficiency (SCID) 3T3/w cells (*left-hand side*) and human GM637 and AT5BI-VA cells (*right-hand side*) after exposure to cobalt 60 γ rays (*open symbols*) or americium 241 α particles (*closed symbols*), respectively. Data points represent mean values of three independent experiments, each $\pm$SD. Curves were fitted by means of a computer program according to the model by Chadwick and Leenhouts (1981)

Table 1. Survival curve parameters after exposure to cobalt 60 γ or americium 241 α rays

Cell line	Species	^{60}Co γ-irradiation			^{241}Am α-irradiation		
		Dq(Gy)	D_0(Gy)	D_{10}(Gy)	D_{37}(Gy)	D_{10}(Gy)	RBE[a]
GM637	Human	1.6	1.1	4.1	0.4	0.8	5.1
AT5BI-VA	Human	0	0.8	1.9	0.4	0.8	2.4
BALB/c	Mouse	4.1	1.9	8.3	0.9	1.9	4.4
SCID 3T3/w	Mouse	0.7	0.9	2.8	0.9	1.9	1.5

[a] Calculated at 10% survival

to mechanisms in bacteria and yeast the mechanisms of double-strand-break repair in mammalian cells are still obscure. A defect in recombinational repair processes has been suggested by Hendrickson et al. (1991) and Powell et al. (1993) to be responsible for the high γ-ray sensitivity of SCID and AT cells. The normal sensitivity of these cells to α particles, however, suggests this type of repair plays a much less important role in the elimination of α-particle-induced

damage. Such an idea is in agreement with the hypothesis that the clustered DNA damage that results from densely ionizing particle exposures may not provide a suitable template for recombination-mediated rejoining (Ritter et al. 1992).

A defective recombination (error prone) is also thought to be responsible for the high genomic instability of these radiosensitive cells showing up as spontaneous chromosome aberrations (Meyn 1993) and a higher incidence of gene amplification in comparison to cells with normal radiosensitivity. An increased capacity for gene amplification has been demonstrated recently for AT5BI-VA cells (Lücke-Huhle et al. 1987) and for ultraviolet-hypersensitive baby hamster kidney cells (Giulotto et al. 1991).

