DNA Double-Strand Break Induction and Repair in Irradiated Lymphoblastoid, Fibroblast Cell Lines and White Blood Cells from ATM, NBS and Radiosensitive Patients

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Background and Purpose: DNA double-strand breaks (dsbs) in lymphoblastoid cell lines (LCLs), fibroblasts and white blood cells from healthy donors, cancer patients with and without late effects of grade 3–4 (RTOG) as well as donors with known radiosensitivity syndromes were examined with the aim to detect dsb repair ability as a marker for radiosensitivity.
Material and Methods: LCLs from six healthy donors, seven patients with a heterozygous or homozygous genotype for ataxia-telangiectasia (ATM) and Nijmegen breakage syndrome (NBS), two patients with a late toxicity of grade 3–4 (RTOG), and one cell line with a ligase IV$^{-/-}$ status and its parental cell line were examined. Furthermore, fibroblasts from patients with ATM, NBS, two healthy control individuals, and leukocytes from 16 healthy and 22 cancer patients including seven patients with clinical hypersensitivity grade 3 (RTOG) were examined. Cells were irradiated in vitro with 0–150 Gy. Initial damage as well as remaining damage after 8 and 24 h were measured using constant field gel electrophoresis.
Results: In contrast to cells derived from patients homozygous for NBS, impaired dsb repair ability could be detected both in fibroblast and lymphoblastoid cells from ATM and ligase IV$^{-/-}$ patients. The dsb repair ability of all 38 leukocyte cell lines (patients with grade 3–4 late effects and controls) was similar, whereas the initial damage among healthy donors was less.
Conclusion: Despite showing a clinically elevated radiosensitivity after irradiation, the DNA repair of the patients with clinical hypersensitivity grade 3 (RTOG) appeared to be normal. Other mechanisms such as mutations, altered cell cycle or defective apoptosis could play a critical role toward determining radiosensitivity.

Key Words: Radiation-induced dsbs · Individual radiosensivity · Hypersensitivity · LCLs · Leukocytes

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Induktion und Reparatur von DNA-Doppelstrangbrüchen in bestrahlten lymphoblastoiden Zelllinien, Fibroblasten sowie Leukozyten von ATM- und NBS-Patienten sowie erhöht strahlenempfindlichen Patienten

Hintergrund und Ziel: DNA-Doppelstrangbrüche in lymphoblastoiden Zelllinien (LCLs), Fibroblasten und Leukozyten von Probanden, Krebspatienten mit und ohne Spättoxizität Grad 3–4 (RTOG) sowie von Spendern mit Strahlenempfindlichkeitssyndromen wurden mit dem Ziel untersucht, Doppelstrangbruch-Reparaturfähigkeit als einen Marker zur Detektion von Strahlenempfindlichkeit zu verwenden. Dies basiert auf der Hypothese, dass erhöht strahlenempfindliche Zellen auf In-vitro-Bestrahlung eine erniedrigte Reparaturfähigkeit von Doppelstrangbrüchen zeigen.
Material und Methodik: Epstein-Barr-Virus-(EBV-)transformierte LCLs von sechs gesunden Spendern, sieben Patienten mit heterozygotem oder homozygotem Genotyp für Ataxia teleangiectatica (ATM) und Nijmegen-Breakage-Syndrom (NBS), zwei Patienten mit Spättoxizität Grad 3–4 (RTOG) und eine Zelllinie mit einem Ligase-IV$^{-/-}$-Status mit ihrer Mutterzelllinie wurden untersucht. Des Weiteren wurden Fibroblasten von Patienten mit ATM bzw. NBS und zwei gesunden Kontrollpersonen sowie Leukozyten von 16 Gesunden und 22 Krebspatienten einschließlich sieben Patienten mit nachgewiesener Strahlenempfindlichkeit untersucht. Die Zellen wurden in vitro mit einer Einzeldosis zwischen 0 und 150 Gy bestrahlt. Der Initialschaden sowie der Restschaden nach einer Reparaturzeit von 8 bzw. 24 h wurden mit Hilfe der Gelelektrophorese gemessen.
Ergebnisse: Im Gegensatz zu den Zellen, die von NBS-Patienten stammten, ließ sich sowohl in den Fibroblasten als auch in den lymphoblastoiden Zelllinien, bei den ATM-Zellen und der Ligase-IV$^{-/-}$-Zelllinie eine erniedrigte Doppelstrangbruch-Reparaturfähigkeit nachweisen. Die Doppelstrangbruch-Reparaturfähigkeit der Leukozyten aller 38 Patienten und Kontrollpersonen war sehr ähnlich, während die Leukozyten der Kontrollpersonen erniedrigte Initialschäden aufwiesen. Entsprechend konnten keine strahlenempfindlichen Krebspatienten durch erniedrigte Doppelstrangbruch-Reparaturfähigkeit identifiziert werden.

