Activation of multiple DNA repair pathways by sub-nuclear damage induction methods

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Summary
Live cell studies of DNA repair mechanisms are greatly enhanced by new developments in real-time visualization of repair factors in living cells. Combined with recent advances in local sub-nuclear DNA damage induction procedures these methods have yielded detailed information on the dynamics of damage recognition and repair. Here we analyze and discuss the various types of DNA damage induced in cells by three different local damage induction methods: pulsed 800 nm laser irradiation, Hoechst 33342 treatment combined with 405 nm laser irradiation and UV-C (266 nm) laser irradiation. A wide variety of damage was detected with the first two methods, including pyrimidine dimers and single- and double-strand breaks. However, many aspects of the cellular response to presensitization by Hoechst 33342 and subsequent 405 nm irradiation were aberrant from those to every other DNA damaging method described here or in the literature. Whereas, application of low-dose 266 nm laser irradiation induced only UV-specific DNA photolesions allowing the study of the UV-C-induced DNA damage response in a user-defined area in cultured cells.

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Key words: Pyrimidine dimers, Local DNA damage induction, Double-strand breaks, Living cells, DNA repair kinetics

Introduction
The mammalian genome is protected against the continuous stress of both exogenous and endogenous DNA damaging agents by a number of DNA damage response mechanisms, including different DNA repair pathways. Unresolved DNA lesions may introduce mutations, which can lead to cancer (Mitchell et al., 2003). In addition, un repaired damages may result in disturbed transcription and replication, which eventually causes cell death contributing to aging. The severe clinical consequences associated with hereditary disorders that harbor defects in DNA repair systems underscore the importance of efficient DNA repair (Bootsma and Hoeijmakers, 1994; Hoeijmakers, 2001).

Genetic and biochemical analysis of repair processes have culminated in detailed mechanistic insight into the distinct DNA repair processes. To study the interaction of the different DNA repair processes with each other and with other cellular processes such as transcription and replication, spatiotemporal analysis of different DNA repair systems in intact living cells is required and has been used extensively with the aid of GFP-tagged repair factors (Essers et al., 2002b; Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003). Recently, DNA repair research has been boosted substantially by the development of several methods to locally inflict DNA damage in cultured living cells, enabling the direct visualization of GFP-tagged repair factors accumulating at the sub-nuclear region where the damage is caused. These methods range from irradiating partially shielded cells (Kannoche et al., 2001; Katsumi et al., 2001; Mone et al., 2001; Nelms et al., 1998; Volker et al., 2001) to focusing laser beams inside living cell nuclei (Essers et al., 2006; Lukas et al., 2005).

The kinetics of nucleotide excision repair (NER) have been determined previously by irradiation of cultured cells through a polycarbonate filter with UV-C light, either prior to or after mounting on the microscope stage, and subsequently measuring the accumulation of repair proteins (Hoogstraten et al., 2002; Mone et al., 2004; Politi et al., 2005; Zotter et al., 2006). In addition, alternative methods have been developed where DNA damage is introduced by focused laser beams, at user-defined regions within the nucleus (Cremer et al., 1980; Lan et al., 2004; Meldrum et al., 2003; Walter et al., 2003). This approach allows great flexibility not only with respect to position, but also size and shape of the local damage induced in individual cells.

Tuned localized intense laser irradiation with 365 nm light causes different types of DNA lesions ranging from oxidized base damage, single-strand breaks (SSBs) and up to double-strand breaks (DSBs) (Lan et al., 2004). Another powerful method uses pulsed near infrared laser (multiphoton) technology. In this case two or three lower energy photons are absorbed simultaneously resulting in twice or three times the energy deposition. Meldrum et al. (Meldrum et al., 2003) applied this procedure using a pulsed 750 nm laser (with an effective wavelength of 250 nm) and showed that this method is able to create UV-like DNA lesions in living cells as shown by in situ immunostaining using antibodies against cyclobutane...
pyrimidine dimers (CPDs). Recently, it has been shown that with a pulsed near infrared laser DSBs are created as well (Mari et al., 2006), indicating the broad spectrum of DNA lesions induced with this procedure.

More indirect methods rely on local relatively low energy UV-A irradiation. These methods require cells to be pretreated with halogenated thymidine analogs such as BrdU or IdU, which are incorporated into DNA, and induce SSBs and DSBs when the cells are exposed to UV-A (Lukas et al., 2003; Tashiro et al., 2000). A variant of this method employs DNA-binding dyes such as Hoechst either in combination with (Rogakou et al., 1999; Walter et al., 2003), or without thymidine analogs (Bradshaw et al., 2005). Although a number of these in situ local damage-inducing systems have been applied to study DNA damage response mechanisms the spectrum of DNA lesion induced by these procedures has not been analyzed in great detail.

