Senescence of Human Fibroblasts after Psoralen Photoactivation Is Mediated by ATR Kinase and Persistent DNA Damage Foci at Telomeres[□]

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Cellular senescence is a phenotype that is likely linked with aging. Recent concepts view different forms of senescence as permanently maintained DNA damage responses partially characterized by the presence of senescence-associated DNA damage foci at dysfunctional telomeres. Irradiation of primary human dermal fibroblasts with the photosensitizer 8-methoxypsoralen and ultraviolet A radiation (PUVA) induces senescence. In the present study, we demonstrate that senescence after PUVA depends on DNA interstrand cross-link (ICL) formation that activates ATR kinase. ATR is necessary for the manifestation and maintenance of the senescent phenotype, because depletion of ATR expression before PUVA prevents induction of senescence, and reduction of ATR expression in PUVA-senesced fibroblasts releases cells from growth arrest. We find an ATR-dependent phosphorylation of the histone H2AX (γ -H2AX). After PUVA, ATR and γ -H2AX colocalize in multiple nuclear foci. After several days, only few predominantly telomere-localized foci persist and telomeric DNA can be coimmunoprecipitated with ATR from PUVA-senesced fibroblasts. We thus identify ATR as a novel mediator of telomere-dependent senescence in response to ICL induced by photoactivated psoralens.

INTRODUCTION

Cellular senescence is a loss of proliferative capacity that is likely linked to aging. Several lines of evidence suggest that different forms of senescence, similar to apoptosis, function as programs to suppress tumorigenesis. Hence, senescence is also considered antagonistically pleiotropic, because senescent fibroblasts release various factors that contribute to tissue degradation in extrinsic aging processes (Toussaint *et al.*, 2002) as well as to the promotion of growth of adjacent neoplastic and proneoplastic epithelial cells (Krtolica *et al.*, 2001; Parrinello *et al.*, 2005).

Recent concepts regard different forms of senescence as permanently maintained DNA damage responses (DDR) characterized by focalization of DNA damage response factors such as the kinase *Ataxia teleangiectasia* mutated (ATM), phosphorylated histone H2AX (γ -H2AX), and different DNA repair proteins in senescence-associated DNA damage foci

(SDF) mediating the signaling for the permanent growth arrest in the vicinity of different DNA lesions (d'Adda di Fagnana *et al.*, 2003; Takai *et al.*, 2003; Herbig *et al.*, 2004).

Depending on the localization of SDF, current definitions of senescence distinguish telomere-independent and telomere-dependent forms (von Zglinicki *et al.*, 2005). The former, stress-induced premature senescence (SIPS) is considered a shortened replicative life span because of widespread nontelomeric DNA damage in cells exposed to acute intense or chronic genotoxic stress (Toussaint *et al.*, 2002). The latter, even though most likely overlapping, are further differentiated into replicative and accelerated telomere-dependent senescence. Both result from progressive structural telomere changes, which are caused by multiple cell divisions (replicative) or by different forms of cellular stress that lead to telomeric damage (accelerated) (von Zglinicki *et al.*, 1995).

Replicative senescence of human cells in culture was shown to be mediated by ATM that is activated by structurally altered telomeres and then signals for a permanent G₁-arrest (Herbig *et al.*, 2004). ATM, DNA-dependent protein kinase (DNA-PK), and *Ataxia teleangiectasia* and Rad3-related protein (ATR) belong to the superfamily of phosphoinositide 3-kinase related kinases (PIKK) and play critical roles in early signal transmission after DNA damage. They share several substrates and have partially overlapping but distinct functions (Shiloh, 2003; Yang *et al.*, 2003). ATM and DNA-PK respond primarily to DNA double-strand breaks, whereas ATR is predominantly activated by stalled replication forks (Abraham, 2001; Shiloh, 2003). DNA interstrand cross-links (ICL) are potent inducers of stalled replication forks. Recently, it was shown that in response to ICL formation, ATR controls an S-phase checkpoint in SV40-transformed human fibroblasts and HeLa cells (Pichierri and Rosselli, 2004).

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Abbreviations used: 3-CP, 3-carbethoxypsoralen; 8-MOP, 8-methoxypsoralen; ATM, *Ataxia teleangiectasia* mutated kinase; ATR, ATM and Rad3-related protein; CPD, cumulative population doubling; ChIP, chromatin immunoprecipitation; DDR, DNA damage response; ICL, DNA interstrand cross-links; PIKK, phosphoinositide 3-kinase related kinase; PUVA, 8-methoxypsoralen plus UVA irradiation; SDF, senescence-associated DNA damage foci; UVA, ultraviolet A radiation.

Depending on the irradiation conditions or derivative, photoactivated psoralens are capable of inducing ICL. In photomedicine, the combination of psoralens plus broad band UVA irradiation (PUVA therapy) is commonly used for the treatment of different skin disorders under ICL inducing conditions. Long-term PUVA-treated patients suffer from accelerated skin aging and an increased incidence of skin cancers (Peritz and Gasparro, 1999). On the cellular level, we could previously show that a single noncytotoxic PUVA treatment induces premature cellular senescence in human dermal fibroblasts (Herrmann *et al.*, 1998). In the present study, we used this model for further analysis of the underlying damage and signaling events and find that senescence after psoralen photoactivation depends on the DNA damage kinase ATR and persistent SDF at telomeres. We thus identify another PIKK as an important mediator of senescence in human cells in response to ICL.