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Schlussfolgerung: Trotz erhöhter klinischer Strahlenempfindlichkeit konnte keine signifikant erhöhte Anzahl von verbleibenden DNA-Doppelstrangbrüchen in den Leukozyten dieser Patienten gefunden werden. Andere Mechanismen, wie erlittene Mutationen oder gestörte Regelmechanismen in Zellzykluskontrolle oder Apoptose, könnten dafür ursächlich sein.

Schlüsselwörter: Strahleninduzierte Doppelstrangbrüche · Individuelle Strahlenempfindlichkeit · Überempfindlichkeit · Lymphoblastoide Zelllinien · Leukozyten

Introduction

Differences in individual radiosensitivity are clinically well known. Rare syndromes such as ataxia-telangiectasia (ATM), Nijmegen breakage syndrome (NBS1), Bloom's syndrome and recently detected mutations in hMRE11 [29] and ligase IV [25] are associated with a substantially increased radiosensitivity. A much higher proportion of the patient population receiving radiotherapy is predisposed to complications due to a moderately increased radiosensitivity. In contrast to the known gene defects underlying radiosensitivity syndromes, presently, it is not clear which specific mechanisms may lead to a hypersensitivity in nonsyndromic patients that causes radiation treatment-related complications. It is assumed that low penetrance genes may cause increased radiosensitivity [2, 27]. The identification of sensitive individuals is a primary and clinically important goal. Many different functional assays have been used to this end, for example, testing DNA repair, misrepair, cell-cycle control, micronuclei formation, chromosomal aberrations, and cell inactivation. While it is possible to identify radiosensitivity syndromes with most of the functional assays, it is much more difficult to identify nonsyndromic radiosensitive individuals.

Repair of DNA damage in mammalian cells is lesion-specific, consequently, several repair pathways with about 130 different known human proteins are used [31]. The DNA double-strand break (dsb) is considered the most toxic among the different types of DNA lesions [18]. Therefore, repair of dsbs is regarded to be essential for cellular integrity and this is the reason why dsb repair capacity is frequently used to examine cellular radiosensitivity. Two major pathways for DNA dsb removal are used in a cell-cycle-dependent way. Nonhomologous end joining (NHEJ) is predominantly used in G0/G1 and homologous recombination (HR) in S/G2 phase. The NHEJ pathway basically consists of damage recognition and protein protection which leads to rejoining of the two DNA ends by ligase IV [7]. HR has a more complex processing of the DNA dsb using a second DNA molecule with sequence homology to restore the damaged DNA molecule [28].

The intention of this work was to identify individuals with a known increased radiosensitivity using constant field gel electrophoresis by measuring the initial DNA dsbs and remaining DNA damage during DNA dsb repair. In general, previous studies have measured DNA dsbs in fibroblasts. Since it requires time to generate fibroblasts, we attempted to use lymphocytes to study dsb damage and repair [13, 24]. First, we compared fibroblasts from controls and radiosensitivity syndromes to lymphoblastoid cell lines (LCLs). Then, we analyzed leukocytes derived from healthy donors, cancer patients, and cancer patients with previously identified cellular and clinical radiosensitivity.

