After double-strand break induction by UV-A, homologous recombination and nonhomologous end joining cooperate at the same DSB if both systems are available

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Accepted 10 June 2004
Journal of Cell Science 117, 4935-4945 Published by The Company of Biologists 2004
doi:10.1242/jcs.01355

Summary
After induction of DNA double-strand breaks (DSB) two repair systems, the error-prone 'nonhomologous end joining' (NHEJ) and the more accurate 'homologous recombination repair' (HRR) can compete for the same individual DSB site. In the human keratinocyte cell line, HaCaT, we have tested the spatial co-localisation and the temporal sequence of events. We used UV-A (365 nm) as a damaging agent, which can be applied in clearly defined doses and can lead to rare DSBs via propagation of clustered single-strand breaks (SSBs). DNA fragmentation and repair was measured by the Comet assay and persisting DSBs were quantified by the micronucleus assay. Direct DSB detection was performed by immunohistochemical labelling of γ-H2AX, a phosphorylated histone that is assumed to form one foci per DSB. Intra- and inter-pathway interactions were quantified by co-localisation, FRET imaging and by co-immunoprecipitation (Co-IP) of XRCC4, DNA-PK and Ku70 as representatives of NHEJ, Rad51 and Rad52 for HRR and γ-H2AX, Mre11 and Rad50 as representatives of both pathways. In G2 cells, where both systems are available, the temporal sequence after irradiation is: (1) γ-H2AX (2) Mre11 (3) DNA-PK Rad51 (4) XRCC4. That is, the first two proteins involved in both pathways 'label' the damaged site and initiate repair, followed by the NHEJ, which is temporally overlapping with HRR activity. Taking all these observations together we suggest that a cell tries to repair DSBs with a combination of both HRR and NHEJ, if available.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/117/21/4935/DC1

Key words: DNA double strand break, Gamma-H2AX, Homologous recombination repair, Nonhomologous end joining, Rad51 foci, Ultraviolet radiation

Introduction
After induction of DNA double-strand breaks by various sources, e.g. radiation, the two principal repair pathways nonhomologous end joining (NHEJ) and homologous recombination repair (HRR) can repair these lesions. It is known that the two pathways can interact even on the same individual DSB site (Frank-Vaillant and Marcand, 2002; Richardson and Jasin, 2000; Saintigny et al., 2001). This crosstalk is not well understood in terms of the proteins involved, because these processes have either been studied in yeast (Frank-Vaillant and Marcand, 2002), or by the resulting DNA structures (Richardson and Jasin, 2000). Other studies have used a limited number of proteins (Saintigny et al., 2001). The recruitment of the initiating proteins to DSB sites has been studied using co-localisation of γ-H2AX, H2AX histones phosphorylated at SER 139 (Rogakou et al., 1998), together with Rad50/Rad51 and BRCA1 (Paull et al., 2000). But the closer interactions and the time course of interaction during repair thereafter has not been studied. The interaction pattern within the HRR or the NHEJ pathway is known in some detail (intra-pathway interaction) (Hoeijmakers, 2001; Valerie and Povirk, 2003), but knowledge of the inter-pathway interaction at the level of different repair proteins involved is poorly understood. It is known that γ-H2AX serves as a marker for DSBs and recently it has been published that each H2AX focus represents an individual DSB (Rothkamm and Lobrich, 2003). These foci contain several thousands of phosphorylated histones and are supposed to label the chromatin for subsequent repair.

The Rad50/Mre11/NBS complex serves as an initial factor and has been reported to participate both in the NHEJ and in the HRR pathway (Jackson, 2002). In the NHEJ, the complex is assumed to modify the ends of the DSB site by its endo- and exo-nuclease activity (Trujillo et al., 1998). In HRR, the complex acts as an exonuclease to produce single-strand overhangs (Jackson, 2002). The central proteins of the NHEJ pathway are the Ku70/80-heterodimer and DNA-PC (Allen et al., 2002), which rejoin the broken ends. Finally the Ku protein recruits the XRCC4, a cofactor of Ligase IV, which is a phosphorylation target of DNA-PK (Leber et al., 1998) that, in turn, enhances the recruitment process to close the strand break.
HRR is also initiated by the NBS1/Mre11/Rad50 complex that incises the two strands and produces 3’ overhangs that are bound by Rad52. The single-stranded DNA is bound by multiple Rad51 molecules (Haaf et al., 1995; Raderschall et al., 2002). Strand invasion and recombination are enhanced by Rad51, its paralogues and Rad54 (Hoeijmakers, 2001; Valerie and Povirk, 2003) and finally ligated by ligase I.