MATERIALS AND METHODS

Reagents

8-Methoxypsoralen (8-MOP) and Lectin from *Phaseolus vulgaris* (PHA) were obtained from Sigma-Aldrich (St. Louis, MO), and phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I) was from Stratagene (La Jolla, CA). 3-Carbethoxypsoralen (3-CP) was kindly provided by D. Averbeck (Institut Curie, Orsay, France) (Averbeck *et al.*, 1978).

Cells and Media

Primary dermal fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 1% glutamine, and antibiotics. Cells were used for the experiments at cumulative population doublings (CPD) 8–20 (Bayreuther *et al.*, 1992). SV40-transformed embryonic lung fibroblast cell lines WI26 VA4 and WI38 VA13 (Knaup *et al.*, 1978) were obtained from American Type Culture Collection (Manassas, VA). Primary human epidermal keratinocytes were isolated from neonatal foreskin, cultivated with a feeder layer as described previously (Watt, 1994). Primary human CD3⁺ cells were isolated from buffy coats by depleting non-CD3⁺ cells with the Human T-Cell Enrichment Cocktail/RosetteSep (Stem Cell Technologies, Vancouver, Canada). CD3⁺ T-cells were stimulated with 4 μ g/ml PHA, cultured for 1 wk in RPMI 1640 medium supplemented with 10% FCS, 2% glutamine, and antibiotics. For synchrony in G₁, cells were incubated in DMEM without FCS for 24 h.

Irradiation

Crystalline 8-MOP and 3-CP were dissolved in dimethyl sulfoxide at 1 mg/ml and added to the growth medium of the cells at 50 or 100 ng/ml, respectively, for 2 h before irradiation. These concentrations are similar to those detected in dermal suction blister fluid of patients receiving systemic PUVA therapy (de Wolff and Thomas, 1986). Cells were irradiated in phosphate-buffered saline (PBS) containing 8-MOP or 3-CP in a temperature-controlled water bath. UVA irradiation was performed with a high-intensity UVA source (UVASUN 3000 equipped with the UVASUN safety filters) emitting wavelengths in the 320- to 460-nm range (Mutzhas *et al.*, 1981; Herrmann *et al.*, 1993) with 9 J/cm², if not indicated otherwise. Fluences were determined with a UVA-UV meter (Dr. K. Hönle AG, Gräfelfing, Germany). Monochromatic irradiation (0.75 J/cm²) was performed with a 1000-W xenon high-pressure UV source in conjunction with a monochromator with holographic grating (Dermolum, Müller GmbH, Moosinning, Germany), as described previously (Brenneisen *et al.*, 1996). The band width (at half-maximum intensity) was 10 nm. UVA or monochromatic irradiation without addition of psoralens served as control in all experiments. γ -Irradiation was performed at a dose of 10 Gy.

Senescence-associated β -Galactosidase (SA- β -gal) Staining

SA- β -gal staining was performed as described previously (Dimri *et al.*, 1995). Briefly, cells were fixed in 4% paraformaldehyde, rinsed with PBS, and incubated at 37°C with fresh SA- β -gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl β -D galactoside [X-Gal] per milliliter, 40 mM citric acid/Na₂PO₄, pH 6.0, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂).

Immunoblotting

Cells were scraped in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Nonidet NP-40, 0.5% sodium deoxycholate, and 0.1% SDS plus protease inhibitors). Cell lysates (20–60 μ g) were subjected to 5–15% SDS-PAGE and transferred to a nitrocellulose membrane

(GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Equal loading was confirmed by Ponceau Red staining of the membrane. Blots were incubated with primary antibodies against ATR (1:500, N-19; Santa Cruz Biotechnology, Santa Cruz, CA), ATR (1:1000, ab2905; Abcam, Cambridge, United Kingdom), or phospho-ATR (S1981) (1:1500, ab2888; Abcam), followed by addition of the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Signals were visualized with ECL reagent (PerkinElmer Life and Analytical Sciences, Boston, MA).

Immunoprecipitation and Kinase Assays

Cell lysate (1.4 mg) was prepared with RIPA buffer (plus 4 mM EDTA, 0.2% *n*-dodecyl- β -maltoside, 20 μ M aprotinin, 20 μ M leupeptin, 2 μ M phenylmethylsulfonyl fluoride, 100 μ M Na₃VO₄, and 10 mM NaF), and precleared with protein A/G plus agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. After incubation with anti-ATR (N-19) or anti-ATR antibody (ab91; Novus Biologicals, Littleton, CO) for 1 h at 4°C, incubation with protein A/G plus agarose beads was performed overnight at 4°C. After extensive washing with RIPA and kinase buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 4 mM MnCl₂, 6 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, and 100 μ M Na₃VO₄), beads were solubilized in 30 μ l of kinase buffer containing 10 μ Ci of [γ -³²P]ATP and 1 μ g of the artificial substrate PHAS-I. Probes were incubated for 30 min at 30°C, boiled after the addition of 10 μ l of 4 $\times$ Laemmli sample buffer, and separated by 15% SDS-PAGE. Gels were dried and exposed to x-ray films.