Fig. 2 shows that not only AT5BI-VA cells, but also SCID cells can amplify

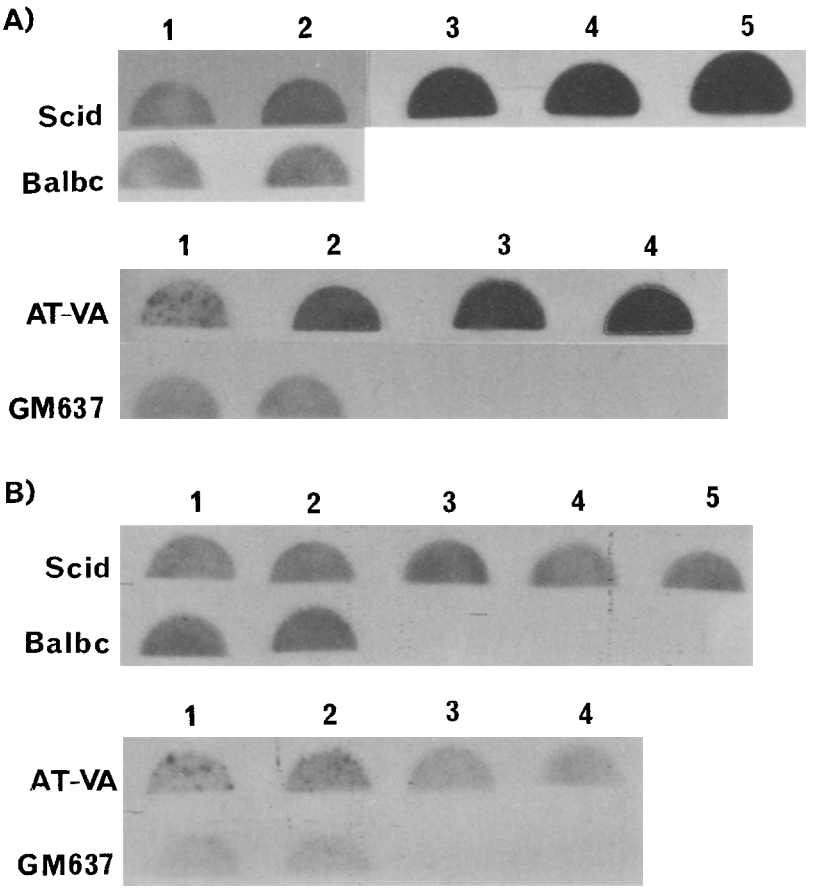

Fig. 2. Autoradiograms of dispersed cell blots, hybridized either with a ^{32}P-labeled dihydrofolate reductase (DHFR) gene probe (**A**) or an α-actin gene probe (**B**), show an increase in DHFR gene copy number for methotrexate (MTX)-resistant SCID and AT cells. The 5×10^5 cells have been blotted onto each round nitrocellulose filter that afterward were cut in half to allow hybridization with two different genes (e.g., DHFR and α-actin). The numbers 1–5 stand for MTX concentrations the cells can grow in: without MTX (1), 10^{-3} mM MTX (2), 10^{-2} mM MTX (3), 10^{-1} mM MTX (4), and 1 mM MTX (5)

their DHFR gene under MTX selection stress. Through stepwise increases in the concentration of MTX in the culture medium, SCID cell clones were obtained which finally grew in concentrations of 1 mM MTX; this was five orders of magnitude higher than the MTX concentration in which the original cells survived. At this point, MTX-resistant SCID clones have increased their DHFR gene copy number 30-fold (Table 2). Human AT cells show lower amplification rates. They are only able to grow in MTX concentrations up to 10^{-1} mM, and increased their gene copy number by a factor of five- to sixfold.

That the increase in signal intensity in Fig. 2A is not due to a general polyploidization but reflects the specific increase in DHFR gene copy number is demonstrated by equal signal intensity between control and MTX-selected cells after hybridizations of the corresponding filter halves to an α-actin probe (Fig. 2B).

In contrast to the radiosensitive cell lines, the radioresistant cell lines – BALB/c and GM637 – do not show any sign of DHFR gene amplification and stop growing at low concentrations of MTX. The finding that normal cells do not give rise to the formation of drug-resistant colonies on selection with drugs inducing gene amplification is in agreement with observations made in several other laboratories (Tlsty 1990; Wright et al. 1990).

A more detailed analysis of genomic DNA by *Eco*RI digestion and Southern blotting supports the finding obtained with the dispersed cell assay: SCID and AT cells show increasing amplification of their DHFR gene fragments in association with increased MTX resistance (Fig. 3). The MTX-resistant SCID cells show amplification of four characteristic *Eco*RI fragments of 20, 6.2, 5.8, and 3.8 kb in size (Fig. 3A, left-hand side). In the MTX-resistant AT cells, fragments of 1.8, 6, 1.65, and 4 kb are amplified corresponding to the exons 1, 2+3, 4, and 5 of the DHFR structural gene, respectively (Anagnou et al. 1984). The increase in MTX concentration from 10^{-2} to 10^{-1} mM did not increase the DHFR gene copy number in AT cells any further (Table 2, Fig. 3A). At this high concentration many cells do not divide any longer and exhibit a more general polyploidization, as indicated by the amplification of some α-actin gene fragments (Fig. 3B). Amplification of the DHFR gene leads in all cases to an

Table 2. Amplification of DHFR-specific DNA in MTX-resistant SCID and AT cells based on the evaluation of the dispersed cell assays in Fig. 2

Cell line	Resistance of cells to MTX concentrations (mM)	DHFR gene (copies per cell)
SCID 3T3/w	0	2[a]
	10^{-3}	4
	10^{-2}	10
	10^{-1}	26
	1	60
AT5BI-VA	0	2[a]
	10^{-3}	6
	10^{-2}	12
	10^{-1}	10