We have systematically analyzed and compared three different procedures to locally inflict DNA damage in cultured cells. We show that pulsed 800 nm irradiation introduces a broad variety of DNA lesions at which proteins involved in different pathways accumulate. The combination of Hoechst 33342 incorporation and 405 nm irradiation induced a cellular response that differed strongly from the response to other damaging methods. In addition, we have developed a microscope setting using focused UV-C (266 nm) laser irradiation, which induces predominantly UV-C-specific photolesions such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP).

Results
Experimental setup

We have investigated local DNA damage induction in cultured living cells with confocal microscopy using lasers of different wavelengths: 800 nm, 405 nm and 266 nm. The types of damages created with these methods and the assembly of different repair proteins after local irradiation were first analyzed using immunocytological procedures directed against lesions (CPD, 6-4PP and TUNEL) or the consequences of lesions (accumulation of phosphorylated H2AX, phosphorylated DNA-PKcs, PARP-1) as well as protein-GFP fusions. The results of these studies are summarized in Tables 1 and 2. In addition, the kinetics of protein interaction with DNA damage complexes were analyzed in living cells expressing fluorescently tagged repair factors involved in both early and late steps of the reaction of both nucleotide excision repair (involving XPC and XPA proteins) and DSB repair (MDC1and Rad54 proteins). All cell lines expressing GFP- or YFP-tagged proteins have previously been characterized and published (see Materials and Methods section and references therein).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUNEL</th>
<th>γH2AX</th>
<th>γPKcs</th>
<th>MDC1</th>
<th>Rad54</th>
<th>Ku70</th>
<th>PARP-1</th>
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*DSB repair proteins that do not accumulate in foci but in a homogenous pattern; †UV-C irradiation without attenuation resulted in positive TUNEL staining and Ku-GFP accumulation; ‡at higher UV-C doses γH2AX accumulation can be found; §accumulation of DSB repair proteins on UV-C damage is dependent on ongoing replication.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CPD</th>
<th>6-4PP</th>
<th>XPC</th>
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<td>Pulsed 800 nm laser</td>
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Response of the NER machinery to pulsed 800 nm irradiation

To investigate the types of DNA damage created by pulsed near infrared (NIR) laser irradiation, cells were subjected to high intensity 800 nm laser pulses. To provide an internal control for the immunofluorescent detection of pyrimidine dimers, we irradiated XPC-GFP expressing cells with UV light before irradiation with a NIR laser. Pulsed 800 nm laser irradiation resulted in the formation of CPDs (Fig. 1A), as reported previously (Meldrum et al., 2003). In addition to CPDs, also 6-4PPs were formed (Fig. 1B; arrowheads). XPC-GFP (Politi et al., 2005) accumulated in areas irradiated with a UV lamp through a micro-porous filter as well as areas irradiated with a pulsed 800 nm laser (Fig. 1A,B). GFP-XPA (Rademakers et al., 2003) also accumulated with both methods, but there was a much stronger response to UV lamp irradiation than to pulsed 800 nm irradiation (Fig. 1C).

Response of the DSB repair machinery to pulsed 800 nm irradiation

To determine whether DSBs are induced by a pulsed 800 nm laser we stained locally irradiated nuclei of XPC-deficient fibroblasts (XP4PA) expressing XPC-GFP (Politi et al., 2005) with an antibody against phosphorylated DNA-PKcs (γPKcs). DNA-PKcs is the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), which is autophosphorylated in response to ionizing radiation (Chan et al., 2002). The presence of γPKcs suggested the formation of DSBs by a pulsed 800 nm laser (Fig. 2A). In addition, γH2AX (Fig. 2B) and Ku80-GFP (Mari et al., 2006) were also found at these sites, indicative of the presence of DSBs. Under similar conditions local UV-C irradiation through pores in a filter failed to induce DSBs as indicated by the absence of γPKcs positive signal (Table 1) and γH2AX staining (Fig. 2B).

Rad54 is implicated in multiple steps of DSB repair through homologous recombination (HR). Previous research has shown that in response to DSB induction by ionizing radiation HR proteins accumulate in nuclear foci (Essers et al., 2002b; Rouse and Jackson, 2002; van Veelen et al., 2005a; van Veelen et al., 2005b). Accordingly, Rad54-GFP accumulated in a focal pattern at the damaged area (Fig. 2C, right panel), similar to what has been described for multiple HR proteins after DNA damage.
induction (Bekker-Jensen et al., 2006). Whereas HR is thought to be predominantly active during the S and G2 phases of the cell-cycle, we found accumulation of Rad54-GFP in virtually all cells. Similarly, Rad51 was found to accumulate at locally damaged areas regardless of cell-cycle phase (Kim et al., 2005). These observations suggest that part of the HR machinery is loaded onto DSBs in G1. However, this recruitment might not reflect ongoing repair. Interestingly, the BRCT domain of MDC1 tagged with YFP [YFP-MDC1(BRCT)] also accumulated on pulsed 800 nm laser-induced damage, but with faster kinetics and in much bigger foci than Rad54 (Fig. 2D, right panel, versus Fig. 2C, right panel). These large foci are likely indicative of interaction between MDC1 and γH2AX (Bekker-Jensen et al., 2006).