Material and Methods

Cell Lines and Patients

Lymphoblastoid and fibroblasts cell lines were used to prove the feasibility of the assay and compare the radiosensitivity of homozygous *ATM* (p104, p106, L117), heterozygous *ATM* (L115), homozygous *NBS1* (p122, p122-F, ms2), and heterozygous (ms4) cells with controls. The NBS1 cell lines have the typical founder mutation 657del5. The LCL, p122, the corresponding primary fibroblast cell line, p122-F, and the ATM cell lines have been described previously [11, 30]. A DNA dsb repair-deficient cell line with a homozygous ligase IV deficiency (N114) and its parental leukemia cell line (Nalm6) served as a positive control. Two LCLs (p105, p109) were derived from patients with increased radiosensitivity having severe late effects of grade 3 (RTOG). Two primary fibroblast lines (SBL2-F and SBL3-F) and six LCLs from healthy individuals were used as control cell lines. Heparinized blood samples [8, 26] were obtained from 22 cancer patients (Table 1) and 16 healthy controls. Among the cancer patients seven individuals suffered from increased late complications (Table 1) after radiotherapy. Age ranged from 29 to 78 years with a mean of 55 in the cancer patients group, 47 in the sensitive group, and 52 in the healthy individuals group. The study was carried out in a blinded manner.

DNA Double-Strand Breaks

Fibroblasts were grown in F12 medium (15% FCS, 1% L-glutamine, and 1% Pen/Strep) in a humidified incubator at 37 °C and 5% CO_2 [1, 5, 23]. All experiments were done using confluent cultures. Cells were irradiated and incubated for 8 or 24 h, trypsinized and mixed with agarose to get 40,000 cells per plug (1 × 8 × 8 mm) having about 600 cells per mm^3. To evaluate initial DNA dsbs, the fibroblasts were first trypsinized and molded into agarose plugs and then irradiated on ice and held at 0 °C.

Heparinized blood was diluted to a ratio of 1 : 4 in medium (RPMI with 15% FCS, 1% L-glutamine, and 1% Pen/Strep). To determine the initial DNA dsbs, blood was mixed 1 : 1 with 1% agarose, embedded in plugs and held on ice to

Table 1. Identification number, age and clinical characteristics of patients including dose, dose fraction, time interval between therapy, and DNA double-strand break measurement, tumor type, time of follow-up after the end of therapy, as well as grade and type of late event.

Tabelle 1. Identifikationsnummer, Alter und klinische Charakteristika der Patienten in Bezug auf Dosis, Dosisfraktionierung, Zeitintervall zwischen Therapie und Messung der DNA-Doppelstrangbrüche, Tumorart, Dauer der Nachbeobachtung sowie Art und Typ des Schweregrads.

ID	Age (years)	Tumor type	Dose (Gy)	Dose/ fraction (Gy)	Interval therapy – measurement (months)	Grade of late effect (RTOG)	Follow-up time (years)	Type of late event
P200	50	Bladder cancer	55.8	1.8	18	3	6	Bladder contracture
P201	68	Esophageal cancer	64.8	1.8	–	1	5	
P202	37	Oropharyngeal cancer	66	2.0	62	3	8	Osteonecrosis
P203	48	Oral cavity cancer	68.5	2.0	–	1	7	
P204	65	Breast cancer	56	1.8	76	2	9	
P205	72	Lung cancer	66	1.8	–	1	5	
P206	51	Bladder cancer	55.8	1.8	6	0	6	
P207	55	Oral cavity cancer	60	2.0	–	1	5	
P208	72	Oral cavity cancer	60	2.0	24	3	9	Fibrosis
P209	46	Anal carcinoma	55.8	1.8	52	3	8	Bladder contracture
P210	61	Cervical carcinoma	50.4	1.8	–	2	5	
P211	73	Meningioma	55.8	1.8	–	1	5	
P212	44	Breast cancer	56	1.8	28	3	7	Fibrosis
P213	41	Rectal cancer	50.4	1.8	–	1	5	
P214	62	Non-Hodgkin's lymphoma	40	2.0	–	2	5	
P215	60	Rectal cancer	55.8	1.8	–	2	5	
P216	63	Lung cancer	66	1.8	15	2	6	
P217	69	Larynx cancer	59.4	1.8	–	1	5	
P218	29	Oral cavity cancer	71	2.0	18	3	6	Osteonecrosis
P219	41	Rectal cancer	55.8	1.8	–	1	5	
P220	51	Oral cavity cancer	71	2.0	35	3	8	Fibrosis
P221	57	Hypopharyngeal cancer	66	2.0	–	1	5	