Presently it is assumed that in mammalian cells the NHEJ system is the predominant one, whereas the HRR is active in late S and G2 only. While this relationship has been recently studied in hamster cells (Rothkamm et al., 2003) the data for human cell systems are more controversial (Valerie and Povirk, 2003). Also, it is not defined to which degree the two repair pathways contribute to the overall DSB repair and to DSB repair in specific cell-cycle phases.

For a more-detailed insight into DSB repair and HNEJ/HRR crosstalk, knowledge on network-like interactions in spatial and temporal terms is required. We have measured the interaction in the repair-proficient human cell line, HaCaT, with eight proteins from the two pathways. The interaction matrix of several pairs of repair protein allows us to propose that the co-activation of both DSB repair pathways on the same DSB is a general response of the cell in the S or G2 phases, if both systems are available.

Materials and Methods

Cell culture, cell synchronisation and irradiation

HaCaT cells (Boukamp et al., 1988) were cultured in RPMI including 10% FCS using standard conditions. For G1 synchronisation confluent cultures were incubated for 5 days in isoleucin depleted Eagles F1 medium (Biochrom, Germany) including 5% dialysed FCS. For the last 24 hours the cells were additionally incubated with l-mimosine (150 μM final concentration). S phase synchronisation was achieved with a double thymidine block following standard procedures (16 hours release, 16 hours with a double thymidine block following standard procedures (16 hours block with 1 mM final concentration, 16 hours release, 16 hours with a double thymidine block following standard procedures (16 hours block with 1 mM final concentration). S phase synchronisation was achieved with a double thymidine block following standard procedures (16 hours block with 1 mM final concentration, 16 hours release, 16 hours block) and G2 cells were enriched by a 9 hour release from the thymidin block. Synchronised cells were controlled by flow cytometry (Coulter Eclipse) following DNA staining with Propidium iodide. For irradiation we used a 300 W Ultravitalux (Osram, Germany) light bulb. The peak at 365 nm was filtered with a combination of two colour glass filters UG1 und KG1 (both obtained from Schott, Germany). Thorous definition of the wavelength is mandatory because there is a strong dependence of light induced DNA damages on wavelength (Mohanly et al., 2002; de Wit and Greulich, 1995). The resulting fluence rate of 535 W/m2 was measured with a SolarScope UV Radiometer (SolarTech, UK) and it was assured that no irradiation below 340 nm passes through the filters. The cells were irradiated in pre-warmed, sterile PBS. Temperature during irradiation was controlled by two air-flow venting systems, resulting in a temperature increase from 34.2±1.2°C to 38.1±0.9°C during 40 minutes of irradiation. For post incubation after irradiation the slides have been transferred into fresh culture medium and were incubated as described above.

Comet assay

Comet assay was performed according to the alkaline version described by Singh and Tice (Bauer et al., 1998; Singh et al., 1988; Tice et al., 2000). In short, the incubation times were as follows: 1 hour lysis at 4°C, 1 hour unwinding at 4°C and 30 minutes electrophoresis at 1 V/cm also at 4°C. The neutral Comet assay was performed according to the procedure described elsewhere (Olive and Banath, 1993), with an additional proteinase K digest for 4 hours at 37°C. Also, electrophoresis was performed at 4°C in 1× TBE at 1 V/cm for 30 minutes. Image acquisition and analysis was performed with the KOMET 4.0 system (Kineticimaging, UK). 4×2 slides with 60 Comets each of individual preparations were scored per sample point.