To determine a dose of pulsed 800 nm radiation with which one specific repair pathway was induced and not another, we lowered the laser intensity. At slightly lower doses than used above, both GFP-XPA (NER) and Rad54-GFP (HR) remained undetectable in the irradiated areas (data not shown). This indicates that under the conditions used we did not observe preferential formation of one type of lesion over the other by changing the applied dose.

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Fig. 1. The NER response to local pulsed 800 nm laser irradiation. (A) XPC-GFP-expressing cells were irradiated through a filter with UV-C light (spots indicated by arrows) and subsequently treated with pulsed 800 nm laser pulses (lines indicated by arrowheads). Induction of CPDs is shown by staining with the CPD antibody (red, right panel) both on UV-C and pulsed 800 nm locally irradiated areas. In both areas XPC-GFP accumulated (green, left panel). (B) XPC-GFP-expressing cells were treated as in panel A and stained for the presence of 6-4PPs (red, right panel). Pulsed 800 nm irradiation is able to induce 6-4PP-formation as shown by the lines indicated by the arrowheads (right panel). The bar graph indicates fluorescence intensities of the nucleus (1), pulsed 800 nm induced local damage (2) and UV-C induced local damage (3). (C) GFP-XPA accumulates to a limited extent on pulsed 800 nm induced damaged areas (arrowhead) compared to UV-C irradiated areas (arrow). The bar graph indicates fluorescence intensities of the nucleus (1), pulsed 800 nm induced local damage (2) and UV-C induced local damage (3).

Fig. 2. The DSB repair response to local pulsed 800 nm laser irradiation. (A) XPC-GFP-expressing cells were treated with pulsed 800 nm irradiation and presence of DSBs is shown by immunohistochemical staining with a γPKcs antibody (lines in right panel indicated by arrowheads). The bright spots outside the damaged area in the right panel are nucleolar structures of unknown origin and it is unknown if they exist in a living cell as well. (B) XPC-GFP-expressing cells were treated as in panel A and stained for the presence of phosphorylated histone H2AX (γH2AX). Accumulation of γH2AX at areas irradiated by the pulsed 800 nm laser confirms the presence of DSBs (right panel, arrowheads). No accumulation of γH2AX is found on UV-C irradiated spots (arrows). Earlier it was shown that phosphorylation of H2AX takes place after UV-C irradiation (Marti et al., 2006; O’Driscoll et al., 2003) and we have found this as well in other experiments (data not shown). It is possible that in this case the specific immunohistochemical staining of γH2AX at UV-C damage was not strong enough to be detected over background signals. (C) Rad54-GFP expressing cells were irradiated in an area of approximately 5 μm² with pulsed 800 nm light and the redistribution of fluorescence was studied in time. The boxed area is two times enlarged in the left bottom of both panels. Rad54-GFP accumulates in small foci at the damaged area. (D) YFP-MDC1(BRCT) expressing cells were irradiated in a rectangular line through the nucleus and fluorescence redistribution was followed in time. YFP-MDC1(BRCT) accumulates in large foci at the damaged area (boxed area, left panel).
UV-C irradiated through a filter as an internal control for the procedure. XPC-GFP-expressing cells were prompted us to further analyze the types of DNA lesions not shown). This localization of the UV damage sensor XPC nm laser required more than tenfold higher laser intensity (data not shown). This localization of the UV damage sensor XPC detected 6-4PPs, and to a lesser extent CPDs, induced by UV-C (<300 nm), was targeted to 405 nm (UV-A)-irradiated areas. XPC-GFP accumulated on both areas irradiated through a filter with UV-C light (arrows) and irradiated with 405 nm in combination with Hoechst 33342 (arrowheads). (B) Treatment as in panel A, here cells were stained with an antibody that recognizes 6-4PPs (right panel). Surprisingly, no 6-4PP-staining can be detected on laser-irradiated areas (lines indicated by arrowheads), while the UV-C treated areas show a clear induction (arrows). The bar graph indicates fluorescence intensities of the nucleus (1), 405 nm combined with Hoechst 33342 treatment induced local damage (2) and UV-C induced local damage (3). (C) GFP-XPA accumulates at a low level on local damage induced by 405 nm laser irradiation in combination with Hoechst 33342 treatment (arrowhead) compared with local UV-C irradiated areas (arrow). The bar graph indicates fluorescence intensities of the nucleus (1), 405 nm combined with Hoechst 33342 treatment induced local damage (2) and UV-C induced local damage (3).