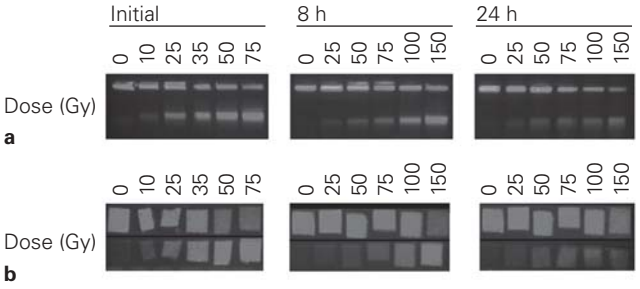
prevent repair [4, 15, 17, 24]. The blood-medium mixture was irradiated and incubated for 8 or 24 h to allow repair and subsequently embedded in agarose and cooled to 4 °C. Plugs were incubated in a solution of proteinase K (1 mg/ml proteinase K, 2% sodium lauryl sulphate) in lysis solution (100 mM EDTA, 50 mM Tris, 50 mM NaCl, pH 8) and kept on ice for 2 h and then incubated at 50 °C for 24 h. The plugs were then dialyzed for 2 h in TBE and inserted into the wells of a 0.5% agarose gel. Electrophoresis gels were run for 16 h at 0.85 V/cm and were stained in ethidium bromide (0.1 µg/ml) for 24 h due to the thick gels [19] (Figure 1).

Statistical Methods

Patients were compared to controls using the nonparametric Mann-Whitney test. Correlations were calculated and given as Spearman's coefficient of rank correlation (rs; SPSS for Windows, Version 11.5, Munich, Germany) [12, 21].

Results

Use of LCLs enables the testing of a large number of cells from individuals with increased radiosensitivity, which would not ordinarily be possible to obtain from the general popula-



Figures 1a and 1b. Example of an ethidium bromide-stained electrophoresis gel showing initial and postirradiation low-molecular-weight DNA that have entered the gel matrix. Leukocytes of an individual were irradiated in vitro with different doses and DNA damage was assessed. The plugs and the "migrated" DNA band were initially photographed directly (a), then cut out, turned 90° and rephotographed (b).

Abbildungen 1a und 1b. Ausschnitte eines Elektrophoresegels, welches den Anteil der initial freigesetzten DNA und den Anteil nach einer Reparaturzeit von 8 bzw. 24 h nach Bestrahlung anzeigt. Leukozyten einer Person wurden in vitro mit verschiedenen Dosen bestrahlt. Das Gel lief 16 h, anschließend wurde für 24 h mit Ethidiumbromid gefärbt. Die Reihen mit den Plugs und der migrierten DNA wurden direkt photographiert (a), anschließend zu jeweils 8 mm dicken Blöcken geschnitten, um 90° gedreht und erneut photographiert (b).