[a] Per definition

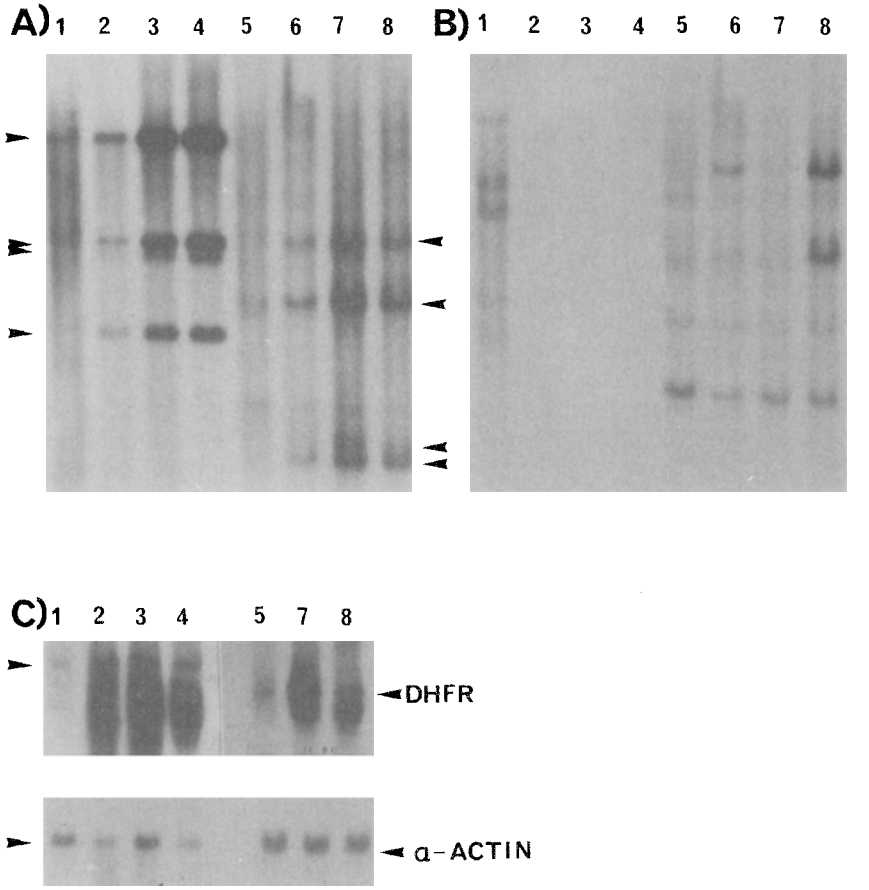

Fig. 3. Autoradiograms of DNA and RNA blots from control and MTX-resistant cell lines. *Eco*RI-digested DNA was either hybridized to ^{32}P-labeled DHFR gene probe (**A**) or to ^{32}P-labeled α-actin (**B**). *Eco*RI-digested SCID DNA (lane 1:10 μg control DNA; lanes 2–4: 1 μg DNA, each of cells resistant to 10^{-2}, 10^{-1}, and 1 mM MTX, respectively) yields four characteristic fragments of 20, 6.2, 5.8, and 3.8 kb (*left-hand arrows from top to bottom*). DNA from AT cells exhibits *Eco*RI fragments of 6, 4.2, 1.65, and 1.8 kb in size: lanes 5–8, 10 μg DNA each; control AT5BI-VA cells (lane 5) or AT cells resistant to 10^{-3}, 10^{-2}, and 10^{-1} mM MTX, respectively. Northern blot analysis of poly A^+ mRNA (**C**) shows increased expression of the DHFR gene in all MTX-resistant SCID cells (lanes 2–4) and AT cells (lanes 7 and 8) in comparison to the corresponding controls (lanes 1 and 5, respectively). Lanes are numbered as in **A** and **B**. Hybridization of the same blot with α-actin served as a control to ensure that equal amounts of RNA had been blotted

increased expression of this gene, as demonstrated in the Northern blot of Fig. 3C.

In conclusion, the observed correlation between the capacity of gene amplification in SCID and AT cells and the defect of these cells being responsible for the low survival after γ irradiation makes it very likely that similar proteins may be involved in both pathways. Repair of α-particle-induced DNA damage seems to utilize different repair mechanisms.

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References

Anagnou NP, O'Brien SJ, Shimada T, Nash WG, Chen MJ, Nienhuis AW (1984) Chromosomal organisation of the human dihydrofolate reductase genes: dispersion, selective amplification and a novel form of polymorphism. Proc Natl Acad Sci USA 81:5170–5174

Biedermann KA, Sun J, Giaccia AJ, Tosto LM, Brown JM (1991) Scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc Natl Acad Sci USA 88:1394–1397

Blöcher D, Sigut D, Hannan MA (1991) Fibroblasts from ataxia telangiectasia (AT) and AT heterozygotes show an enhanced level of residual DNA double-strand breaks after low dose-rate γ-irradiation as assayed by pulsed field gel electrophoresis. Int J Radiat Biol 60:791–802

Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. Nature 301:527–530

Budach W, Hartford A, Gioioso D, Freeman J, Taghian A, Suit HD (1992) Tumors arising in SCID mice share enhanced radiation sensitivity of SCID normal tissues. Cancer Res 52:6292–6296

Carbonari M, Cherchi M, Paganelli R, Giannini G, Galli E, Gaetano C, Papetti C, Fiorilli M (1990) Relative increase of T cells expressing the gamma/delta rather than the alpha/beta receptor in ataxia-telangiectasia. N Engl J Med 322:73–76

Chadwick KH, Leenhouts HP (1981) The molecular theory of radiation biology. Springer, Berlin Heidelberg New York

Coquerelle TM, Weibezahn KF, Lücke-Huhle C (1987) Rejoining of double strand breaks in normal human and ataxia-telangiectasia fibroblasts after exposure to 60Co γ-rays, 241AM α-particles or bleomycin. Int J Radiat Biol 51:209–218

Fulop GM, Phillips RA (1990) The scid mutation in mice causes a general defect in DNA repair. Nature 347:479–482

Gasser CS, Simonsen CC, Schilling JW, Schimke RT (1982) Expression of abbreviated mouse dihydrofolate reductase genes in cultured hamster cells. Proc Natl Acad Sci USA 79:6522–6526

Giulotto E, Bertoni L, Attolini C, Rainaldi G, Anglana M (1991) BHK cell lines with increased rates of gene amplification are hypersensitive to ultraviolet light. Proc Natl Acad Sci USA 88:3484–3488

Hendrickson EA, Qin X-Q, Bump EA, Schatz DG, Oettinger M, Weaver DT (1991) A link between double-strand break-related repair and V(D)J recombination: the scid mutation. Proc Natl Acad Sci USA 88:4061–4065

Hinrichs S (1986) Methotrexat Resistenz durch Genamplifikation in menschlichen Hautfibroblasten. Diplomarbeit, Universität Karlsruhe

Itoh M, Hamatani K, Komatsu K, Araki R, Takayama K, Abe M (1993) Human chromosome 8 (p12 → q22) complements radiosensitivity in the severe combined immune deficiency (SCID) mouse. Radiat Res 134:364–368

Komatsu K, Yoshida M, Okumura Y (1993) Murine scid cells complement ataxia-telangiectasia cells and show a normal post-irradiation response of DNA synthesis. Int J Radiat Biol 63:725–730

Lavi S, Etkin S (1981) Carcinogen-mediated induction of SV40 DNA snythesis in SV40 transformed Chinese hamster embryo cells. Carcinogenesis 2:417–423

Lücke-Huhle C, Comper W, Hieber L, Pech M (1982) Comparative study of G2 delay and survival after ^{241}Americium-α and ^{60}Cobalt-γ irradiation. Radiat Environ Biophys 20:171–185

Lücke-Huhle C, Hinrichs S, Speit G (1987) DHFR gene amplification in cultured skin fibroblasts of ataxia telangiectasia patients after methotrexate selection. Carcinogenesis 8:1801–1806

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Meyn MS (1993) High spontaneous intrachromosomal recombination rates in ataxia-telangiectasia. Science 260:1327–1330

Minty AJ, Caravatti M, Robert B, Cohen A, Daubas P, Weydert A, Gros F, Buckingham ME (1981) Mouse actin messenger RNAs. J Biol Chem 256:1008–1014

Painter RB, Young BR (1980) Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc Natl Acad Sci USA 77:7315–7317

Powell S, Whitaker S, Peacock J, McMillan T (1993) Ataxia telangiectasia: an investigation of the repair defect in the cell line AT5BIVA by plasmid reconstitution. Mutat Res 294:9–20

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase. J Mol Biol 113:237–251

Ritter S, Kraft-Weyrather W, Scholz M, Kraft G (1992) Induction of chromosome aberrations in mammalian cells after heavy ion exposure. Adv Space Res 12:119–125

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Tlsty TD (1990) Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc Natl Acad Sci USA 87:3132–3136

Wright JA, Smith HS, Watt FM, Hancock MC, Hudson DL, Stark GR (1990) DNA amplification is rare in normal human cells. Proc Natl Acad Sci USA 87:1791–1795