Micronucleus assay

After irradiation, cells grown on cover slips were incubated for 24 hours in the presence of cytochalasin B to block cell, but not nuclear, division at a final concentration of 2 μg/ml in RPMI medium to identify binucleated cells. After fixation, DNA was stained with Hoechst dye and the cytoskeleton (to identify the cytoplasm) was labelled with phalloidin-rhodamin diluted 1:1000 in PBS for 30 minutes. Specimens were analysed by combined fluorescence and phase contrast microscopy. 4×500 binucleated cells were analysed per sample point of two individual preparations.

Immunohistochemistry

Cells were grown on microscopic glass slides and washed once in PBS. Fixation was done with 3.7% formaldehyde and permeabilization with 0.7% Triton X-100 using standard conditions. After blocking with 3% BSA in PBS for 1 hour, primary antibodies (rabbit anti-Rad51: H-92, goat anti-Rad51-C-20, both from Santa Cruz Biotech, USA; rabbit anti-γ-H2AX, Travigen, USA; mouse anti-γ-H2AX, Upstate, USA; mouse anti-DNA-PKcs, Kamiya, USA; rabbit anti-Rad50 H-300, goat anti-Mre11 C-16, rabbit anti-Rad52; goat anti-XRCC4, all from Santa Cruz Biotech, USA; mouse anti-Ku70, Sigma-Aldrich, Germany and mouse anti-Mre11, R&D Systems, USA) were added, at a dilution of 1:200 in PBS containing 2% BSA and incubated for 1 hour at room temperature. For single staining secondary antibodies [anti-rabbit FITC, anti-mouse-FITC or anti-goat-FITC (all Sigma Aldrich), diluted 1:200 in PBS containing 2% BSA] were used for the detection. For two colour immunohistochemistry donkey anti-goat, donkey anti-rabbit and donkey anti-mouse conjugated with either Alexa488 or Alexa594 (all from Molecular Probes) were used at a dilution of 1:400 and were applied as described above.

Microscopy and imaging

Microscopic imaging was done using an Axiostop (Zeiss, Jena) equipped with a cooled CCD camera (Photometrix, Quantix) and a HBO50 illumination. High numerical objectives were used together with immersion oil: 40× NA1.3; 63× NA1.25; 100× NA1.3 (Planneofluar series, Zeiss, Germany). The camera was controlled by the Qips software package (Vysis, Germany) and by Openlab (Improvision, UK). For fluorescence excitation/emission, high-quality band-pass filters (Chroma, USA) were used. Images were captured as 12 bit black and white images and stored in TIF format. Contrast and brightness were adjusted using Adobe Photoshop before images were merged as pseudo-colour. Confocal imaging was done using a LSM 510 (Zeiss, Jena) equipped with an Ar ion and an HeNe laser, controlled by the LSM 3.0 software (Zeiss, Germany). Single images were scanned 4× per pixel and a median filter was applied during acquisition to reduce noise and then exported as TIF images for each channel individually, and processed in Photoshop as described above. FRET imaging was done using the LSM 510 by exciting the Alexa488 (donor) dyes at 458 nm to avoid direct excitation of the acceptor dye (Alexa594). Emission of the acceptor was detected by a LP 580 nm filter. For representation the images were binarised to black and white and exported in single TIF files.

Western blotting

Western blotting was performed after total protein isolation with RIPA...
buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.25% SDS, 100 μg/ml PMSF, 1 tablet of protease inhibitors (Complete Mini, Roche) following standard procedures. 50 μg of total protein were separated on a 10% PAA gel and transferred to a nirocellulose membrane. The same primary antibodies as mentioned above were used at a dilution of 1:1000 in PBS including 4% BSA and 0.05% Tween 20. Secondary antibodies (HRP conjugated) were used together with the ECL Plus chemoluminiscnt detection kit (Amersham Pharmacia).

**Results**

To quantify the UV-A-induced DSBs, DNA fragmentation was monitored and the repair kinetic of SSBs and DSBs was monitored on the level of DNA fragmentation. After the induction of DSBs has been established, the persistence of genomic DNA damages has been measured by the micronucleus assay.

