Published in final edited form as:

Mech Ageing Dev. 2007 ; 128(11-12): 637–649. doi:10.1016/j.mad.2007.09.005.

Increased ROS generation in subsets of OGG1 knockout fibroblast cells

Attila Bacsi^{a,§}, Grzegorz Chodaczek^a, Tapas K. Hazra^b, David Konkel^b, and Istvan Boldogh^{a,*}

^a Department of Microbiology and Immunology, University of Texas Medical Branch at Galveston, 301 University Blvd, Galveston, Texas 77555, USA

^b Department of Biochemistry & Molecular Biology, University of Texas Medical Branch at Galveston, 301 University Blvd, Galveston, Texas 77555, USA

Abstract

Oxoguanine DNA glycosylase (OGG1) is a major base excision repair protein responsible for excision of the mutagenic 8-oxoguanosine (8-oxoG) lesions from the genome. Despite OGG1's importance, the moderate phenotype of *Ogg1*-null (*Ogg1*^{-/-}) mice is not well understood. This study addresses a mechanism by which *Ogg1*^{-/-} cells limit accumulation of 8-oxoG in their genome. Our data reveal that a subset of *Ogg1*^{-/-} cells shows higher ROS levels (^HROS cells), while ~85% of *Ogg1*^{-/-} cells exhibit physiological levels of ROS (^LROS cells). *Ogg1*^{-/-} cells were sorted based on their DCF fluorescence intensity to obtain ^LROS and ^HROS cell cultures. ^LROS cultures proliferated at a rate comparable to *Ogg1*^{+/+} and gradually accumulated cells exhibiting increased ROS and 8-oxoG levels. ^LROS cells show a 2.8-fold increase in 8-oxoG level vs. ^HROS cells (7- to 27-fold). Mitochondria of ^HROS cells released more H₂O₂ than ^LROS and *Ogg1*^{+/+} cells and were eliminated by apoptotic-like processes. These findings suggest that in the absence of OGG1, a surveillance system is activated that removes cells with extreme 8-oxoG levels from *Ogg1*^{-/-} cultures. Whether similar mechanism exists in tissues of *Ogg1*^{-/-} mice is the focus of future investigations.

Keywords

Ogg1 null fibroblast; ROS; 8-oxoguanine; mitochondria

Introduction

Steady-state increases in oxidative DNA base modifications in the nuclear and mitochondrial DNA of aged cells and tissues have been extensively documented both in animal models and human subjects, as reviewed previously (Hamilton et al., 2001b; Van Remmen et al., 2003). Among DNA bases, guanine has the lowest redox potential, and so it is easily oxidized to 8-oxo-7,8-dihydroguanine (8-oxoG) via two-electron oxidation (Shukla et al., 2004). The key repair enzyme that excises 8-oxoG from cellular DNA (8-oxoG: C base pairs) is the 8-oxoguanine DNA glycosylase (OGG1) (Hazra et al., 1998). If the damaged base is not removed,

*Corresponding author. Tel.: +1 409 772 914; fax +1 409 474 6869. E-mail: E-mail: sboldogh@utmb.edu.

§Permanent address: Institute of Immunology, School of Medicine, University of Debrecen, Debrecen, H-4012 Hungary

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

8-oxoG may pair with adenine, causing G:C → T:A transversions after DNA replication (Cheng et al., 1992; Mitra et al., 1997). These mutations are often found in coding regions of tumor-promoting and tumor suppressor genes (Bos, 1989; Sigal and Rotter, 2000). Mutations may occur in the human *Ogg1* gene leading to polymorphisms and altered 8-oxoG and FapyG (ring-opened guanine) excision capacity of OGG1, thereby OGG1 appears to be etiologically involved in accumulation of mutations in the coding regions of DNA in head, neck, lung and kidney cancers (Blons et al., 1999; Shinmura and Yokota, 2001). The lack of efficient removal of 8-oxoG from DNA in tissues of patients with Cockayne syndrome implicates 8-oxoG in accelerated aging (Stevnsner et al., 2002).

Recently, *Ogg1*^{-/-} mice were generated (Klungland et al., 1999; Minowa et al., 2000). It has been shown that the levels of 8-oxoG in the nuclear and mitochondrial DNA of OGG1-deficient mice increases continuously over the whole life-span, especially under oxidative stress conditions (Arai et al., 2002; Klungland et al., 1999; Minowa et al., 2000; Nishimura, 2002; Osterod et al., 2001). Despite the increase in this potentially mutagenic, miscoding DNA lesion, *Ogg1*^{-/-} mice are only moderately prone to cancer, and show no marked age-associated pathological changes (Klungland et al., 1999; Sakumi et al., 2003). There are no significant differences in life-span between wild-type and OGG1-deficient mice, which appear phenotypically normal (Klungland et al., 1999).

Fibroblast cultures developed from *Ogg1*^{-/-} mice showed no major morphological changes and their cell cycle dynamics were similar to those of wild-type counterparts (Osterod et al., 2001). Up to date there are no studies addressing how *Ogg1*^{-/-} cells maintain steady state ~3-fold increase in 8-oxoG level in their nuclear DNA in the absence of OGG1, although they are continuously being damaged by exogenous/endogenous ROS. Here we show that in cultures of *Ogg1*^{-/-} there is a balance between low- and high-ROS-containing cells. High-ROS cells containing 7- to 27-fold increase in 8-oxoG levels exhibit mitochondrial dysfunction, and are eliminated by process(es) that share characteristics with apoptosis. These results suggest that a surveillance mechanism(s) is activated in response to supraphysiological levels of ROS and 8-oxoG, which limit the lifespan of *Ogg1*^{-/-} cells. These data raise the possibility that similar mechanism(s) could exist in tissues of OGG1 knockout mice.