Small-interfering RNA (siRNA) Transfection

Cells were transfected with 2 μ g of either ATR or ATM smart pool siRNA (Dharmacon, Chicago, IL). Transfection of primary fibroblasts was carried out by electroporation using the NHDF nucleofector kit (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's protocol. Transfection efficiencies were determined using a green fluorescent protein (GFP)-expressing plasmid included in the kit. Fibroblast cell lines and senescent primary fibroblasts were transfected with the TransMessenger Transfection Reagent (QIAGEN, Hilden, Germany), according to the manufacturer's protocol for adherent cells. To increase transfection efficiencies, primary senescent cells were twice transfected at days 14 and 16 after PUVA. Nonsilencing control siRNA (QIAGEN), or Silencer Cy3 labeled Negative Control #1 siRNA (Ambion, Austin, TX) were used to rule out unspecific siRNA effects and to determine transfection efficiencies.

Immunofluorescence and Fluorescence In Situ Hybridization (FISH)

Cells were seeded on glass coverslips, allowed to recover for 24 h, incubated with 50 ng/ml 8-MOP for 2 h, and irradiated with 9 J/cm² UVA. At the indicated time points after irradiation, cells were fixed for 10 min at room temperature with 4% paraformaldehyde-buffered solution, followed by permeabilization with 0.5% Triton X-100 for 5 min. Cell preparations were blocked for 45 min at room temperature in 0.5% bovine serum albumin and 0.1% fish gelatin/phosphate-buffered saline. Cells were incubated with anti-ATR (1:2000, N-19; Santa Cruz Biotechnology), fluorescein isothiocyanate-conjugated anti-phospho H2AX (1:500; Novus), or anti-phospho H2AX (1:500; Upstate Biotechnology, Lake Placid, NY) for 1 h at 37°C, with anti-lamin A (1:25) for 1 h at room temperature, or with anti-TRF1 (1:500, ab1423; Abcam) overnight at 4°C. Appropriate fluorescence-conjugated secondary antibodies (Santa Cruz Biotechnology and Invitrogen, Carlsbad, CA) were applied for 1 h at 37°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Staining was analyzed using a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan). Confocal images were taken with an inverted Leica TCS-SP laser-scanning microscope (details available in Supplemental Material). All images were prepared for publication using Adobe Photoshop (Adobe Systems, Mountain View, CA).

FISH was carried out using the Telomere PNA FISH kit/Cy3 (DakoCytomation Denmark, Glostrup, Denmark), according to the manufacturer's instructions with slight modifications (available in Supplemental Material).

Chromatin Immunoprecipitation Assay (ChIP)

ChIP was carried out using the ChIP assay (Upstate Biotechnology) essentially as described by the manufacturer. Briefly, 1 $\times$ 10⁶ primary fibroblasts were cross-linked for 10 min with 1% formaldehyde at 37°C followed by lysis with SDS-lysis buffer supplemented with protease inhibitors. DNA was sheared by sonication, and extracts were cleared by centrifugation and incubated for 1 h at 4°C with protein A/G plus agarose beads (Santa Cruz Biotechnology) to reduce unspecific background. Precleared chromatin was incubated overnight at 4°C with anti-ATR antibody (N-19) or with rabbit-serum IgG (Santa Cruz Biotechnology), followed by 1-h incubation with A/G plus agarose beads at 4°C. After washing, immune complexes were eluted from the beads as described by the manufacturer. Cross-linking was reversed at 65°C for 4 h in 0.2 M NaCl. Proteins were removed by proteinase K treatment. The DNA was purified, denatured in 0.4 M NaOH at 100°C for 10 min and dot-blotted onto a Zeta-Probe GT blotting membrane (Bio-Rad, Hercules, CA). Hybridization was performed with a digoxigenin-labeled probe specific for telomeric repeats from the TeloTAGGG telomere length

Figure 1. Induction of senescence in human dermal fibroblasts depends on ICL. (A) CPDs of primary human fibroblasts irradiated in the presence of 8-MOP with 350 or 400 ± 10 nm monochromatic irradiation (0.75 J/cm²). At the indicated time points after irradiation, cell numbers were determined in triplicates for each experimental group and equal cell numbers were transferred to a new tissue plate for further observation. SD were <10% for all values. (B) Cultures of primary fibroblasts were irradiated with broad-spectrum UVA in the presence of 8-MOP or 3-CP. Experimental conditions as described in A. SD were <10% for all values. (C) Expression of SA-β-gal. At day 28 after irradiation with UVA or monochromatic irradiation (wavelengths as indicated, irradiation conditions as in A and B), 8-MOP- or 3-CP-treated fibroblasts were fixed and assayed for SA-β-gal. Magnification 20×.

assay kit (Roche Diagnostics, Basel, Switzerland). Signals were visualized via chemiluminescence reaction according to the manufacturer's instructions. After stripping, an rDNA probe (Chikaraishi *et al.*, 1983) was used to determine unspecific DNA binding.