DNA single-strand breaks are induced 3.5-fold and repaired more than six times faster than double-strand breaks

Induction and repair of DNA single- and double-strand breaks as measured by the Comet assay (Fig. 1) together with sample micrographs of typical Comets (Fig. 1D-E). DNA single-strand breaks together with alkali-labile sites, such as abasic sites, are induced in a dose-dependent (i.e. fluence-dependent) manner as measured with the alkaline version of the Comet assay. The DNA damages increase linearly with exposure time and with fluence. Strictly this increase should follow an exponential function, such as described in Eqn 2 (below). Because obviously DNA damage is far from saturation, a linear approximation is adequate. After irradiation, the DNA damages recover following an exponential decay (Fig. 2B) (in particular, see inset Fig. 2B).

\[ f = a \cdot \exp \left(-\frac{t}{\tau}\right) \]

The time constant \( \tau \) for the single-strand break repair can be calculated as \( \tau \approx 110 \) minutes. From the time constant the time period of the SSBs in the alkaline Comet assay can be calculated to \( T_G = 75 \) minutes.

With the neutral Comet assay, double-strand breaks are detected exclusively. This version of the Comet assay omits...
the DNA denaturation step and, therefore, only fragments generated by DSBs can migrate in the electric field. As with the induction of single-strand breaks, the induction of DSBs is linearly dependent on the fluence. Repair of the DSBs is found to be slower than repair of the SSBs. The residual level of DNA damage after 5 hours of repair is still significantly higher than the control level (one-sided Anova, Dunnett’s method, \(P<0.05\)). For the DSBs it takes 24 hours post-irradiation until the level of damaged DNA is reduced to the control level. The initial repair of DSBs follows Eqn 1 with \(\tau=670\) minutes and a half-life period of approximately 460 minutes (Fig. 1B). Although single-strand breaks highly exceed double-strand breaks, the latter are clearly detectable.

The linear dependence of DNA damage induction on the fluence (or exposure time) reveals information about the repair process. Even in a region far from saturation, a decrease in the detected SSBs is expected, because DNA repair should counteract damage induction. This discrepancy, at a first glance, can be explained by the fact that the Comet assay does not only detect radiation-induced DNA damages, but also the nicks and gaps that are generated during base excision repair (BER) and nucleotide excision repair (NER) (Asaeda et al., 1998; Collins and Squires, 1986; de With and Greulich, 1995; Kleinau et al., 1997; Olive and Banath, 1993). The repair time measured in our experiments is slow compared with other reports in the literature (Hamilton et al., 2001; Terris et al., 2002), where half-life times of a few minutes to 30 minutes (for SSBs and base damages) have been reported. A possible reason for this is the high UV-A fluences of 960 kJ/m² used for the repair study. This high fluence induces a large number of base modifications (mainly 8-oxo-G) and SSBs. The repair of these damages involves NER and BER, two systems that induce further nicks, which are not discriminated by the Comet assay from initial damage. Therefore, it seems reasonable that a prolonged repair time is detected because the removal of SSBs is overlaid by repair processes that induce further nicks.

So far the dose relationship of DNA damages has been described in time constants. The following data will be described in terms of fluence constants, which are related to the time constants via the fluence rate.

Induction of micronuclei

The induction of micronuclei is accepted as a further indication for DNA double-strand and/or chromatin breaks (Kasahara et al., 1992; Stopper et al., 1994). Micronuclei (MN) are formed during mitosis, when chromatin fragments are not distributed into daughter nuclei. We examined induced micronuclei as a function of irradiation time and fluence (Fig. 2A). The inset of Fig. 2A shows a sample micrograph with a micronucleus (arrow). The MN frequency in control cells is 2.7±0.4% and increases significantly to 5% after a fluence of 320 kJ/m². After 30 minutes irradiation (corresponding to a fluence of 960 kJ/m²) the MN frequency reaches the saturation value of 10.6±0.5%. The MN frequency does not increase further but remains on the same level after 40 minutes of irradiation. An exponential increase to a maximum can be described according to the following equation (Eqn 2)

\[
f = M - M_0 \cdot \exp \left( -\frac{t}{\tau} \right),
\]

where \(M\) is the maximum value, with \(M=12.7\). The fluence constant is calculated to \(F=784.8\) kJ/m² or can also be expressed as time constant \(\tau\), because the fluence is directly proportional to the time.