2. Materials and Methods

2.1. Cell cultures

Ogg1^{-/-} and *Ogg1*^{+/+} primary mouse embryo fibroblast (MEF) cultures were established by standard procedures from individual C57BL/6J 129 embryos on embryonic day 13.5 (Klungland et al., 1999). Cells were cultured in DMEM/Ham's F-12 (3:1) with 10% FBS, genotyped by Southern hybridization, and permanent cell lines were established from individual transformed clones arising spontaneously after repeated passage in culture (Klungland et al., 1999). Immortalized *Ogg1*^{-/-} and *Ogg1*^{+/+} cell lines were kindly provided by Dr. Barnes (Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, United Kingdom). NTH1 (thymine glycol-DNA glycosylase)-knockout (*Nth1*^{-/-}) MEF cells were derived from individual C57BL/6J 129 *Nth1*^{-/-} embryos and continuously passaged to obtain spontaneously immortalized cells (Takao et al., 2002). *Nth1*^{-/-} and *Nth1*^{+/+} cell lines were kindly provided by Dr. Elder (Cancer Research UK, Carcinogenesis Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 4BX, United Kingdom). In our laboratory the cells were cultured in DMEM/Ham's F-12 (3:1) (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.2 mM glutamine (Elder and Dianov, 2002; Takao et al., 2002). *Ogg1*^{-/-} and *Ogg1*^{+/+} cells were cloned to establish single cell colonies that were further propagated to develop permanent cell lines. Individual clonal cell lines were further characterized. There were no differences between parent unselected

Ogg1^{-/-} fibroblast cultures and clonal cell lines in morphology, replication dynamics, cellular ROS and 8-oxoG levels. *Ogg1*^{-/-} L¹ROS and H¹ROS cell cultures were isolated by sorting (FACSaria, Becton Dickinson, Mountain View, CA) as we described below (2.5. third paragraph). These cells were maintained in DMEM/Ham's F-12 (3:1) (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.2 mM glutamine. At passage second the doubling time of L¹ROS and H¹ROS cells were 18.2±1.2 and 19.3±0.7 h, respectively. Since we did not know the exact number of population doubling levels (PDLs), the initial PD was arbitrarily set at zero. Cultures were initiated by seeding 2 × 10⁴ cells per cm² and at 90% confluence, the cells were removed by trypsinization, counted, and PDs calculated (PD = log(number of cells obtained at subculture per 2 × 10⁴)/log 2) (Szczesny et al., 2003).

2.2. Western blot analysis

Equal amounts of protein-containing cell lysates were electrophoresed on SDS-PAGE; transferred to a nitrocellulose membrane PROTRAN (Schleicher and Schuell BioScience, Keene, NH), and blocked in TBST (20 mM Tris-HCl {pH 7.6}, 137 mM NaCl, and 0.5 % Tween 20) containing 5% dry milk. The membranes were incubated with primary antibody against mouse OGG1 (Szczesny et al., 2003), at 1:500 dilutions in TBST+5% milk. After washing in TBS-T horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 anti-rabbit IgG-HRP; Amersham Biosciences, Piscataway, NJ) was added. Subsequently, membranes were washed and incubated in ECL Western blotting detection reagent (Amersham Biosciences). Chemiluminograms were analyzed using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

2.3. Immunohistochemistry

Cells were grown to 50% confluences on glass cover slips (25 mm), washed with PBS, dried, and fixed in acetone-methanol 1:1 (v/v). After exposure to pre-immune serum (0.1 µg in PBS containing 0.05% Tween 20 and 0.5% BSA; PBS-T), cells were incubated with primary antibody (anti-OGG1 or anti-FLAG) for 60 min. A fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, Inc) was then applied to cells for 60 min at 37°C and washed in PBS-T. Nuclei of cells were stained for 15 min with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; 10 ng/ml, Molecular Probes, Eugene, OR). Extensively PBS-T-washed cells were dried and then mounted in anti-fade medium (Dako Inc. Carpinteria, CA) on microscope slides (Boldogh et al., 2001). Fluorescent images were captured at magnification of ×60 using a Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope.

2.4. 8-oxoG assays

The 8-oxoG in nuclear DNA was quantified as we previously described (Bhakat et al., 2006). Briefly, cells on coverslip cultures were washed with PBS, air-dried, and fixed in acetone-methanol (1:1), rehydrated in PBS for 15 min, then sequentially treated with 100 µg/ml pepsin in 0.1 N HCl for 15 to 30 min at 37°C, 1.5 N HCl for 15 min, and sodium borate for 5 min. The cells were incubated with nonimmune IgG (100 µg/ml) for 30 min and washed in PBS containing 0.5% bovine serum albumin, 0.1% Tween 20 (PBS-T). After incubation with anti-8-oxoG antibody (Trevigen, Gaithersburg, MD; 1:200 dilutions) (Bespalov et al., 1999) for 30 min, the cells were washed three times with PBS-T for 15 min then exposed to fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. Cells were washed with PBS-T for 15 min (3-times) and their DNA stained with DAPI (10 ng/ml) for 15 min. The cells were air dried and mounted in anti-fade medium (Dako North America, Inc., Carpinteria, CA) on a microscope slide. The fluorescence intensities of a minimum of 40

cells per plate were determined using a Zeiss LSM510 META system, operated via MetaMorph software version 6.06r (Universal Imaging Corporation, Downingtown, PA).