RESULTS

PUVA-induced Senescence Depends on ICL Formation

We have previously demonstrated that a single 8-MOP + UVA irradiation of primary human dermal fibroblasts under nontoxic conditions resulted in cellular senescence. This phenotype was characterized by long-term growth arrest, expression of SA-β-gal and upregulation of matrix-degrading metalloproteinases (Herrmann *et al.*, 1998). Irradiation of 8-MOP-incubated cells with broad-spectrum UVA (320–400 nm) efficiently induces ICL (Bethea *et al.*, 1999). Therefore, we wanted to define the role of ICL in 8-MOP + UVA-induced senescence. The formation of ICL by 8-MOP requires irradiation with wavelengths around 340–365 nm, whereas irradiation with 390–405 nm results almost exclu-

sively in monoadduct generation (Tessman *et al.*, 1985; Sage and Moustacchi, 1987; Calsou *et al.*, 1996). Other psoralen derivatives such as 3-CP lack the ability to form ICL upon broad-spectrum UVA irradiation (Averbeck *et al.*, 1978). Refer to Figure S1 for details on the chemical structure of the different psoralen derivatives. Fibroblasts were irradiated under conditions of comparable toxicity, resulting in >80% cellular survival (Figure S2). Induction of senescence was determined by observation of growth-arrest, morphological alterations, and SA-β-gal expression. Senescence of human primary fibroblasts was induced in 8-MOP-preincubated cells after irradiation with broad-spectrum UVA or monochromatic irradiation with 350 ± 10 nm representing conditions that benefit ICL formation (Figure 1, A–C). Generation of monoadducts, either by monochromatic irradiation with 400 ± 10 nm of 8-MOP-preincubated cells (Figure 1, A and C) or by broad-spectrum UVA irradiation of 3-CP-preincubated cells (Figure 1, B and C) did not result in senescence. Thus, induction of senescence after PUVA correlates with

Figure 2. ATR is activated in response to PUVA treatment. (A) Kinase assays of ATR activity in different human primary cells after 8-MOP + UVA. CD3⁺ T-lymphocytes, G₁-arrested fibroblasts, or keratinocytes were treated as indicated and recovered for 2 h. Harvesting of the cells was followed by immunoprecipitation with anti-ATR (N-19). Radioactive kinase assays were performed using the artificial substrate PHAS-I. Then, 15 μ l of kinase reactions was separated by 15% SDS-PAGE and exposed to x-ray films. (B) Time course of ATR activation in human fibroblasts. Cellular extracts were prepared at the indicated time points after psoralen photoactivation. Kinase assays of ATR activity were performed as described in A. (C) Detection of activated ATM kinase. Total cellular extracts were prepared from 8-MOP + UVA-treated fibroblasts at the indicated time points, from replicative senescent fibroblasts with >70 cumulative population doublings (CPD > 70), and from γ -irradiated fibroblasts 1 h after irradiation. Lysates were separated by 5% SDS-PAGE and immunoblotting was performed with phosphospecific anti-ATM (S1981) antibody. Equal

ICL formation of the photoactivated psoralen derivative. Additional growth curves of primary fibroblasts from different donors are presented in Figure S3.

Activation of ATR in Human Primary Cells after PUVA Treatment

Recently, ATR kinase was shown to signal for an S-phase checkpoint in response to ICL formation in SV40-transformed lymphoblast and fibroblast cell lines (Pichierri and Rosselli, 2004). Different cell types display varying sensitivities in response to psoralen photoactivation (Gasparro 1996; Johnson *et al.*, 1996). Additionally, primary cells differ in their reactions towards UV or γ -irradiation compared with the corresponding cell lines, including cellular survival or production of signaling molecules (Petit-Frère *et al.*, 2000). We therefore addressed the question whether ATR is also activated in human primary cells in response to 8-MOP + UVA, independent of the affected cell type. ATR was immunoprecipitated from cellular extracts at various time points after irradiation. ATR activation was analyzed using a kinase assay that bases on the phosphorylation of the artificial substrate PHAS-1. Incubation with 8-MOP without irradiation, or UVA irradiation alone did not have any effect on ATR activation in fibroblasts, keratinocytes, or CD3⁺ T-cells. Two hours after 8-MOP + UVA, we detected an activation of ATR in all cell types (Figure 2A), indicating that activation of ATR in response to psoralen DNA ICL is a general mechanism in human primary cells that is independent of SV40 transformation. Time-course experiments of synchronized human fibroblasts showed a maximum activation of ATR between 1 and 6 h, which returned to background levels 7 h after 8-MOP + UVA treatment (Figure 2B).

So far, ATM has not been implicated in the DNA-damage signaling in response to ICL, and ATM-deficient AT-cells are not sensitive to ICL-inducing agents (Andreassen *et al.*, 2004; Pichierri and Rosselli, 2004). However, models for the repair of ICL propose an intermediate state of double-strand break formation (Bessho, 2003; Niedernhofer *et al.*, 2004; Rothfuss and Grompe, 2004), a DNA lesion that is known to mainly activate ATM (Shiloh, 2003). To reinvestigate ATM activation in response to psoralen photoactivation, we analyzed ATM phosphorylation at S1981 in PUVA-treated fibroblasts with the appropriate phosphospecific antibody. Autophosphorylation of ATM at S1981 corresponds to the activation of the kinase (Bakkenist and Kastan, 2003). ATM phosphorylation was not detectable after 8-MOP + UVA treatment 2 h, 6 h, or up to 31 d after irradiation, whereas replicative senescent (CPD > 70) and γ -irradiated fibroblasts used as positive controls showed an activation of ATM (Figure 2C). Accordingly, ATM activation after PUVA could not be detected in kinase assays after precipitation of ATM (Figure 2D). Therefore it seems unlikely that ATM is involved in the signaling induced by psoralen DNA ICL. Specificity of the anti-ATR and anti-ATM antibodies is shown in Figure S4, A and B.