The obtained values confirm previous data (Phillipson et al., 2002), where comparable values for MN induced by UV-A have been reported and where it was demonstrated that these damages persist up to 21 days post exposure. The flattening of the curve at fluences above 1000 kJ/m² can be explained with a reduced viability. To verify the induction of DSBs, the formation of γ-H2AX foci were monitored by immunofluorescence.

Immunohistochemical detection of DSB sites

The number of cells with γ-H2AX signal is plotted against the irradiation time (Fig. 2B). Sample micrographs of the detected foci in control cells and after 30 minutes of irradiation are shown in the inset of Fig. 2B. The number of cells with γ-H2AX foci increases from 11.1±1.2% (controls) to 43.0±1.7% after only 5 minutes of UV-A exposure (corresponding to 180 kJ/m²). The number of cells with γ-H2AX signal reaches 85.2±9.3% after 10 minutes of irradiation. Further exposure does not significantly increase the number of cells with γ-H2AX signal (Phillipson et al., 2002). According to Eqn 2 the induction yields \(M=93.5\) and \(F=223\) kJ/m². In terms of exposure time, \(\tau\) is found to be 6.2 minutes. The involvement of NHEJ in UV-A-induced DNA double-strand-break repair has recently also be demonstrated (Fell et al., 2002).

NHEJ activity after UV-A irradiation

NHEJ was monitored by the formation of DNA-PKcs foci. The time-dependent formation of the foci was quantified as cells that exhibit foci after irradiation. Values for the induction, together with two sample micrographs showing non-irradiated cells (left) and after 20 minutes UV-A (right), have been plotted (Fig. 2C). In non-irradiated cells almost no foci are found 1.6±0.4%, but a diffuse labelling instead. After 5 minutes of exposure, 23.4±5.3% of the cells show foci. The number increases further to 64.2±15.3% after 10 minutes and reaches the maximum after 20 minutes of exposure at 84.6±11.9%. Further irradiation does not increase the number of cells with foci. According to Eqn 2, the time constant \(\tau\) for the induction of DNA-PKcs foci is 9.1 minutes, which equals a fluence constant of \(F=290\) kJ/m², with \(M=85.6\).

Activation of homologous recombination repair after UV-A exposure

To follow the repair of DSBs, the HRR activation was studied by the visualisation of Rad51 foci. To quantify this, the number of cells showing Rad51 foci is quantified in unsynchronised cells. The percentage of cells with Rad51 foci, together with two example micrographs showing typical cells before and after the exposure to UV-A, was plotted (Fig. 2D). Rad51 foci formation is thought to represent HRR (Haaf et al., 1995). The initial level of Rad51 foci before irradiation is 2.7±1.9%, increases after 10 minutes to 25.7±5.5% and reaches saturation after 30 minutes at 42.2±2.9% of the cells showing Rad51 foci. The curve can again be described by Eqn 2 with \(M=42.7\), and \(F=360\) kJ/m² (e.g. \(\tau=11.25\)). For a direct comparison of the
Cell-cycle-dependent activation of repair pathways

The saturation limit of approximately 40% of the cells that show Rad51 foci after UV-A exposure correlates with the potential activation of the HRR pathway during the cell cycle. It has been reported that HRR is especially active in late S and G2 cells, where the homologous sequences have been already replicated (Rothkamm et al., 2003; Hoeijmakers, 2001). To test this hypothesis we exposed cell-cycle-synchronised cultures to a single fluence of 960 kJ/m², and analysed the number of cells with Rad51 and γ-H2AX foci. We found that in G1 only 7.2±4.1% of the cells show Rad51 DNA-PKcs foci, whereas 86.3±3.8% of the cells were Rad51-positive in cultures after 9 hours following a thymidine block. By contrast, the number of cells with γ-H2AX and DNA-PKcs foci is found to be independent of the cell-cycle position, and foci were found in 90.1±5.8% and 80.7±4.2% of the cells, respectively, in all examined cell-cycle phases.

The values for the frequency of Rad51, DNA-PKcs and γ-H2AX foci in unsynchronised G1- and G2-enriched cultures are shown (Fig. 3A). Sample images (Fig. 3B-D) and the cell-cycle distribution of the cultures after DNA staining and flow cytometry analysis (Fig. 3E).