The comet assay was performed according to the manufacturer's instructions with some modifications as we described previously (Bacsi et al., 2005; Boldogh et al., 2003). Briefly, cells were suspended in 0.75% low-melting point agarose (Trevigen, Inc., Gaithersburg) in PBS and spread on microscope slides (Trevigen, Inc., Gaithersburg). The cells in solidified agarose were lysed for 1 h at 4°C in lyses buffer (10 mM Tris-HCl, pH 10, 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100). DNA was released in the presence of NaI (Hamilton et al., 2001a) and treated with bacterial DNA formamidopyrimidine DNA glycosylase (Fpg, 1 µg/ml; New England Biolabs, Beverly, MA) in digestion buffer (40 mM HEPES, pH 8.0, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA) for 1 h at 37°C (Trzeciak et al., 2004). Electrophoresis was performed in alkaline electrophoresis buffer at 4°C for 30 min at 1 V/cm. The slides were then neutralized (0.4 M Tris-HCl, pH 7.5), washed, air-dried, and stained with 10 ng/ml of SYBR Green I (Trevigen, Inc., Gaithersburg). The comet moment images were recorded with a Nikon TE200 epifluorescence UV microscope photomicrographic system (Photometric CoolSNAP Fx camera). Fifty or more images were randomly selected for each data points and the tail moments were analyzed.

2.5. Measurement of Reactive Oxygen Species

2',7'-dichlorodihydro-fluorescein diacetate (H₂DCF-DA; Invitrogen Co., Carlsbad, CA), a redox-sensitive probe was used to determine cellular ROS levels (Bacsi et al., 2005; Boldogh et al., 2003). Cells on microscope cover-slips were placed in a thermo-controlled microscopic chamber and loaded with 10 µM (final concentration) H₂DCF-DA for 15 min. Cells were washed with medium pre-warmed to 37°C and fluorescent images captured using a Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope. Fluorescence intensities of >200 cells were determined by Metamorph™ Version 6.06r software (Universal Imaging Corporation).

To define the intracellular site(s) of ROS generation the cells were loaded with 2 µM dihydroethidium (H₂Et; Invitrogen Co., Carlsbad, CA) for 10 min and placed in a thermo-controlled microscopic chamber (Bacsi et al., 2006). MitoTracker Red (Invitrogen Co., Carlsbad, CA), a cell-permeable fluorescent probe that accumulates in active mitochondria was used to stain mitochondria at a final concentration of 25 nM. Images were captured with Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope. The microscope was operated and superimpositions were performed with Metamorph™ Version 6.06r software.

In selected experiments cells in suspension were loaded with H₂DCF-DA (2.5 µM, final concentration) for 15 min and changes in fluorescence intensities were then determined by flow cytometry (FACScanto, Becton Dickinson, Mountain View, CA). 15,000 cells were collected and analyzed for each sample. To generate nearly homogenous ^HROS and ^LROS cultures, *Ogg1*^{-/-} cells were loaded with 2.5 µM H₂DCF-DA and sorted using Becton-Dickinson FACS Aria cell sorter (Becton Dickinson, Mountain View, CA). Cells were cultured and purities of populations were assessed after 2 population doublings by flow cytometry.

Amplex Red assays were used to measure H₂O₂ production of isolated mitochondria (Bacsi et al., 2006). Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes) reacts with H₂O₂ in the presence of horseradish peroxidase to generate a stable fluorescent product, resorufin (Zhou et al., 1997). Briefly, isolated mitochondria (100 µg/ml) were suspended in 50 µl (per well) reaction buffer containing pyruvate (5 mM) plus malate (5 mM) as well as succinate (10 mM) and incubated at room temperature (25°C) for 15 min with 0.25 U/ml of Amplex® Red (determined in preliminary studies) and 1 U/ml of horseradish peroxidase. The

change in fluorescence (excitation 563 nm; emission 587 nm) was measured using a microplate reader (SpectraMass M2, Molecular Devices Corporation, Downingtown, PA). In selected experiments, 3-nitropropionic acid (3-NPA; 1 mM, pH balanced) – inhibitor succinate dehydrogenase in complex II, rotenone (5 μ M) – inhibitor of NADH-decylubiquinone reductase activity of complex I and antimycin A (0.4 μ M) – inhibitor of cytochrome *b* reoxidation in complex III, were added alone or in combination to the reaction mixtures. In control, the H₂O₂ production was linear with mitochondrial protein concentration. Reactions were carried out $\pm$ exogenously added superoxide dismutase (SOD) that converts superoxide anion (O₂^{•-}) into H₂O₂. The addition of catalase (400 U/ml) decreased H₂O₂ levels by ~90 percent. As a positive control, increasing dilutions (0 to 4000 pM) of H₂O₂ were used.

2.6. Simultaneous analysis of cell cycle and cellular ROS levels

To sort out whether the increased ROS levels were associated with a particular cell cycle stage we loaded cells with Vybrant DyeCycle Violet stain (Invitrogen Co., Carlsbad, CA) (Telford et al., 2007) and H₂DCF-DA simultaneously as previously described (Boldogh, 2003). Vybrant DyeCycle Violet stain (excitation/emission maxima ~396/437 nm) is cell membrane permeant dye and nonfluorescent until bound to double-stranded DNA. Briefly, actively replicating Ogg1^{+/+}; Ogg1^{-/-}, H⁺ROS and L⁺ROS cells (2 $\times$ 10⁶ cells per ml) were suspended in Hanks' balanced saline solution containing 2% FBS and 2 mM HEPES (pH: 7.4) and loaded with Vybrant DyeCycle Violet (10 μ g per ml) and H₂DCF-DA (5 μ M, final concentration) for 15 min. Extracellular probes were removed by washing cells in Hank's solution and cells were analyzed by Flow cytometry (FACScanto, Becton Dickinson, Mountain View, CA). 30,000 cells were collected and analyzed for each sample.