Role of ATR in DNA Damage Signaling and Cellular Senescence after Psoralen Photoactivation

To investigate signaling events downstream of ATR, we analyzed phosphorylation of histone H2AX (γ -H2AX) in

loading was verified by Ponceau S staining (our unpublished data). (D) Kinase assays of ATM activity in human primary fibroblasts at 2 h after irradiation. Kinase assays were performed as described in A, immunoprecipitation was with anti-ATM (ab91).

Figure 3. ATR is essential for DNA damage signaling after PUVA. (A) Quantification of γ -H2AX-positive cells. Unirradiated, UVA-irradiated, or 8-MOP + UVA-treated human primary fibroblasts were stained with γ -H2AX antibody at the indicated time points. Cells with more than one γ -H2AX focus were considered positive. At least 300 cells were scored in each experimental group. Bars represent average of three independent experiments, including standard deviations. (B) Inhibition of ATR expression by ATR siRNA transfection. Fibroblasts were transfected with either ATR siRNA or control siRNA (Co siRNA). Cellular extracts were prepared at the indicated time points after transfection, separated by 5% SDS-PAGE, and subjected to immunoblotting with anti-ATR (ab2905). (C) Quantification of γ -H2AX-positive cells after ATR-depletion and subsequent 8-MOP + UVA treatment. Left, percentage of γ -H2AX-positive cells after ATR depletion or treatment with control siRNA. Cells were counted 24 h after 8-MOP + UVA irradiation. Bars represent average of three independently conducted experiments with >300 cells counted plus standard deviations. Right, representative images of either control siRNA- or ATR siRNA-transfected cells immunostained with an antibody against γ -H2AX. (D) ATR is necessary for the induction of senescence. Quantification of cell numbers at the indicated time points after different treatments. Cell numbers at the time point of irradiation (day 0) were set as 1. Determination of cell numbers in the different experimental groups was performed on the indicated days, and cell numbers are presented as -fold alteration compared with day 0. Embedded immunoblot analysis shows ablation of ATM expression by siRNA at 24 h after transfection. (E) Quantification of NF after ATR siRNA and 8-MOP + UVA treatment. Left, visualization of NF in ATR siRNA-/8-MOP + UVA-treated cells using an anti-lamin A antibody and DAPI staining (magnification 100 $\times$). Right, quantification of NF at different time points after 8-MOP + UVA.

response to psoralen photoactivation. H2AX phosphorylation by PIKK in the vicinity of DNA lesions is an early DNA damage signaling event. γ -H2AX formation in response to DNA double-strand breaks depends mainly on ATM (Rogakou *et al.*, 1998), whereas phosphorylation of H2AX in response to hydroxyurea or UVC was shown to be mediated by ATR (Ward and Chen, 2001). We visualized γ -H2AX formation by immunostaining with an antibody specific for the phosphorylated form. Numbers of cells staining positive for γ -H2AX in primary human fibroblasts increased from 11% in untreated to 82% in PUVA-treated cells at 24 h after irradiation (Figure 3A). In accordance with findings of others who observed a dose-dependent increase of γ -H2AX foci in response to UVA irradiation alone (Rapp and Greulich, 2004), we detected increased numbers of γ -H2AX-positive

cells 4 h after UVA. Twenty-four hours after UVA, numbers of γ -H2AX-positive cells almost returned to control levels, whereas γ -H2AX foci could still be detected in >90% of the cells at day 28 after PUVA irradiation (Figure 3A).

To test whether H2AX phosphorylation after PUVA depends on ATR, we ablated ATR expression in primary human fibroblasts by siRNA. ATR expression was almost abolished 24 h after transfection (Figure 3B). We chose this time point for subsequent irradiation. Transfection efficiencies were between 70 and 80%, as determined by transfection of a GFP-expressing plasmid (our unpublished data). Twenty-four hours after 8-MOP + UVA irradiation of ATR siRNA-treated cells, the portion of γ -H2AX-positive cells was reduced to 35% compared with 80% in nonsilencing control siRNA-transfected cells (Figure 3C). Considering transfection

tion efficiencies of 70–80%, and 11% γ -H2AX-positive cells in the unirradiated control group, we can quantitatively explain the remaining 35% γ -H2AX-positive cells. Moreover, γ -H2AX foci formation correlated with cells in that did not show foci formation of ATR after ATR siRNA transfection followed by psoralen photoactivation (Figure S4, D). We conclude from this that H2AX phosphorylation after psoralen photoactivation is ATR dependent. The amount of γ -H2AX-positive cells in the control siRNA-treated group was comparable with the 82% observed in the untransfected, 8-MOP + UVA-treated cells (Figure 3, A and C).

To study the influence of ATR on the induction of senescence in response to 8-MOP + UVA, we monitored irradiated cells for periods up to 11 d. To rule out effects of ATM kinase, we also used cells in which ATM expression was ablated by ATM siRNA (Figure 3D). Knockdown of ATR or ATM expression without subsequent irradiation did not alter cellular viability over a period of 11 d, compared with cells that were only subjected to the transfection procedure (Figure 3D). Fibroblasts subjected to transfection procedures only, cells transfected with control siRNA, or ATM siRNA equally growth-arrested in response to 8-MOP + UVA, which again argues against a role for ATM in the signaling in response to psoralen DNA ICL formation (Figure 3D). In contrast, cell numbers of ATR siRNA-treated cells permanently decreased after PUVA, until after 11 d only 21% of the cells survived compared with corresponding cell numbers of PUVA-irradiated control siRNA- or ATM siRNA-treated cells (Figure 3D). SA- β -gal analysis of the surviving cells at day 11 after PUVA revealed equivalent portions of senescent cells in control siRNA- and ATR siRNA-transfected groups (our unpublished data). In conjunction with transfection efficiencies, this suggests that the few surviving cells in the ATR-siRNA- and PUVA-treated group represent untransfected cells.