These results, together with the finding that the number of Rad51-positive cells is dependent on the cell cycle rather than on the UV-A fluence, explain the saturation at 40% of cells with foci in unsynchronised cultures. By contrast, the number...
of foci per cell (data not shown) is correlated to the fluence and, therefore, to the number of induced breaks, as recently shown for γ-H2AX (Rothkamm and Lobrich, 2003).

Protein interactions confirm active DSB repair complexes
To verify whether DSB is activated, and Rad51, DNAPKcs and γ-H2AX foci represent HRR and NHEJ, co-localisation of the foci together with a selection of other repair proteins of the two pathways were studied after a single exposure to 960 kJ/m² (30 minutes) without post incubation. Spatial neighbourhood was detected by the co-localisation of pairs of two proteins by laser scanning microscopy. Additionally, close spatial co-localisation was detected by the FRET technique, which detects whether two chromophores (proteins) are in a range of approximately 10 nm or closer (Periasamy, 2001; Sekar and Periasamy, 2003; Hink et al., 2002). We present the co-localisation results of Rad51 (green) and Rad52 (red) (Fig. 4A). Not only an optical co-localisation (arrow) but also FRET signals were detected. This indicates a close neighbouring of the two factors, or even molecular interaction, during HRR. Also the Mre11/Rad50 can be found after UV-A exposure (Fig. 4B) with a positive FRET effect. In addition to the optical detection of spatial co-localisation the interaction of Rad51/Rad52 and Mre11/Rad50 can be found by Co-IP (Fig. 4C). Taken together these results show that DSB repair is activated in response to UV-A irradiation within the 30 minutes of UV-A exposure and that Rad51 foci represent HRR repair sites and the intra-pathway interactions are confirmed.

Spatial crosstalk of the NHEJ and HRR repair pathways
To study the interaction of the two DSB repair pathways (inter-pathway interaction) G2-synchronised cell cultures were used, in which both systems are active. After a single dose of 960 kJ/m² the interaction was studied on the level of optical co-localisation (in the 200 nm range), by the FRET effect (in the 10 nm range) and direct protein-protein interaction by Co-IP. Sample images of optical co-localised data (Fig. 4), where yellow colour (arrows) indicate optical co-localisation and the white lines indicate the border of the nucleus. The co-localisation of γ-H2AX together with Rad52 (Fig. 4D), and the co-localisation of Rad51 together with Rad50 (Fig. 4E). The co-localisation of γ-H2AX together with XRCC4 (Fig. 4F) indicates that the two proteins show simultaneous retention to a DSB site, although γ-H2AX is a very early participant and XRCC4 is a late participant in the repair pathway.

**Fig. 3.** Cell-cycle dependence of γ-H2AX, DNA-PKcs and Rad51 foci after irradiation with 960 kJ/m². (A) Frequencies of γ-H2AX, DNA-PKcs and Rad51 foci in cell-cycle-synchronised cells. The Rad51 foci are mainly formed in G2 phase, whereas there are only a small number of cells with Rad51 foci in G1. By contrast, the DNA-PKcs and γ-H2AX foci are not related to the cell-cycle stage. (B-D) Sample micrographs of Rad51 foci, DNA-PKcs foci and H2AX foci in synchronised cells. The Rad51 foci are prevalent in S and G2 phases, whereas the γ-H2AX (right) and DNA-PKcs (middle) are not. Each experiment has been independently repeated and 2x200 cells were scored. (E) Flow-cytometric analysis of cell-cycle position after propidium iodide staining of total DNA.
We can see the presence of both pathways at the same DSB site by the co-localisation of Rad51 together with XRCC4 (Fig. 4G). Co-localisation data of Ku70 are difficult to interpret, because we have not found a focus formation of this protein, but a more-or-less homogeneous distribution. We also see Ku70 together with XRCC4 (Fig. 4H). γ-H2AX together with Rad51, indicates the known sequestering of Rad51 to the DSB site (Fig. 4I). Activity of both pathways on single DSB sites is also visible (Fig. 4J), shown as the co-localisation between Rad51 and DNA-PKcs.