2.7. Mitochondria Isolation

Cells were propagated in large volumes, collected by centrifugation (800 $\times$ g), and mitochondria were isolated as we described previously (Bacsi et al., 2006). Briefly, cell pellets were incubated in 10 $\times$ volume of hypotonic buffer (10 mM KCl, 20 mM MOPS, and 1 mM EGTA; ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid) for 20 min. Sucrose (200 mM) and mannitol (50 mM) were added to the swollen cells, which were then Dounce-homogenized. The homogenate was centrifuged at 800 $\times$ g and the supernatants re-centrifuged at 10,000 $\times$ g to collect mitochondria. Mitochondrial pellets were washed, and resuspended in 10 mM KCl, 20 mM MOPS and 1 mM EGTA containing 200 mM sucrose, 50 mM mannitol. In selected experiments fresh mitochondrial suspensions were purified on a continuous sucrose gradient (0.25M to 1.5M) and used immediately for determining the levels of released H₂O₂.

2.8. Oxygen Consumption

The oxygen consumption rates of mitochondria were determined at 30°C with a Clark-type oxygen electrode (Strathkelvin Oxygen System Model 782, Strathkelvin Instruments, United Kingdom) as we previously described (Bacsi et al., 2006). Purified mitochondria (0.2 mg) were suspended in 1 ml respiration medium (125 mM KCl, 20 mM HEPES; pH 7.4), 5 mM potassium phosphate, $\pm$ substrates (5 mM pyruvate plus 5 mM malate as well as 10 mM succinate). The signal from the oxygen sensor was recorded on a computer at sampling intervals of 0.5 seconds with the aid of software from Strathkelvin Instruments (782 System V3.0). Respiration was measured without ADP (state IV) and with 0.5 mM ADP (state III) (Bacsi et al., 2006; Chen et al., 2003). Mitochondria suspension showing a higher than 3 respiratory control ratio were used.

2.9. Transfection

cDNA encoding human OGG1 was originally cloned into *E. coli* expression plasmid pRSETB (Dr. Sankar Mitra, University of Texas Medical School, Galveston, TX). OGG1 encoding sequences were re-cloned into the pcDNA3.0-neo expression plasmid (He et al., 2002) and kindly provided by Dr. MR Kelley (Herman B. Wells Center for Pediatric Research, and Biochemistry and Molecular Biology, Indiana University Medical School, Indianapolis, Indiana). Sequence identity of OGG1 was confirmed by direct sequencing (Molecular Genomic Core Facility University of Texas Medical School, Galveston, TX). At 75% confluence, *Ogg1*^{-/-} and *Ogg1*^{+/+} cells were transfected with 0.25 µg of OGG1-encoding expression vector. As control, parallel cultures of cells were transfected with 0.25 µg of empty plasmid DNA (pCDNA.3.0-neo). The cells were subcultured 24 h after transfection and fresh medium containing 350 µg/ml of G418 (Invitrogen Co., Carlsbad, CA) was added. *Ogg1*^{-/-} cell colonies resistant to G418 were grown and screened for OGG1 expression by immunostaining.

2.10. Annexin V Assay

Flow cytometric analyses with annexin V-PE and 7AAD-stained cells were performed as we described previously (Boldogh et al., 2003). Briefly, cells were trypsinized, washed in PBS and resuspended in cold annexin V binding buffer (10 mM HEPES pH 7.4, 0.14 M NaCl, 0.25 mM CaCl₂·2H₂O, 0.1% BSA (w/v); anti-annexin V-PE-conjugated antibody (Becton Dickinson) was then added to the cells for 30 min. Cells were sedimented and 7AAD was added (5 µg/ml per sample). The cells were analyzed on a FACScanto flow cytometer (Becton Dickinson, Mountain View, CA). Each data point represents the mean fluorescence for 12,000 cells from three or more independent experiments; expressed ±SEM.

2.11. Assessment of ATP levels

Cellular ATP levels were measured by a bioluminescence assay employing the luciferase enzyme using the ATP Determination Kit (Invitrogen Co., Carlsbad, CA). Prior to measurement of ATP levels annexin V positive cells were removed from the cell population by flow cytometric sorting. The cells (3 × 10⁶/ml) were collected by centrifugation and ATP was then extracted by adding 100 µl of boiling distilled water to the pellet and heated at 100°C (Isotemp 125 D, Fisher Scientific, Pittsburg, PA) for 3 min. The cell lysates were then centrifuged at 12,000 × g for 5 min at 4°C (Silent SPIN, Continental Lab Products, Edison, NJ). Luminescences corresponding to ATP concentrations in the supernatant fluid were determined in a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA).

2.12. Caspase activity measurement

Cells were grown to 80% confluence washed and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% {v/v} Triton X-100 and 10 mM sodium pyrophosphate). The lysates were incubated with 20 µM Ac-DEVD-AMC (Becton-Dickinson) in reaction buffer (20 mM HEPES, 10% (v/v) glycerol, 2 mM dithiothreitol) at 37°C for 1 h. Changes in fluorescence intensity were determined at 380 nm (excitation) and 435 nm (emission) wavelengths in an FLx800 microplate reader (Bio-Tek Instruments, Winooski, VT). Caspase-3-like activities were expressed as pmoles of AMC (7-amino-4-methyl coumarin) released from milligram of protein per 60 min (Boldogh, 2003).