A recent report describes nuclear fragmentation (NF) as a response of lymphocytes harboring a splicing mutation of ATR (ATR-Seckel cells) in response to various genotoxic agents. This phenotype is characterized by chromatin condensation and micronucleation, which is surrounded by remnants of the nuclear envelope (Alderton *et al.*, 2004). To check whether ATR siRNA treatment followed by 8-MOP + UVA elicits a similar phenotype, we performed DAPI staining to visualize the chromatin and lamin A immunostaining to label the nuclear membrane. We detected increased frequencies of chromatin condensation and micronucleation with remnants of the nuclear membrane in 8-MOP + UVA irradiated/ATR siRNA-treated cells (Figure 3E). This phenotype is consistent with nuclear fragmentation, and we observe similar NF frequencies as in ATR-Seckel cells after genotoxic stress that results in stalled replication forks (Alderton *et al.*, 2004). In summary, ATR kinase is indispensable for initiating the DDR and the induction of cellular senescence in primary fibroblasts after psoralen photoactivation.

To analyze whether ATR is also required for the maintenance of senescence, we decided to deplete ATR or ATM in PUVA-senescent fibroblasts at days 14 and 16 after irradiation (Figure 4). Repeated transfection increased transfection efficiencies of primary senescent cells to around 60%, as determined with Cy3-labeled control siRNA (our unpublished data). We constantly monitored cellular morphologies and observed that cells of the ATR siRNA-treated group rounded up and lost contact to the Petri dish. Two days after the second transfection, SA- β -gal staining was performed to determine numbers of senescent cells. The amount of SA- β -gal-positive cells was markedly reduced in the ATR siRNA-treated group compared with the ATM- or control siRNA-

Figure 4. Reduction of ATR expression in senescent primary fibroblasts reduces SA- β -gal-positive cells. At days 14 and 16 after 8-MOP + UVA treatment, equal cell numbers of senescent primary fibroblasts were repeatedly transfected with either ATR siRNA, ATM siRNA, or Cy3-labeled control siRNA (Co siRNA). Four days after initial transfection, SA- β -gal-positive cells were quantified. Each spot represents one visual field at 10 $\times$ magnification.

treated cells (Figure 4). From this, we conclude that depletion of ATR releases PUVA-treated fibroblasts from senescence. Notably, similar results were obtained in SV40-transformed WI26 VA4 fibroblasts, which also senesce after psoralen photoactivation (Figure S5, A–C).

ATR Colocalizes with γ -H2AX Foci in Fibroblasts after Psoralen Photoactivation

We then monitored the nuclear distribution of ATR in PUVA-treated primary fibroblasts and observed the formation of multiple nuclear ATR foci 24 h after irradiation. Consistent with the ATR-dependent H2AX phosphorylation, we could colocalize ATR and γ -H2AX (Figures 5A and S4D). Quantification of nuclear γ -H2AX foci 24 h after irradiation revealed that 80% of the primary fibroblasts displayed >50 foci, consistent with random nuclear damage (Figure 5B). Colocalization of ATR and γ -H2AX was not observed in untreated mitotic (CPD < 12), postmitotic replicative senescent fibroblasts (CPD > 70) (Figure 5A) or in fibroblasts treated with UVA or 8-MOP alone (our unpublished data). Further observation of the cells up to 28 d after treatment revealed gradual changes of the nuclear staining pattern of ATR and γ -H2AX. The number of foci permanently decreased until in most of the cells only few foci persisted. Figure 5A shows ATR and γ -H2AX colocalization at day 10 after 8-MOP + UVA. Quantification of γ -H2AX foci at day 14 and 28 after PUVA showed that 75% of the primary fibroblasts contained <50 nuclear foci and 50% of the cells had <20 nuclear foci (Figure 5B). The observation of ATR/ γ -H2AX colocalization implies a persistent ATR activation in response to psoralen photoactivation at these sites. In contrast to replicative senescent fibroblasts, where the kinase ATM has been shown to signal for senescence from dysfunctional telomeres (Herbig *et al.*, 2004), we could not detect a colocalization of activated ATM(S1981) with γ -H2AX foci in PUVA-senescent primary fibroblasts (Figure S6). Consistent with observations that the association of DNA-PK with telomeres in replicative senescent cells depends on ATM and not on ATR (Herbig *et al.*, 2004), we could also not colocalize DNA-PK with γ -H2AX in these cells (our unpublished data).