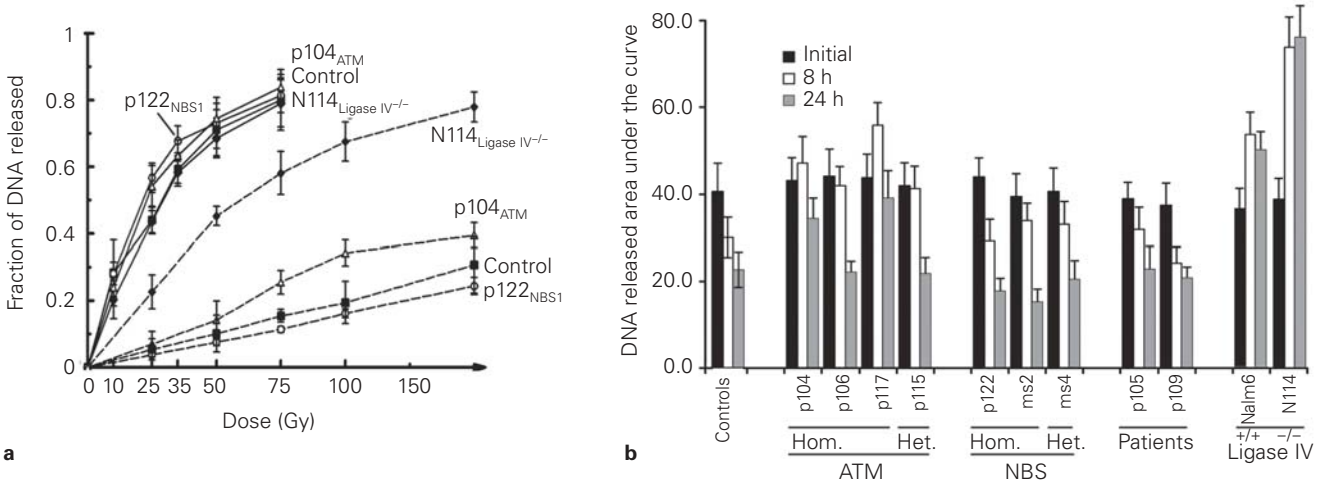
tion or patients of a clinical department because of the low frequency at which they occur. Using these cell lines, it is possible to assess differences between control patients and patients with increased radiosensitivity. Figure 2a depicts typical dose-response curves at initial times and after 24 h of repair for selected cell lines having a distinct increased radiosensitivity.

Values for initial dsbs were very similar between all LCLs (Figure 2b). However, distinct differences in the amount of remaining migrated DNA in the cell lines were observed. All three cell lines homozygous for ATM showed increased remaining dsbs after 8 h, yet after 24 h, one ATM cell line had levels comparable to controls. The ligase IV-deficient N114 cell line had very high remaining dsb values which were significantly different from control values after 8 and 24 h ($p < 0.001$). Its parental cell line, Nalm6, had significantly increased values for remaining dsbs, but significantly different ($p < 0.001$) from the ligase IV-defective line. Despite the known radiosensitivity, NBS1-deficient cells had a DNA dsb rejoining efficiency comparable to control cells. Likewise, no significant difference was observed in lymphoblastoid cells from patients with increased clinical radiosensitivity (p105, p109).

To compare fibroblast data to lymphoblastoid data, we used four fibroblast cell lines derived from the same individuals as the lymphoblastoid cell lines (Figure 3). The initial damage revealed a homogeneous distribution between all cell lines. DNA dsbs values for ATM p104_F and controls were significantly different after 8 h ($p < 0.001$) and 24 h ($p < 0.001$) whereas there was no difference between the NBS1 p122_F fibroblasts and controls ($p = 0.83$). Data from fibroblasts corresponded to that of lymphoblastoid cells from the same individuals showing that among these four individuals, lymphoblastoid cells reflect fibroblast results.

A typical curve for initial and DNA damage is seen in Figure 4a. Leukocytes of cancer patients had significantly ($p < 0.001$) increased initial DNA dsbs released compared to the control individuals (Figure 4b). After repair times of 8 h ($p = 0.21$) and 24 h ($p = 0.11$), there was no further difference. Results for the seven cancer patients with clinical hypersensitivity having grade 3 (RTOG) late effects were not different from cancer patients ($p > 0.54$) and healthy individuals with the exception of the initial DNA dsbs (Figure 4b).