NER response to Hoechst 33342 + 405 nm damage induction
The DNA binding agents Hoechst 33258 and 33342 are known to induce DNA breaks when activated by UV-A irradiation (Lecoeur, 2002). Surprisingly, also the NER protein XPC-GFP, which detects 6-4PPs, and to a lesser extent CPDs, induced by UV-C (<300 nm), was targeted to 405 nm (UV-A)-irradiated lines in Hoechst 33342-containing cells (Fig. 3A). In the absence of Hoechst 33342, DNA damage induction with a 405 nm laser required more than tenfold higher laser intensity (data not shown). This localization of the UV damage sensor XPC prompted us to further analyze the types of DNA lesions introduced by this procedure. XPC-GFP-expressing cells were UV-C irradiated through a filter as an internal control for the pyrimidine dimer antibody staining before addition of Hoechst. After Hoechst 33342 treatment, a rectangular area of the nucleus of these cells was exposed to 405 nm irradiation. This resulted in abundant CPD formation (Fig. 3A) identical to pulsed 800 nm-induced damage (Fig. 1C). Remarkably, no 6-4PPs were found (Fig. 3B). Apparently this method specifically induced minor helix distorting lesions such as CPDs but not the more severely helix distorting 6-4PPs. Similar to its response to pulsed 800 nm irradiation, XPC-GFP responded very strongly to these damages (Fig. 3A,B) but GFP-XPA accumulation was much less intense on Hoechst + 405 nm-irradiated areas than on UV lamp-irradiated areas (Fig. 3C). This suggests that XPC responds to a wider variety of lesions than only those typically repaired by NER.

Response of the DSB repair machinery to Hoechst 33342 + 405 nm damage induction
In Hoechst 33342-sensitized CHO9 cells locally irradiated at 405 nm, YFP-MDC1(BRCT) as well as the non-homologous end-joining (NHEJ)-specific Ku80-GFP quickly accumulated in the irradiated areas in very high numbers (Fig. 4A,B). See also Fig. S1 in supplementary material for colocalization of XPC-mCherry and YFP-MDC1(BRCT), indicating that DSBs were present. The presence of phosphorylated H2AX confirmed the creation of DSBs (Table 1). DNA-PKcs is recruited to DNA damage by Ku proteins (Downs and Jackson, 2004) and damage-induced autophosphorylation of DNA-PKcs is regulated by MDC1 (Lou et al., 2004) so we expected to find γPKcs on local Hoechst 33342 + 405 nm damage. In contrast to its response to pulsed 800 nm irradiation, γPKcs did not localize to Hoechst 33342-induced DNA damage in any of the irradiated cells above background levels of the immunohistochemical staining (Fig. 4C). Apparently the types of lesions created with this method are not a good substrate for γPKcs. This indicates an activity of Ku70/Ku80 that is independent of DNA-PKcs as was previously described for its proposed function at telomeres (Hsu et al., 2000).

Furthermore YFP-MDC1(BRCT) accumulation did not show a focal pattern but rather was homogenously distributed within the damaged area (Fig. 4A, right panel). Similarly Rad54-GFP accumulated on damages induced by 405 nm in combination with Hoechst 33342, albeit in low numbers (bar graph Fig. 4D), but it did not appear in foci (Fig. 4D, right panel), not even after 40 minutes (data not shown). Together with the absence of γPKcs at irradiated areas this indicates that the combination of Hoechst 33342 sensitization and 405 nm light triggers a hitherto unknown response of DSB repair proteins.

NER and DSBs upon local UV-C irradiation
To induce local UV damage, we installed a pulsed 2 mW 266 nm laser on a confocal microscope adapted for UV-C transmission with all-quartz optics. Local UV irradiation through a micro-porous filter to inflict light-induced DNA damage is technically fairly easy, but includes a number of drawbacks that are overcome with the use of a laser. First, unless a set-up is used where irradiation takes place on the microscope stage (Mone et al., 2004), irradiation through a micro-porous filter is unsuitable for the study of accumulation rates. Even with the use of the on-the-microscope-stage set-up, early or quick assembly rates are hard to monitor because of the relatively long irradiation times required (>12 seconds).
Second, irradiation through a filter induces damage in all cells in the preparation simultaneously, making it very difficult to monitor protein accumulations in multiple cells in one experiment. Laser irradiation provides much more flexibility, allowing local damage infliction at specific locations in individual cells, e.g. specific sub-nuclear hetero- or euchromatic regions or even multiple irradiations in one cell or different doses in different cells in the same view, which is not possible with filter irradiation.