2.13. Reagents

Anti-OGG1 antibody (OGG1-2672) was generated against the purified OGG1 polypeptide (Alpha Diagnostic International, San Antonio, TX). This affinity-purified antibody reacts with both human and mouse OGG1 in Western blot and immunohistochemical analysis (Bhakat et al., 2006; Szczesny et al., 2003). Antimycin A, ATP, catalase, H₂O₂, malate, pyruvate, rotenone, succinate and SOD were purchased from Sigma-Aldrich (St. Louis, MO). 3-NPA

(Aldrich Chemical Co., Milwaukee, WI) was dissolved in PBS and the pH was adjusted to 7.4 with 1 M NaOH (Bacsi et al., 2006). The osmolality of pH-balanced 3-NPA solution was determined using an Advanced Osmometer (SIM International Co).

2.14. Statistical Analysis

Results were analyzed for significant differences using ANOVA procedures and Student's t-tests (Sigma Plot 6.0). Data are expressed as the mean $\pm$ SE. Results were considered significant at $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

3. Results

3.1. A subset of *Ogg1*^{-/-} cultures show extreme levels of 8-oxoG

Ogg1^{-/-} cells showed a 3.2 $\pm$ 0.2-fold increase in 8-oxoG levels in the nuclear DNA compared to the corresponding wild-type *Ogg1*^{+/+} cells. In *Nth1*^{-/-} cells, which are devoid of NTH1 glycosylase involved in the repair of oxidized pyrimidines (Ikeda et al., 1998) the 8-oxoG level was similar to *Ogg1*^{+/+} cells (Fig. 1A). These results are consistent with the permanent absence of OGG1 (Fig. 1A, inset) in *Ogg1*^{-/-} cells. Interestingly, 8-oxoG levels were increased by 7- to 27-fold in subsets (12.4 $\pm$ 1.5%) of *Ogg1*^{-/-} cells compared to the *Ogg1*^{+/+} cells.

Representative images of 8-oxoG-mediated fluorescence are shown in Fig. 1B. The low- and high 8-oxoG-containing cells appear to be in equilibrium, because their percentages did not change with increasing passage number. These data raise the possibility that *Ogg1*^{-/-} fibroblast cultures are heterogeneous in regard to maintenance of 8-oxoG in their genome. Therefore, we isolated cell lines of single cell origin. In all eight clonal *Ogg1*^{-/-} cell lines, 11 to 12.5 percent of the cells showed increased 8-oxoG levels (7- to 27-fold, compared to *Ogg1*^{+/+}) similarly to the parent *Ogg1*^{-/-} cells as determined by fluorescent imaging (data not shown). Data shown are from experiments with the parent *Ogg1*^{-/-} fibroblast cells.

To confirm increased levels of 8-oxoG in DNA of *Ogg1*^{-/-} cells, we used single cell DNA electrophoresis (Boldogh et al., 2003; Olive et al., 1991) combined with Fpg digestion (Banath et al., 1999; Trzeciak et al., 2004). In control (without Fpg), only small differences were observed between comet tail moments of *Ogg1*^{-/-} and *Ogg1*^{+/+} cells (Fig. 1C). Treatment of DNA with Fpg resulted in 4.2-fold difference between Comet tail moments of *Ogg1*^{-/-} and *Ogg1*^{+/+} cells (Fig. 1C). These data suggest that the increased tail moment was due to 8-oxoG and FapyG (2,6-diamino-4-hydroxy-5-formamidopyrimidinine; a ring opened 8-oxoG product) lesions in the DNA and not to increased levels of DNA single-strand breaks in the *Ogg1*^{-/-} cells. In repeated experiments, 11.4 $\pm$ 2% of Comet tail moments were larger (Fig. 1D, double arrow head) compared to the majority of Comet moments of DNA from *Ogg1*^{-/-} cells (Fig. 1D, single arrow head). When compared to DNA from *Ogg1*^{+/+} cells the Comet tails moments showed 7 to 27-fold increase (Fig. 1E). Data from Comet assays are consistent with the increased reactivity of anti-8-oxoG antibody with the oxidized guanine base lesions in the DNA.

3.2. Increased ROS levels in subsets of *Ogg1*^{-/-} cells

To investigate whether the excessive levels of 8-oxoG in subsets of *Ogg1*^{-/-} cultures were due to increased levels of ROS, cells were loaded with H₂DCF-DA and fluorescence intensities were assessed microscopically. We observed that 16 $\pm$ 5% of the cells showed increased DCF fluorescence (high-ROS: ^HROS cells), while ~84% of the cells had lower oxidative stress levels (low-ROS: ^LROS cells), which were similar to *Ogg1*^{+/+} cells (Fig. 2A,B). The percentage of ^HROS and ^LROS cells did not significantly change during subculturing. In *Nth1*^{-/-} and *Ogg1*^{+/+} cultures only <1% of cells showed increased DCF fluorescence (Fig. 2B). When ROS levels were assessed by flow cytometry there were no significant differences in overall ROS levels between *Ogg1*^{-/-} and *Ogg1*^{+/+} cells (Fig. 2C and D upper panel). However; there was

a distinct *OggI*^{-/-} cell population showing increased DCF fluorescence. Frequencies of ^HROS cells were nearly identical in the parent *OggI*^{-/-} and in sub-cell lines of single cell origin. The increased intracellular oxidative stress levels showed no correlation with cell cycle stages as determined by simultaneous measurements of DNA content and DCF fluorescence (data not shown).