Among the three different cell types used herein (LCLs, leukocytes, and fibroblasts), the initial number of DNA dsbs was very similar with an area under the curve (AUC) of 37.1 ± 0.6 AUC ± SD (standard deviation) for the control fibroblasts, 40.7 ± 6.3 AUC for the LCLs, and 35.8 ± 2.8 AUC for the leukocytes from healthy controls ($p > 0.1$). Only the initial values for cancer patients with an AUC of 45.1 ± 5.2 were



Figures 2a and 2b. Fraction of DNA released in lymphoblastoid cell lines of healthy individuals (squares) and patients with known radiosensitivity syndromes (ATM triangles, NBS circles, ligase IV$^{-/-}$ diamonds). The initial fraction of released DNA (solid lines) due to dsbs and after a repair time of 24 h (dashed lines) is plotted (a). Fraction of DNA released in gel of lymphoblastoid cell lines, after 0, 8, and 24 h. The mean of six cell lines from healthy individuals (controls) were compared to known radiosensitivity syndromes (ATM, NBS, N114 [ligase IV$^{-/-}$]), a leukemia cell line (Nalm6), and patients (p105, p109) with clinical hypersensitivity having late effects of grade 3–4 (RTOG). Values were obtained for each individual by calculating the area under each curve exemplified in a (b).

Abbildungen 2a und 2b. Anteil der freigesetzten DNA in lymphoblastoiden Zellen von gesunden Personen (Quadrate) und Patienten mit bekanntem Strahlenempfindlichkeitssyndrom (ATM Dreiecke, NBS Kreise, Ligase IV$^{-/-}$ Rauten). Die Werte zeigen den Anteil der initial gewanderten DNA (durchgezogene Linien) aufgrund Doppelstrangbrüchen sowie nach einer Reparaturzeit von 24 h (gestrichelte Linien; a). Freigesetzte DNA aus lymphoblastoiden Zelllinien nach 0, 8 und 24 h. Der Durchschnittswert von sechs Zelllinien gesunder Personen (Kontrollen) wurde mit bekannten Strahlenempfindlichkeitssyndromen (ATM, NBS, N114 [Ligase IV$^{-/-}$]), einer Zelllinie eines Leukämiepatienten (Nalm6) und Patienten (p105, p109) mit klinisch erhöhter Strahlensensibilität (Spätschäden Grad 3–4 RTOG) verglichen. Die Werte erhält man durch Errechnen der jeweiligen Fläche unter den in a exemplarisch dargestellten Kurven (b).

distinctly higher ($p = 0.001$). DNA dsbs' remaining (24 h) among healthy individuals was conspicuously different between the different cell types with 16.1 ± 0.3 for the fibroblasts, 22.7 ± 4.0 for the lymphoblastoids, and 60.7 ± 6.2 for the leukocytes ($p < 0.008$).

Discussion

Initial and remaining DNA damage were studied in LCLs, primary fibroblast cell lines, and leukocytes. Initially, we investigated whether it is possible to detect elevated radiosensitivity in LCLs or leukocytes with a similar reliability as found by others using fibroblasts [3, 6, 9, 10, 13, 14, 20]. For the homozygous ATM cells lines for which we had a matching pair (p104 and p104-F), we observed a slower rate of DNA dsbs. This indicated that the lymphoblastoid cells behaved similar to fibroblasts as shown previously [24]. We also observed a slower dsb repair time in a second homozygous ATM LCL (L117), whereas a third homozygous ATM cell line (p106) showed no increase in DNA dsb repair after 24 h relative to the control cell lines indicating that different mutations in ATM cell lines can behave quite differently. Consistent with the idea that repair kinetics were similar among cell lines of divergent origin, the two homozygous NBS1 cell lines (p122 and ms2) for both fibroblasts and LCLs had very similar dsb repair kinetics. The DNA dsbs were also rejoined with comparable kinetics to controls for both of these lines. This is in line with previous reports where no difference in repair kinetics between an NBS cell line and a control cell line was observed [22]. Furthermore, we have shown previously, that the V(D)J recombination, which is known to be mediated by