Interpathway co-localisation of Rad50/Rad52, Rad51/XRCC4, Ku70/Rad51 and DNA-PKcs/Rad51 showed an incomplete optical co-localisation with approximately 20% of the foci co-localised but no FRET signal. Taken together these data suggest that in some, but not all, DSB foci both repair systems are active, but do not have a direct interaction. Table 1 summarises the results from the crosstalk studies. Each protein pair has been studied independently. A minimum of 25 cells per protein pair have been analysed in replicated experiments. The interactions were classified as strong (+), weak (0) or not detectable (−).

**Temporal sequence: focus and complex formation**
The temporal choreography of the two repair pathways was studied using the focus formation of Rad51, Mre11, γ-H2AX,
Table 1: Interaction map of DSB repair proteins detected by optical co-localisation, FRET and IP

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Proteins of the two pathways have been tested pairwise for optical co-localisation (opt. coloc.), FRET effect and co-immunoprecipitation (Co-IP). Results were scored as positive detectable (+), only weak detectable (0) or not detectable (–). All co-localisation results with the Ku70 protein are given in brackets, because this protein is distributed ubiquitously and is known to participate in several processes other than DSB repair. n.t., not tested as antibody pairs are not compatible with each other.

DNA-PKcs and XRCC4. The foci were counted in 2×50 cells 1–24 hours after exposure to 980 kJ/m² UV-A. We plotted the frequency of foci formation for each of the four examined repair proteins (Fig. 5A). The persistence of the foci allows an interpretation of the duration of the repair process. The γ-H2AX foci provide fastest signal observed, even after 5 minutes of UV-A exposure a significant increase becomes visible (compare with Fig. 2). After 30 minutes of exposure, foci of Rad51, Mre11, DNA-PKcs and XRCC4 are visible as well. Nevertheless, the number of Rad51 foci continues to increase even after the exposure has ended. The Mre11 and the γ-H2AX foci disappear, or are reduced significantly, within the first 5 hours. Also the number of cells with DNA-PKcs foci is reduced, but at a slower rate. The number of cells with γ-H2AX foci reaches the control level after 5 hours. The Rad51, DNA-PKcs and XRCC4 foci are persistent longer. The control level is reached after 24 hours, although after 5 hours a significant decrease is already observed.

The formation of protein-protein complexes after UV-A exposure was monitored the same way (Fig. 5B). The rate of protein-protein complex formation depends on the cellular environment (De Gruijl et al., 2001; Griffiths et al., 1998). The fastest response found after exposure to UV-A was the phosphorylation of H2AX. Even after small doses, foci become visible within 5 minutes. It has been demonstrated that the γ-H2AX foci label all DSBs (Rothkamm and Lobrich, 2003). Because the H2AX phosphorylation is a modification of existing histones, it is reasonable to assume that there is no saturation limit in the number of foci per cell. By contrast, the subsequently activated repair systems are limited in number by the available proteins. From our results we can conclude that the number of HRR repair sites is exceeded by the number of NHEJ sites, even in G2 cells. Therefore, it can also be concluded that the NHEJ system is active throughout the entire cell cycle in human cells and is not decreased in its activity by the HRR in G2. While the co-localisation data show a recruitment of all examined proteins to DSB sites (i.e. a co-

Discussion

In light of the discussion about whether UV-A induces DNA double-strand breaks, we have shown here that DSBs are indeed induced and repaired, both at the level of DNA fragmentation and by the activation of DSB repair pathways. It is assumed that these DSBs are generated by clustered SSBs that occur in close proximity in the nucleus (Jenner et al., 2001), at sites where the endogenous photosensitisers are localised. In contrast to cell-free systems, where no DSBs are found after UV-A exposure (Song et al., 2002), the DSB induction is strictly dependent on the cellular environment (De Gruijl et al., 2001; Griffiths et al., 1998).