Figure 5. Colocalization of ATR with γ -H2AX foci and telomeres in 8-MOP + UVA-treated senescent fibroblasts. (A) Untreated mitotic (CPD < 12), replicative senescent (CPD > 70), or 8-MOP + UVA-treated primary fibroblasts were sequentially immunostained with antibodies against ATR (N-19) and γ -H2AX. Twenty-four hours and day 10 indicate time points after 8-MOP + UVA. (B) Quantification of γ -H2AX foci in human fibroblasts. PUVA-treated primary fibroblasts were immunostained with γ -H2AX at the indicated time points, and nuclear foci were counted in at least 500 cells. Bars represent fractions of cells containing an indicated range of γ -H2AX foci. Experiments were performed in triplicates and SD were <10% for all values. (C) Colocalization of γ -H2AX and TRF1 using confocal microscopy. 8-MOP + UVA-treated primary fibroblasts and WI38 VA13 fibroblasts were sequentially immunostained at day 14 after treatment with antibodies against TRF1 and γ -H2AX. (D) Percentage of γ -H2AX foci that colocalize with TRF1 was determined in 50 cells at day 14 after PUVA irradiation. (E) Immuno-FISH analysis of human WI38 VA13 fibroblasts with a PNA-probe (PNA-Telo) specific for telomeric DNA, followed by immunostaining against γ -H2AX at the indicated time points. (F) Percentage of γ -H2AX foci that colocalize with telomeric DNA (PNA-Telo) was determined in 50 cells at day 28 after PUVA irradiation in WI38 VA13 cells. (G) Immuno-FISH analysis with PNA-Telo followed by immunostaining against ATR (N-19) at day 28 after PUVA.

However, the pattern of the remaining ATR/ γ -H2AX foci in 8-MOP + UVA-treated senescent fibroblasts was reminiscent of the γ -H2AX pattern observed in replicative senescent fibroblasts (Figures 5A and S6). We therefore investigated whether these foci are likewise telomere associated. We both used human primary fibroblasts and SV40-transformed WI38 VA13 fibroblasts, which also undergo senescence after 8-MOP + UVA treatment (Figure S5, D). Confocal immunofluorescence analysis of cells containing <50 nuclear H2AX foci (>80% of the cells in both cell types) revealed that SDF foci colocalize with the telomere-associated protein TRF1 in both cell types (Figure 5C). In the fibroblast cell line, 61% of the remaining γ -H2AX foci colocalize with TRF1 (Figure 5D). Additionally, using a combination of immunofluores-

cence and FISH analysis with PNA-probes complementary to telomeric DNA, we found that in these cells at day 28 after PUVA 77% of the γ -H2AX foci colocalize with telomeric DNA (Figure 5, E and F). Similarly, ATR could be colocalized with telomeric DNA at day 28 after PUVA (Figure 5G).

To confirm the association of ATR with telomeres in PUVA-senescent primary fibroblasts by an alternative method, we performed telomere ChIP. Consistent with the immunofluorescence data, anti-ATR antibodies coimmunoprecipitated telomeric DNA only from PUVA-irradiated primary fibroblasts and not from unirradiated or replicative senescent fibroblasts (Figure 6).

In summary, these observations suggest that, similar to the function of ATM in replicative senescence, ATR main-

Figure 6. ATR is enriched at telomeres of PUVA-senesced primary fibroblasts. Dot blot analysis of DNA coimmunoprecipitated with anti-ATR antibodies (N-19) or rabbit IgG (control) of cross-linked chromatin from PUVA-treated and untreated primary fibroblasts (control) at day 28 after treatment or from replicative senescent fibroblasts (CPD >70). Ovals indicate areas where DNA was dropped onto the membrane using a micropipette. Blots were sequentially hybridized with telomeric (Telo-probe; top) and rDNA probes (bottom). 500 ng of genomic DNA was used as a positive control for hybridization (DNA control).

tains accelerated senescence after psoralen photoactivation by a persistent DNA damage response at telomeres. Moreover, experiments with WI26 VA4 and WI38 VA13 fibroblast cell lines suggest that PUVA-induced senescence is independent of SV40 transformation.

DISCUSSION

In this study, we used the psoralen derivatives 8-MOP and 3-CP in conjunction with various irradiation conditions to induce different types of DNA lesions. Neither 3-CP nor 8-MOP photoexcited under conditions that lead to the formation of DNA lesions that only affect one DNA strand could induce senescence in human fibroblasts. This argues against a role of DNA monoadducts in mediating senescence after psoralen photoactivation. Irradiation of 3-CP or 8-MOP results in singlet oxygen and superoxide anion formation. 3-CP generates similar amounts of superoxide anions and up to fourfold higher amounts of singlet oxygen compared with 8-MOP (Joshi and Pathak, 1984). Because senescence could not be induced by photoexcitation of 3-CP, this argues against an involvement of these reactive oxygen

species as mediators of the phenotype. It is therefore likely that psoralen DNA ICL are required to induce senescence after 8-MOP + UVA.

Psoralen DNA ICL cause stalled replication forks in S phase (Akkari *et al.*, 2000). The kinase ATR has emerged as a primary mediator of S-phase-dependent cellular responses to stalled replication forks (Hammond *et al.*, 2002; Ward *et al.*, 2004), and, accordingly, a recent report describes an ATR-dependent S-phase checkpoint in response to ICL induced by photoactivated psoralens in immortalized cell lines (Pichierri and Rosselli, 2004). However, ATR activation in the immortalized cell lines has not been studied for >8 h after irradiation. We wanted to study the signaling responsible for cellular senescence, and to rule out effects of immortalization, we initially used primary human cells with intact cell cycle checkpoints. The most commonly used immortalization agent, the SV40 large tumor oncogene, interferes with p53, a central player of the DNA damage response, and SV40-immortalized cells are often phenotypically immature and genetically abnormal (Bryan and Reddel, 1994). We find that, independent of the cell type or cellular sensitivity, ATR activation is a general response of different human primary cells to ICL induced by photoactivated psoralens and thus not different from SV40-transformed cells. Comparing primary fibroblasts and two different SV40-transformed fibroblast cell lines, we find that senescence after psoralen photoactivation is also independent of SV40 transformation. An explanation could be that PUVA-induced senescence is independent of p53, p21, or p16 (Ma *et al.*, 2003).