UV-C light is known to directly induce helix-distorting lesions such as CPDs 6-4PPs but not SSBs or DSBs (Perdiz et al., 2000; Rodrigo et al., 2000). However, at high UV-C intensity positive TUNEL staining was found next to the accumulation of the NER factor XPA (Fig. 5A, arrowhead). In addition, the DSB factor Ku80-GFP accumulated in the irradiated area (footnote Table 1). At ~12-fold lower irradiation intensity, only the NER factors accumulated in the damaged region, indicating that NER-specific lesions were created both at high and at low intensities (Fig. 5A,B). Dose-dependency studies showed that up to 6 seconds irradiation with 12-fold attenuation induces accumulation of GFP-XPA but not of Ku80-GFP and that without attenuation 1-second irradiation was sufficient to induce DSBs (Table S1 in supplementary material). In the remaining experiments, the UV-C dose used was 0.5 seconds with 12-fold attenuation. After local irradiation with this dose, GFP-PCNA-expressing cells (Essers et al., 2005) were still able to go through mitosis (Fig. S2, and Movie 1 in supplementary material), suggesting that under the conditions used we did not trigger apoptosis.

During S-phase, replication forks can stall when they encounter a UV-induced lesion. HR is suggested to be involved in resolving these stalled replication forks. Therefore we examined the response of Rad54 to UV-C laser irradiation at different stages of the cell-cycle in cells expressing both Rad54-GFP and mCherry-PCNA. In cells that showed a homogeneous mCherry-PCNA staining (G1 or G2 phases), no accumulation of Rad54-GFP at damaged areas was found (Fig. 5C). In cells with a focal mCherry-PCNA pattern, Rad54-GFP accumulated at irritated areas (Fig. 5D), suggesting that HR is only activated by UV-C laser irradiation during replication.

Accumulation kinetics with laser assisted DNA damaging methods
We have measured the kinetic behavior of four DNA damage repair proteins, XPC, XPA, MDC1 and Rad54, upon recruitment to the various local laser-damaged areas discussed above. To this end we monitored protein redistribution for up to 20 minutes after local damage induction with either pulsed 800 nm irradiation, 405 nm combined with Hoechst 33342 or 266 nm laser irradiation and compared fold increase of fluorescence in the damaged area over time for these three damaging methods (Fig. 6A-D).

XPC-GFP responded quickly to both the pulsed 800 nm and 266 nm irradiation methods but it accumulated slower at Hoechst 33342 + 405 nm induced damage sites (Fig. 6A). This unexpected behavior of XPC is most likely caused by an inhibitory effect of the presence of Hoechst on XPC mobility (our unpublished work). XPC appeared to very transiently and frequently bind to Hoechst-stained DNA thus limiting the speed of its accumulation in the damaged area.

GFP-XPA was not visibly retarded by Hoechst 33342 addition, but it accumulated to a much lesser extent than XPC-GFP in areas exposed to either pulsed 800 nm irradiation or Hoechst combined with 405 nm irradiation. Both GFP-XPA (Fig. 6B) and XPC-GFP (Fig. 6A) showed a stronger increase in fluorescence intensity with the 266 nm method than with the other two, indicating that a UV-C laser can induce a high concentration of lesions that are specifically repaired by NER without creating DSBs at the same time (Tables 1 and 2). Note that GFP-XPA took much longer to reach a plateau level in
response to 266 nm irradiation than XPC-GFP ($t_{1/2}$ values of ~140 and ~40 seconds, respectively). Two scenarios can explain this difference between XPA and XPC. (1) At individual repair sites XPC is released before repair is complete (Park and Choi, 2006; Riedl et al., 2003; You et al., 2003), whereas XPA remains bound for longer. A consequence of this difference in residence time is that XPC kinetics reach equilibrium between binding and dissociation earlier than XPA. (2) Alternatively, the association of XPA with locally damaged areas is delayed because it depends on the presence or enzymatic activity of an earlier factor (Mone et al., 2004; Politi et al., 2005).

MDC1 has been found to interact with proteins of both the NHEJ and HR pathways (Bekker-Jensen et al., 2005; Lou et al., 2004; Zhang et al., 2005) and is involved in early events in the DSB repair process, serving as an intermediary between the Mre11-Rad50-Nbs1 complex and chromatin (Lukas et al., 2004; Stucki et al., 2005). In agreement with its early association with damage sites, we found rapid accumulation of this protein at both pulsed 800 nm- and Hoechst + 405 nm-irradiated sites (Fig. 6C). Contrary to XPC, MDC1 accumulated faster in Hoechst-treated cells than in 800 nm-irradiated cells.