^LROS and ^HROS cells were separated by cell-sorting based on DCF fluorescence intensity. ROS levels of sorted cells were assessed by flow cytometry at PDL 2. There was an approximately 2-fold increase in oxidative stress levels in ^HROS vs. ^LROS cells (Fig. 2C and Fig. 2D lower panel). When ^HROS cells were propagated further they showed 2.2- and 2.7-fold increase in ROS levels, at PDL-6 and PDL-11, respectively compared to *OggI*^{+/+} cells (Fig. 2E). At PDL-17 the ROS levels in ^HROS exceeded 3.5-fold of *OggI*^{+/+} cells. ^HROS cells proliferated slowly and cultures were lost between PDL-19 and -23. In cells maintained in N-acetyl-L-cysteine (5 mM)-containing media cellular ROS levels were significantly lower (Fig. 2E). Addition of BSO (100 μ M), a known inhibitor of GSH synthesis (Anderson, 1998) to the culture medium increased intracellular levels of ROS and decreased cell viability (data not shown). At PDL-2 ^HROS cells were exhibiting more than 7-fold increase in 8-oxoG levels, while at PDL-17, 8-oxoG levels were more than 22-times higher than in *OggI*^{+/+} cells (data not shown). The increase in 8-oxoG levels in ^HROS cultures correlated well with increased Comet tail moments (Fig. 2F).

In ^LROS cultures, the percentage of cells showing increased DCF fluorescence increased as a function of PDL. For example, at PDL-12, 6.3%; PDL-25, 11.7%; and PDL-32, ~14% of cells showed increased oxidative stress levels (Fig. 2G). Additional culturing of these cells resulted in no further increase in the percentage of ^HROS cells, and now they were similar to that of unsorted *OggI*^{-/-} cell cultures. ^LROS subsets at PDL-2 exhibited 2.8-fold elevation in 8-oxoG levels compared to *OggI*^{+/+} cells. The number of cells showing >7-fold elevation in 8-oxoG levels was proportional to the percentage of ^HROS cells. These data together suggest that ^HROS-high 8-oxoG containing cells are continuously generated (and eliminated), leading to an equilibrium between ^LROS and ^HROS cells.

3.3. Increased ROS production from mitochondria

To identify the site of ROS production, cells were placed in a microscopic culture chamber and loaded with 2 μ M dihydroethidium (H_2Et) (Bacsi et al., 2006). The green fluorescence mediated by H_2Et /superoxide reaction products (Zhao et al., 2003) co-localized with MitoTracker Red suggesting that the mitochondria are the sites of ROS generation (Fig. 3A). Suspensions of *OggI*^{-/-} cultures were sorted for ^LROS and ^HROS subsets and the individual cultures were further propagated. Mitochondria were isolated at PDL-10 to determine the amount of H_2O_2 originating from $O_2\cdot^-$ dismutation by SOD. Intact mitochondria from unsorted *OggI*^{-/-} and *OggI*^{+/+} cells oxidizing from pyruvate/malate (Complex I substrates) plus succinate (Complex II substrate) released nearly equal amounts of H_2O_2 (Fig. 3B). On the other hand, mitochondria from ^LROS generated quantitatively less H_2O_2 than mitochondria from ^HROS subsets (Fig. 3B). Addition of rotenone, an inhibitor of NADH-decylubiquinone reductase activity of complex I, or 3-NPA, an inhibitor of succinate dehydrogenase to mitochondrial suspension only decreased (data not shown), while addition of rotenone and 3-NPA together inhibited H_2O_2 production from mitochondria of ^LROS and ^HROS cells (Fig. 3B). On the other hand, antimycin A, an inhibitor of cytochrome *b* reoxidation in complex III generated significantly higher amounts of H_2O_2 from the mitochondria of ^HROS cells compared to ^LROS cells (Fig. 3B). Addition of catalase decreased H_2O_2 to an undetectable level under our assay conditions.

3.4. ^HROS cells are eliminated from cell cultures

Microscopic observation of cultures showed significantly higher cell death in *Ogg1*^{-/-} cultures than in *Ogg1*^{+/+} or *Nth1*^{-/-} ones. Results show that 9.2±2 % of unsorted *Ogg1*^{-/-} cells bound annexin V (Fig. 4Aa) and of these 24.5±1.1% also incorporated 7-amino actinomycin D (7AAD; data not shown), an indicator for loss of cell membrane integrity. Less than 1% of *Nth1*^{-/-} and *Ogg1*^{+/+} cells bound annexin V and ~0.1% incorporated 7AAD (Fig. 4Aa). Next, *Ogg1*^{-/-} cells were sorted for ^LROS and ^HROS subsets. Percentage of annexin V positive cells was ~1 % in ^LROS cell populations at the time of sorting. Further subculturing of ^LROS cells, resulted in a gradual increase in annexin V positive cells (Fig. 4Ab). For example, at PDL-16 there were 7.5%, while at PDL-24 and higher PDLs it increased to the levels of unsorted *Ogg1*^{-/-} (Fig. 4Ab). When ^HROS cells were further cultured the frequency of annexin V positive cells increased reaching more than 60% by PDL-17 (data not shown). At this or higher PDLs cells did not replicate further and the cultures were gradually lost.