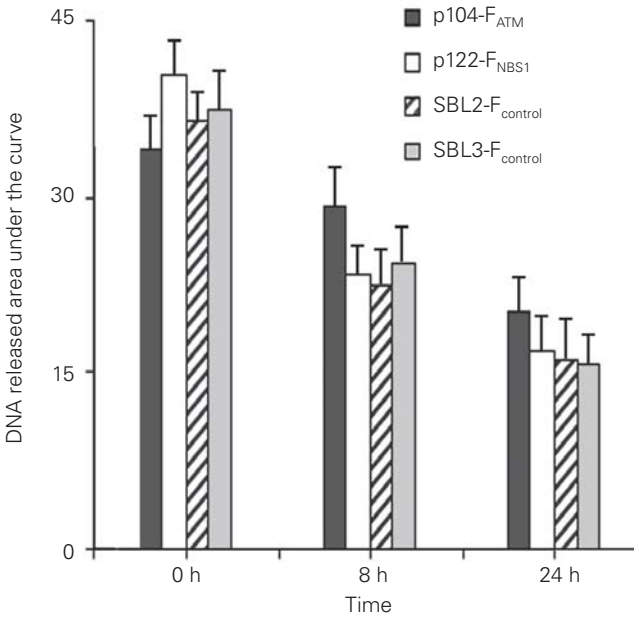
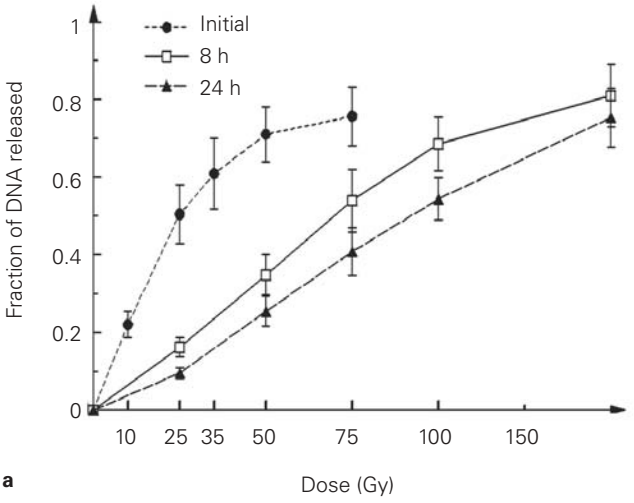
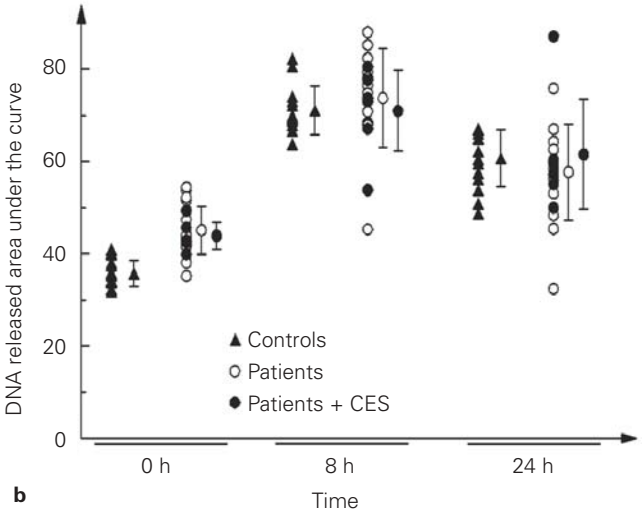


Figure 3. Fraction of DNA released into gel. Initial levels and after allowing 8 and 24 h of DNA repair in four primary fibroblasts derived from two patients with known radiosensitivity syndromes (ATM, NBS1) and two control individuals (SBL2-F, SBL3-F). Values were obtained by calculating the area under each curve (initial damage and repair curve, respectively) as in Figure 2.