Interestingly, Rad54-GFP displayed a delayed response to pulsed 800 nm damage, only visibly accumulating after 10 minutes (Fig. 6D). This is consistent with its proposed function later in the DSB repair process and suggests that the kinetics of HR are slower than that of NER of UV lesions (Essers et al., 2002a; Houtsmuller et al., 1999; Mone et al., 2004). It has been shown previously that Rad51, another HR factor, appears at local damage in a comparable timeframe (30 minutes) after DSB induction by a 532 nm laser and that it is still found at these sites after at least 24 hours (Kim et al., 2005). Rad54-GFP accumulated to a lesser extent but with faster kinetics in areas irradiated at 405 nm in Hoechst-treated cells than in areas irradiated by pulsed 800 nm. The combination of the homogeneous pattern of accumulation of both Rad54 and MDC1 and the absence of detectable γH2AX accumulation suggests that the cellular response to 405 nm irradiation and Hoechst 33342 treatment is very different from the response to pulsed 800 nm irradiation. In addition, it suggests that different types of DNA damage are created with these methods and not just different amounts of the same type of damage.

**Discussion**

We have investigated the response of several DNA repair factors that are involved in either NER or DSB break repair, to different types of DNA damage induction (Tables 1 and 2). We show that the NER factor XPC responds to many different types of lesions. This is illustrated, for example, by the strong response of XPC-GFP to pulsed 800 nm irradiation and 405 nm irradiation after Hoechst treatment, whereas GFP-XPA is recruited to irradiated areas to a much lesser extent. Upon 266 nm irradiation this difference is much smaller. Interestingly, XPC-GFP seemed to be the only NER factor that accumulated faster after irradiation with a 365 nm laser (Lan et al., 2004), confirming that it binds to a wide range of DNA lesions and not only to lesions that are repaired by NER. This is in accordance with previous in vitro DNA binding experiments showing low specificity of XPC for various aberrant DNA structures (Sugasawa et al., 1998). In addition, live cell studies using fluorescence recovery after photobleaching on XPC-GFP-expressing cells exposed to a variety of DNA damaging agents known to induce lesions other than pyrimidine dimers, showed participation of XPC-GFP similar to its behavior after UV-exposure (our unpublished work).

This observed affinity of XPC for a variety of DNA lesions suggests the rapid formation of pre-repair complexes on DNA. Such a quick response may initiate rapid activation of cell-cycle checkpoints after damage detection. The initial, weakly specific response is then followed by a more lesion-specific, but slower acting, damage verification step, which if positive, may, in its turn, activate a fully specific repair pathway required for the type of damage encountered. In addition, rapid exchange of damage recognition proteins with more pathway-specific factors may ensure that a repair pathway can quickly
Laser-induced DNA damage

become completely activated. Recently, such differential dynamic interactions have been suggested to occur during transcription initiation (Hager et al., 2006; Metivier et al., 2006). It was suggested that this prevents slowing down the entire transcription machinery due to too many non-productive long-lasting associations. A bipartite damage-recognition step for NER has been suggested previously (Dip et al., 2004; Sugasawa et al., 2001) with quick binding of a low-specificity initiating factor (XPC) and subsequent lesion verification. Our current data supports this model.

Laser-assisted damaging techniques

Formation of DSBs by a pulsed 800 nm laser has been reported previously (König et al., 2001; Tirlapur and König, 2001) and is thought to be caused by ablation of the DNA at the highly focused laser spot. In metaphase chromosomes this multiphoton ablation introduces gaps of approximately 100 nm corresponding to ~65 kb (König et al., 2001). Most likely such gaps, i.e. DSBs, will be created in interphase chromosomes as well, explaining the accumulation of DSB repair proteins observed here. Recently, also the induction and repair of DSBs in living cultured cells has been described using this DNA damage induction method (Mari et al., 2006).

A pulsed 800 nm laser beam has been shown to efficiently induce CPDs (Meldrum et al., 2003) and here we show that also 6-4PPs are efficiently formed with a pulsed 800 nm laser. The formation of these lesions, which are typically created by UV-C, is likely caused by three-photon absorption on the DNA, the effective wavelength being ~267 nm.