Using kinase assays that base on the precipitation of ATR from cellular extracts and determining its activity by phosphorylating an artificial substrate (Ziv *et al.*, 2000), we were able to detect ATR activation for up to 6 h in synchronized fibroblasts. However, the experimental procedure includes cell lysis and immunoprecipitation and always exhibits some background phosphorylation in the untreated control cells. The assay may therefore not be sensitive enough to detect low amounts of activated ATR at later time points. Moreover, ATR function is considered to rely more on relocalization events than on measurable activation (Abraham, 2001). Monitoring of the nuclear localization of ATR revealed a colocalization with γ -H2AX in randomly distributed nuclear foci 24 h after 8-MOP + UVA, corresponding to patterns observed in cells with stalled replication forks in S phase (Ward and Chen, 2001; Pichierri and Rosselli, 2004). Phosphorylation of H2AX in response to replication stress was shown to be an early event in the DDR and is required to concentrate other damage response and repair proteins in the vicinity of DNA lesions (Ward and Chen, 2001; Fernandez-Capetillo *et al.*, 2002; Celeste *et al.*, 2003). We therefore conclude that the observed colocalization of ATR and γ -H2AX after psoralen photoactivation is indicative for persisting ATR activity.

We observe a decreased phosphorylation of H2AX after 8-MOP + UVA in cells in which ATR expression was ablated by siRNA. Moreover, we find that in the absence of ATR signaling cytotoxicity of PUVA irradiation is strongly increased. These observations are in accordance with both the essential, nonredundant functions of ATR that differ from other PIKK such as ATM (Shiloh, 2003; Yang *et al.*, 2003; Alderton *et al.*, 2004) and the extreme toxicity of ICL. It could be shown that a single, unrepaired ICL was sufficient to kill repair-deficient bacteria or yeast (Magana-Schwencke *et al.*, 1982; Lawley and Philips, 1996). Therefore, if the initiations of the DDR and cell cycle checkpoints that are controlled by ATR are impaired in ATR siRNA-treated cells, it is likely that the cells fail to react to stalled replication forks that are caused by psoralen DNA ICL (Akkari *et al.*, 2000). Instead of entering senescence, cell death eventually

occurs via mitotic catastrophe and nuclear fragmentation, a phenotype recently described in ATR-deficient lymphocytes (ATR-Seckel cells) in response to hydroxyurea, UVC, or nocodazole treatment (Alderton *et al.*, 2004). Nuclear fragmentation is defined as a mitosis-independent event, because the affected cells fail to stain positive for mitotic markers but retain remnants of the nuclear membrane together with micronucleation and chromatin condensation, features that we observe in ATR siRNA- and PUVA-treated cells. ATR therefore seems essential to induce senescence in fibroblasts after 8-MOP + UVA treatment.

In addition to inducing senescence, we also find that ATR is necessary to maintain the senescent phenotype. Constant monitoring of morphology of ATR siRNA-treated senescent fibroblasts showed that the senescent cells did not start forming colonies, but rounded up, lost contact to the culture plate, and eventually died. The absence of colony formation could be because of extensive damage of the cells, probably directly leading to cell death after reversal of senescence. Because ATR siRNA-, ATM siRNA-, and control siRNA-treated cells were recruited from the same pool of 8-MOP + UVA-senesced cells, we exclude that these observations result from differences in the populations before siRNA treatment.

Using immunofluorescence studies, we provide evidence that the majority of the nuclear ATR/ γ -H2AX foci that we observe 28 d after 8-MOP + UVA colocalize or associate with telomeric DNA and telomere-associated proteins. It could be shown that a single telomeric SDF correlated with long-term inhibition of proliferation in replicative senescent cells (Herbig *et al.*, 2004). It is therefore likely that the observed telomere-localized SDF are sufficient to maintain senescence in 8-MOP + UVA-treated fibroblasts. Interestingly, the vertebrate telomeric repeat sequence (TTAGGG) contains the sequence TA, which is a target for the generation of ICL by photoactivated psoralens and fixation of the telomeric t-loop by photoactivated psoralens has been used as a model facilitating electronmicroscopical analysis of this structure in HeLa cells (Griffith *et al.*, 1999). It is therefore likely that 8-MOP + UVA irradiation causes ICL formation at telomeres. However, the frequency of ICL induction, mechanisms of ICL repair, and its kinetics at telomeres are unknown to date. Hence, the few data available regarding DNA repair at telomeres suggest that it is less efficient, or even impaired (Kruk *et al.*, 1995; Petersen *et al.*, 1998).

Current definitions of cellular senescence differentiate telomere-independent (SIPS) and telomere-dependent (accelerated or replicative) forms, even though both result in similar phenotypic markers (von Zglinicki *et al.*, 2005). Common for both forms is that they represent maintained DNA damage response states. Senescence after 8-MOP + UVA cannot be easily categorized into one or the other form of senescence, because immunofluorescence analysis implies characteristics of SIPS early after PUVA-induced senescence, whereas at later time points telomere-mediated signaling may come into play. However, for both forms we identify a new role for ATR kinase in mediating psoralen DNA ICL-induced cellular senescence.

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