Abbildung 3. Freigesetzte DNA aus Fibroblasten von Patienten mit bekanntem Strahlenempfindlichkeitssyndrom (ATM, NBS1) und zwei Kontrollpersonen (SBL2-F, SBL3-F). Die Werte erhält man durch Errechnen der Fläche unter der jeweiligen Kurve (Dosiseffekt- bzw. Reparaturkurve) wie in Abbildung 2.





Figures 4a and 4b. DNA released in leukocytes of a typical patient. Initial and remaining fraction of DNA released after 8 and 24 h at different doses (a). DNA released calculated as the area under the curve in leukocytes of cancer patients without (open circles) and with (filled circles) late effects of grade 3–4 (RTOG) compared to healthy individuals (triangles), each with mean values and SDs, after 0 (75 Gy), 8, and 24 h (150 Gy; b).

Abbildungen 4a und 4b. Initialer sowie verbleibender Anteil von freigesetzter DNA eines Beispielpatienten nach 8 und 24 h bei verschiedenen Strahlendosen (a). Freigesetzte DNA, errechnet als Fläche unter der Kurve, in Leukozyten von Tumorpatienten ohne (offene Kreise) und mit (gefüllte Kreise) Spätschäden Grad 3–4 (RTOG), verglichen mit gesunden Personen (Dreiecke), jeweils mit Mittelwert und Standardabweichung. Die Dosen betrugen 75 Gy für den Initialschaden und jeweils 150 Gy für die verbleibenden Schäden nach 8 und 24 h (b).

NHEJ, is quite normal in NBS1 cells [16], with unimpaired rejoining in both fibroblasts and LCLs compared to control cell lines. Therefore, although the NBS1$^{-/-}$ cells are highly radiosensitive and NBS1 is involved in DNA dsb repair, we cannot easily detect this using the said DNA damage and repair method. The same is true for one of three ATM homozygous cell lines studied here.

A cell line (N114) deficient in ligase IV which is deficient in rejoining dsbs by NHEJ, was confirmed to have deficiency DNA dsb repair. However, within 24 h, a slight decrease of the remaining dsbs was observed which indicates some repair capacity, possibly by HR. The two cell lines from cancer patients with increased radiosensitivity had normal DNA dsb repair.

The initial DNA dsbs were quite similar with the exception that the initial DNA dsbs of the cancer patients were significantly increased compared to the controls. A possible explanation is that radiation treatment was the cause, yet there was a minimum break of 0.5 years between therapy and DNA dsb measurement. The remaining DNA damage of controls did not differ from cancer patients and clinically and cytogenetically sensitive patients. The reason for the similarity could be that only very low numbers of dsbs cause increased radiosensitivity which cannot be easily detected using this method. Previously, it was shown in fibroblast cell lines that only very small variation in dsb repair capacity results in huge differences in cell survival [20]. On the other hand, no increase in remaining dsbs could be detected, in the highly sensitive NBS1 cell lines, one ATM and two sensitive lymphoblastoid cancer cell lines. A possible explanation for not observing a reduced DNA dsb repair capacity in these patient cell lines is that there may be an impaired signal transduction, cell-cycle regulation or programmed cell death which could lead to increased side effects in patients. A further aspect to consider is that treatment regimen differences between individuals including factors such as total dose, fraction size, overall treatment time, radiation quality, and treatment volume may contribute to variation between patients and side effects. We note that there were no apparent variations from commonly used schemes (Table 1).

Conclusion

Some fibroblasts, LCLs and leukocytes with increased radiosensitivity exhibit increased numbers of remaining DNA dsbs, but none of the patients that had severe late toxicity. Increased radiosensitivity in these patients may not be detectable by this way of DNA dsb measurement or may be associated with other deficient cell functions like impaired cell inactivation, cell-cycle regulation, or stable mutations.

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