The fastest response found after exposure to UV-A was the phosphorylation of H2AX. Even after small doses, foci become visible within 5 minutes. It has been demonstrated that the γ-H2AX foci label all DSBs (Rothkamm and Lobrich, 2003). Because the H2AX phosphorylation is just a modification of existing histones, it is reasonable to assume that there is no saturation limit in the number of foci per cell. By contrast, the subsequently activated repair systems are limited in number by the available proteins. From our results we can conclude that the number of HRR repair sites is exceeded by the number of NHEJ sites, even in G2 cells. Therefore, it can also be concluded that the NHEJ system is active throughout the entire cell cycle in human cells and is not decreased in its activity by the HRR in G2. While the co-localisation data show a recruitment of all examined proteins to DSB sites (i.e. a co-
Interaction of combined DSB repair localisation with γ-H2AX foci) the interaction between the single repair factors is different (Fig. 6).

From the temporal sequence of the co-localisation of repair proteins, we can confirm prior data at the protein level (Richardson and Jasin, 2000) (see Fig. S1 in supplementary material). The observation that single factors are recruited to DSB sites leads to the following idea: a DSB is initially marked by the cell with γ-H2AX, then the Mre11/Rad50/NBS complex initiates DSB repair with an activation of NHEJ in G1 cells, followed by the recruitment of DNA-PKcs, and then followed by XRCC4, which remains at the DSB site for several hours. In late S or G2, when both systems are available, the initial sequence of γ-H2AX focus formation to Mre11/Rad50 complex recruitment is followed by a concerted action of both DSB repair pathways. In our experiments, the Rad51/Rad52 complex is formed after DNA-PKcs recruitment, but concomitant with the XRCC4 foci. Therefore the temporal sequence for DSB repair in G2 can be established as follows: (1) H2AX phosphorylation; (2) Mre11/Rad50 complex formation (probably including NBS1 as well); (3) DNA-PKcs recruitment; and (4) association of Rad51 followed by the Rad51/Rad52 complex formation concomitant with ligation by XRCC4.

From the temporal sequence it can also be concluded that the γ-H2AX signal is maintained by the cell until all repair factors are loaded onto the DSB site, whereupon the γ-H2AX label is removed from the DSB site, while the other repair factors stay at the site. In contrast to recent findings, where persisting γ-H2AX foci where found (Rothkamm and Lobrich, 2003; MacPhail et al., 2003), we did not observe such foci, but...
observed a complete repair with an early dephosphorylation of γ-H2AX. These contradictory results are possibly due to the different radiation sources used in these studies and, therefore, different DSB structures are formed, which are known to be cell-type specific (MacPhail et al., 2003). Similarly, the Rad50/Mre11 complex is maintained only during the initiation of the repair, which seems reasonable because the complex is supposed to participate in end processing and stabilisation of the repair, which is not a gaussian distribution.

The absolute numbers of DSB (γ-H2AX), Rad51 and Mre11 foci give the impression that the HRR is nearly always localised with the NHEJ. It is unclear whether the number of proteins is limited to form these foci or whether the DNA structure of the DSB excludes the HRR from some DSB sites.

Recent studies on the effects of BRCA mutations have given further hints to a combined activity of HRR and NHEJ. BRCA1 mutations, which affect HRR activation, do not lead to a reduction in repair capacity, but to a reduced quality of the repair. For example, NHEJ can almost completely repair DSBs alone, but HRR may participate as an additional proofreading step if available. Similar findings have also been reported for the combination of mismatch repair and NHEJ.

This work was supported by the German Research Ministry (BMBF), Grant 13N7506 and 13N8028. The HaCaT cells were kindly provided by P. Boukamp and Fusseneg, German Cancer Research Center (DKFZ) Heidelberg. We would also like to thank R. Greinert, Physical cell biology unit, Dermatology Hospital Buxtehude and J. Thomale, IZB University Essen for discussion. We thank Hellen Morrison for critical reading and H. Dittmar for technical assistance.

References


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Temporal sequence of factor recruitment to dsb sites. First the H2AX is phosphorylated and marks a dsb site. The next factors recruited are the Mre11/Rad50 complexes that remain for a few hours at the dsb site. Then the cell has the choice of HRR or NHEJ factors. If available in late S or G2, the cell recruits both systems. In the tested cell culture the Rad51/Rad52 complex is faster removed than the XRCC4. The lines indicate the duration of the individual proteins at the dsb site as foci.