Many studies have been published in which DNA is sensitized prior to local irradiation. Sensitization of DNA can be accomplished by incorporation of a halogenated thymidine analogue in combination with Hoechst (Limoli and Ward, 1993; Paull et al., 2000; Rogakou et al., 1999), by incorporation of halogenated Hoechst (Martin et al., 1994; Martin et al., 1990) or of halogenated thymidine analogues alone (Lukas et al., 2003; Tashiro et al., 2000). Halogenation is thought to be required for DSB induction. However, Hoechst (either 33258 or 33342) by itself can also sensitize DNA to UV-A irradiation resulting in DSB formation (Bradshaw et al., 2005; Celeste et al., 2003; Kruhlak et al., 2006). Similarly, we have shown here that in the absence of halogen intermediates, irradiation of Hoechst 33342-sensitized cells at 405 nm induced DSBs, although it invokes a different response by Rad54 and γPKcs, i.e. non-focal accumulation and absence at damaged sites, respectively, than those induced by a pulsed 800 nm laser. Another remarkable effect of 405 nm irradiation of Hoechst 33342-sensitized cells is the specific induction of CPDs but not 6-4PPs. Photoisomerization of 6-4PPs results in the formation of the DewarPP, a photoproduct that is not recognized by the 6-4PP antibody (Kobayashi et al., 2001). However, the optimum wavelength for photoisomerization is between 280 and 360 nm, so 405 nm laser irradiation probably does not induce DewarPP formation. Instead, Hoechst binding induces local structural changes in the DNA, which might not allow the bending angle that is necessary for 6-4PP formation (Chen et al., 1993). We and others have noted that pre-sensitization of cells with Hoechst 33343 induces a very broad spectrum of events associated with

Fig. 6. Recruitment of DNA repair factors to various types of DNA damage. (A) XPC-GFP accumulates most efficiently in areas damaged with 266 nm laser light. The presence of Hoechst 33342 causes slower diffusion of XPC thus retarding its recruitment to DNA damage. (B) GFP-XPA also accumulates most efficiently in areas damaged with 266 nm laser light. GFP-XPA responds to a very small extent to pulsed 800 nm irradiation and 405 nm irradiation combined with Hoechst 33342. (C) YFP-MDC1(BRCT) is recruited quicker and in higher numbers to damaged areas in cells irradiated with 405 nm combined with Hoechst 33342 than in pulsed 800 nm-irradiated cells. (D) Rad54-GFP has a delayed response to pulsed 800 nm irradiation but it accumulates to a larger extent to these damages than to 405 nm combined with Hoechst 33342 irradiation.
structural changes in the DNA conformation, ranging from chromosome decondensation (Turner and Denny, 1996) to transcription inhibition (White et al., 2000). The aberrant responses shown here are: (1) absence of phosphorylated DNA-PKcs from damaged areas, whereas DSBs are judged to have formed by accumulation of Ku70-GFP; (2) reduced mobility of XPC; (3) homogenous accumulation of DSB repair proteins, rather than the common focal pattern and (4) very rapid accumulation of YFP-MDC1(BRCT) and Rad54-GFP compared with the response to pulsed 800 nm irradiation. Recently, also an aberrant accumulation of TRF2, a telomere binding protein, in response to local damage inflicted by pre-sensitization with Hoechst combined with high intensity 800 nm laser irradiation has been described, which has not been found using many other local damage techniques (Williams et al., 2007). We conclude that treatment with Hoechst 33343 as a sensitizer for DNA damage induction may have considerable consequences for the cellular response.

Sensitization with halogenated nucleotides instead of Hoechst prior to UV-A irradiation induces a response that is much more similar to ionizing radiation and pulsed 800 nm irradiation as repair proteins accumulate in foci (Lukas et al., 2003; Bekker-Jensen et al., 2006). One striking difference between pulsed 800 nm irradiation and UV-A irradiation of halogenated thymidine-sensitized nuclei is the response of NHEJ factors such as Ku80 and DNA-PKcs, which clearly accumulate in damaged areas created by the former but not by the latter method. Probably, these methods induce a different spectrum of DNA lesions, for example blunt-ended DSBs versus breaks with overhangs. Perhaps the relative concentration of these two types of DSBs determines the extent to which NHEJ or HR becomes activated.

We show that UV-C laser irradiation can induce pyrimidine dimers as well as DSBs, however, the latter only occurs after high intensity irradiation.

Specific DNA damage induction

We show here that UV-C laser irradiation at the appropriate intensity is the most specific method to induce 6-4PPs and CPDs. By contrast, induction of exclusively DSBs seems not possible with currently existing laser-assisted damaging methods. This problem was overcome by a method specifically inducing DSBs using a recombination reporter system involving an HO or I-SceI endonuclease site adjacent to a Lac- or Tet-operon repeat (Lisby et al., 2003; Miyazaki et al., 2004; Rodrigue et al., 2006). After induction of expression of the appropriate endonuclease, accumulation of repair proteins at the single DSB can be studied. This method has provided insight in the nature of repair foci, showing that multiple DSBs can colocalize within one focus in yeast (Lisby et al., 2003). Production of a known amount of well-specified DSBs will become a valuable tool in the study of DSB repair, especially since it has recently been effectively applied in mammalian cells (Rodrigue et al., 2006). However, the study of accumulation kinetics of DSB repair factors may be more complicated with this method because the timing of the activity of restriction enzymes is difficult to control.