Next, we determined changes in caspase-3 activity of ^HROS and ^LROS cells at increasing PDLs. Results summarized in Fig. 4B illustrates that unsorted *Ogg1*^{-/-} cell cultures showed no difference compared to *Ogg1*^{+/+} cells in caspase-3 activity. On the other hand, caspase-3 activity was significantly higher in ^HROS cultures at PDL-12 than at PDL-2 (Fig. 4B). Near the end of the ^HROS cells' life-span (PDL-17), their caspase-3 activity was nearly 12.5-fold higher than in unsorted *Ogg1*^{-/-} cells and 14-fold higher than in *Ogg1*^{+/+} cultures (Fig. 4B). From PDL-12 on, an increasing percentage of ^HROS cells displayed enlargement, irregular, bizarre morphology that did not resemble typical apoptosis (Fig. 4C, left and middle panel). For example, no cellular shrinkage or nuclear fragmentation could be observed (the latter shown by nuclear staining with DAPI, a DNA-binding fluorophore - data not shown). Fig. 4C (right panel) shows the morphology of *Ogg1*^{+/+} cells. Since typical apoptotic processes require ATP (Nicotera and Leist, 1997; Skulachev, 2006), we determined whether a decrease in ATP levels is related to this unusual cell elimination. Figure 4D shows that *Ogg1*^{-/-}, *Ogg1*^{+/+}, ^LROS (PDL-6, PDL-10) and ^HROS (PDL-6, PDL-10) cells contain physiological levels of ATP (62±6 nmol per mg protein). However, ^HROS cells close to the end of their life-span (PDL-17) contained significantly less ATP (Fig. 4D). Prior to measurement of ATP levels annexin V positive cells were discarded after flow cytometric sorting of cell suspensions.

3.5. Transgenic expression of OGG1 decreases 8-oxoG and ROS levels

Parallel cultures of ^LROS *Ogg1*^{-/-} and *Ogg1*^{+/+} cells were transfected with OGG1 expressing or empty vectors and G418-resistant clones were isolated and individually maintained. OGG1 polypeptide primarily localized to nuclei of cells (Fig. 5A, lower panels). Unexpectedly, a large number of OGG1-transfected clones were lost expressing OGG1 protein. A few clones of OGG1-expressing cells were viable up to PDL-8 and then underwent crises, and were lost or reverted back to OGG1 non-expressing phenotype (*Ogg1*^{-/-}). Overexpression of OGG1 in *Ogg1*^{+/+} cells or vector alone in *Ogg1*^{-/-} did not affect cell viability. The cause of this unexpected behavior of OGG1-transfected *Ogg1*^{-/-} clones is currently being investigated. Cells displaying normal morphology were examined for 8-oxoG and ROS levels. The ROS levels in OGG1-expressing cells (PDL-6, -7 and -8) were comparable to *Ogg1*^{+/+} cell cultures as observed by microscopic imaging of H₂DCF-DA-loaded cells (data not shown). In the DNA of individual OGG1-expressing cells, 8-oxoG levels were nearly identical to the level of 8-oxoG found in *Ogg1*^{+/+} cells (Fig. 5B) in line with the physiological levels of ROS.

4. Discussion

8-oxoG and FapyG are the most abundant forms of ROS-induced guanine base damage in the DNA. The relatively high abundance of these DNA base lesions is due to guanine's low redox potential (Candeias and Steenken, 2000). Accumulation of oxidatively damaged guanines in

the DNA has been proposed to play a major role in aging-associated diseases and the aging process itself (Hamilton et al., 2001b; Shigenaga et al., 1994; Van Remmen et al., 2003). Extracts from tissues of homozygous *Ogg1*^{-/-} mice do not excise 8-oxoG so it is accumulated in a tissue- and cell-type specific manner in both nuclear and mitochondrial DNA (Klungland et al., 1999; Minowa et al., 2000). Fibroblast cultures developed from *Ogg1*^{-/-} animals contain a steady-state levels of 8-oxoG; however, there are no studies addressing how the steady-levels of 8-oxoG in nuclear DNA are maintained in the absence of OGG1. Our data show that the *Ogg1*^{-/-} cell line consists of and can be separated into the predominant ^LROS and ^HROS subsets, the latter showing up to 27-fold increase in 8-oxoG levels (compared to the *Ogg1*^{+/+} cells). ^HROS cells showed mitochondrial dysfunction, and a limited life-span. ^LROS *Ogg1*^{-/-} cells proliferate with a rate comparable to *Ogg1*^{+/+} and gradually regenerate cells exhibiting ^HROS phenotype with high 8-oxoG levels.

Due to environmental and endogenous oxidative stress we would expect continuously increasing 8-oxoG levels in the DNA of the *Ogg1*^{-/-} cell populations. Our in depth investigations showed that a subset of the *Ogg1*^{-/-} cells exhibit much higher (up to 27-fold) levels of 8-oxoG in their DNA than the majority (85%) of cells. 8-oxoG levels were determined by two independent but complementary methods. In individual cells, 8-oxoG levels were assessed by a fluorescent immunocytochemical procedure, which employs a well-characterized, 8-oxoG DNA lesion-specific monoclonal antibody (Bespalov et al., 1999). These results were confirmed by comet assay carried out on DNA treated with Fpg, which predominantly excises 8-oxoG and FapyG (Banath et al., 1999).

In the majority of *Ogg1*^{-/-} cells 8-oxoG levels are maintained at a relatively low (~3-fold over *Ogg1*^{+/+}) level. This raises the possibility that NEIL1 and/or NEIL2, recently discovered DNA glycosylases (Dou et al., 2003; Hazra et al., 2002) have a role in maintaining steady-state 8-oxoG/FapyG levels in the DNA. NEIL1 activity has been shown to be present in various organs of *Ogg1*^{-/-} mice (Hu et al., 2005). However, NEIL1's (replication coupled repair protein) activity in cultured *Ogg1*^{-/-} fibroblast may be dispensable because there was no significant accumulation of the 8-oxoG/FapyG observed in *Ogg1*^{-/-} cells when they were kept confluent and proliferation was arrested (Osterod et al., 2001). Moreover, removal of oxidative base modifications induced by photosensitization were 4-fold slower in *Ogg1*^{-/-} than in *Ogg1*^{+/+} cells and no differences were found between confluent and proliferating fibroblasts (Osterod et al., 2001). These results suggest that the NEIL1- or NEIL2-initiated replication or transcription-coupled repair of 8-oxoG and/or FapyG residues is not significant in these cells. Thus, steady-state levels of oxidized guanines cannot be fully explained by the existence of these alternative repair pathway(s) in the absence of OGG1 (Osterod et al., 2001; Russo et al., 2006).