Conclusion

We have shown that most presently available and widely used laser-assisted DNA damaging methods induce a wide response of cellular repair mechanisms. The relative proportion of the induced damages, which determines the extent to which different repair pathways become activated, is shown to differ for the three studied methods. Proteins that respond to a variety of lesions, such as XPC, will exhibit different kinetic behaviors depending on the method used. In future studies, using more than one source of DNA damage to study cellular responses, with accurate analysis of the types of lesions induced with these methods, will greatly help our understanding of DNA repair in vivo.

Materials and Methods

Preparation and culture of cell lines

XPC-GFP and GFP-XPA were expressed in the human cell lines XP4PA-SV and XP2OS-SV, which are deficient in XPC and XPA, respectively (Politi et al., 2005; Rademakers et al., 2003). Rad54-GFP-expressing cell lines were created by stable expression of Rad54-GFP in CHO9 cells as described previously (Essers et al., 2002a). mCherry-PCNA was transfected into this cell line. The YFP-MDC1 (BRCT) cell line was created by stable expression of a construct encoding a YFP fusion to the BRCT domains of human MDC1 in CHO9 cells. This construct was shown to be functional as a marker for MDC1 localization (O’Driscoll et al., 2003). GFP-Ku80 was transfected into Ku-deficient XR-V15B cells (Mari et al., 2006). GFP-PCNA was expressed in CHO9 cells as described previously (Essers et al., 2005). All cell lines were cultured under standard conditions in DMEM-F10 medium supplemented with 10% fetal calf serum and antibiotics at 37°C in 5% CO2.

Local UV induction with UV-C lamps

To induce local UV damage, cells were grown on coverslips, washed with PBS, covered with a polycarbonate filter (5 μm pore size; Millipore), irradiated with 100 J/m2 (overall dose) and incubated in standard growth medium for 30 minutes before fixation or further treatment.

Laser-induction of local damage

A Coherent Mira modelocked Ti:Sapphire laser was used at 800 nm with a pulselength of 200 fs and repetition rate of 76 MHz. Maximum output power on the cells for DNA damage induction was approximately 80 mW.

For the Hoechst + 405 nm treatment a 30 mW 405 nm diode laser supplied by Zeiss was used. Damage was induced at 60% of maximum power. For UV laser irradiation a 2 mW pulsed (7.8 kHz) diode pumped solid state laser emitting at 266 nm (Rapp OptoElectronic, Hamburg GmbH) was connected to a Zeiss LSM 510 confocal microscope with an Axiovolt 200 M housing adapted for UV by all-quartz optics. A special adapter (ZSL-A200, Rapp OptoElectronic) to fit in the aperture slider position of an Axiovolt 200 microscope was developed by Rapp OptoElectronic to focus the laser on a sample. For local UV-C irradiation experiments cells were grown on 25 mm diameter quartz coverslips (010191T-AB, SPI supplies).

Imaging of cells using confocal microscopy

Cells expressing GFP-tagged repair factors were grown on coverslips and imaged at 37°C using a Zeiss confocal microscope setup (Zeiss LSM510). In the case of cells to be treated with a combination of Hoechst and 405 nm light, Hoechst 33342 was added to the medium (final concentration 0.5 μg/ml) shortly before treatment. Cells with an intermediate fluorescence level were selected to be treated with either 405 nm or 800 nm light. All treated cells were analyzed at the same magnification and zoom factor using low laser power to minimize photobleaching during data collection. The region to be damaged was always the same size and shape, and laser treatment was done with calibrated lasers at the same laser output, to exclude variations in dose.

Immunofluorescence analysis

For immunohistochemical analysis, cells were washed with PBS and fixed for 15 minutes in 2% paraformaldehyde in PBS 30-60 minutes after damage induction. Next, the cells were washed with 3% BSA in PBS. In the case of antibodies directed against CPDs (TDM2) (Mori et al., 1991) or 6-4PPs (6-4-M-2) (Mori et al., 1991) cells were treated with 0.07 M NaOH in PBS for 5 minutes at room temperature to denature the DNA. Next, the cells were washed three times with P-buffer (0.1% Triton X-100 in PBS) and washed once using L-buffer (0.1% glycine, 1% BSA in PBS). Then, cells were incubated with primary antibodies (diluted in L-buffer) for 1 hour at 20°C for detection of protein epitopes or 12 hours at 4°C for detection of DNA lesions. The rabbit anti-β2XAX antibody was from Upstate Biotechnology (Charlottesville, VA, USA). After incubation, cells were washed three times using P-buffer, once using L-buffer, and incubated for 1 hour at 20°C with secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 594 (or multiple antibodies for double staining) diluted in L-buffer. Next, cells were washed three times using P-buffer, once with PBS and embedded in Vectashield (Vector
Laser-induced DNA damage 2739

Laboratory). The rabbit anti-


References


Table S1. Dose-dependency of the 266 nm laser

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