Increased DCF fluorescence allowed us to sort *Ogg1*^{-/-} cells to establish cultures containing low or high ROS levels. The fluorescence intensity in ^LROS cells were 2-times lower than in ^HROS ones. When the nearly homogenous ^LROS cells were serially passaged, unexpectedly, the ^HROS cells appeared again with significantly higher 8-oxoG levels (up to 27-fold) and their percentage reached a level similar to unsorted *Ogg1*^{-/-} cells by PDL-32. Microscopic imaging showed that fluorescence mediated by H₂Et/superoxide reaction products (Zhao et al., 2003) were localized primarily to mitochondria. Consistent with these results, we found a markedly increased H₂O₂ release from mitochondria of ^HROS cells vs. ^LROS and unsorted *Ogg1*^{-/-} or *Ogg1*^{+/+} cells. The amounts of H₂O₂ released from mitochondria of unsorted *Ogg1*^{-/-} and *Ogg1*^{+/+} cells were nearly identical. These latter observations are consistent with the functionally normal mitochondria isolated from livers and hearts of *Ogg1*^{-/-} mice, despite their 20-fold higher levels of 8-oxoG (de Souza-Pinto et al., 2001). Thus it appears that without isolating subsets of cells from organs or tissues, mitochondrial respiratory dysfunction cannot be detected.

An increased ROS level alone is sufficient to trigger intrinsic pathways that culminate in mitochondrial dysfunction, release of cytochrome c and other factors leading to cell death (Liu et al., 1996). NAC in the culture medium decreased, while BSO increased ROS levels and the frequency of lost cells, which strongly suggest implication of ROS in cell death processes. ^HROS subset of *Ogg1*^{-/-} cultures exhibited mitochondrial dysfunction indicated by increased formation of H₂O₂. These cells showed increased caspase-3 activity; further implicating ROS in the elimination of these cells from the population. However, these ^HROS cells were not eliminated by typical apoptotic processes, in which caspase-mediated proteolysis and DNase activities result in shrinkage of cells and nuclear fragmentation as shown previously (Zakeri and Ahuja, 1997). Therefore, we termed this type of cell death, apoptosis-like. Apoptosis-like cell death in response to oxidative stress and DNA damage have been described in yeast and mammalian cells as well (Burhans et al., 2003; Malecki, 2001).

It is well established that apoptosis requires ATP for the execution of steps in its ordered program (Nicotera and Leist, 1997). In the presence of dATP apoptosomes are formed, which in turn activate caspase-9 and caspase-3 (Liu et al., 1996). Our data showed significantly lower ATP levels in ^HROS cells near the end of their life-span (e.g., PDL-16, -18). Therefore the absence of typical morphological apoptotic changes (e.g., nuclear fragmentation) is not surprising. It is possible that in the presence of decreased ATP levels in ^HROS cells; the death-pathway share initial common events with apoptosis, such as increase in caspase-3 activity (at higher PDLs of ^HROS cells) until they reach energy exhaustion as shown in other systems (Liu et al., 1996; Nicotera and Leist, 1997).

8-oxoG is one of the most mutagenic lesions in DNA resulting in genomic instability, which may lead to reversal of immortalization in subsets of *Ogg1*^{-/-} cells or accumulation of tumorigenic mutation(s) both inducing senescence-like response (Reed, 1999; Sager, 1991). Indeed, near the end of their lifespan ^HROS cells were enlarged and underwent cell cycle arrest, much like senescent cells. It has been shown that senescent cells become resistant to apoptotic signals (Campisi, 2003), so it is possible that ^HROS subset of *Ogg1*^{-/-} cultures containing extreme levels of 8-oxoG and increased ROS levels is eliminated by senescence-related apoptotic processes.

It has been shown that transgenic expression of OGG1 in *Ogg1*^{-/-} cells restored the wild-type phenotype in response to all pro-oxidants tested (Smart et al., 2006). In our hand, overexpression of OGG1 decreased 8-oxoG levels in the DNA and prevented development of ^HROS cells in the surviving clones of OGG1 expressing cells. These data suggest that supraphysiological levels of 8-oxoG in the DNA could be responsible for increased levels of ROS and cell death. Our most intriguing finding was that we were not able to develop stably-transfected OGG1 expressing cell lines from *Ogg1*^{-/-} cells. These OGG1-expressing cells undergo typical apoptotic processes (including e.g., shrinkage of cells, nuclear fragmentation). On the other hand, the viability of *Ogg1*^{+/+} cells was not affected when stably transfected with the pcDNA3.0-neo expressing OGG1. Similarly, G418 resistant *Ogg1*^{-/-} cells were viable after transfection with empty pcDNA3.0-neo vector. We speculate that OGG1 protein resulted in supraphysiological levels of nicks and single-strand breaks in the DNA of *Ogg1*^{-/-} cells, which cause apoptosis and so led to loss of cell clones. This hypothesis is supported by data showing DNA damage-induced apoptosis (Zabkiewicz and Clarke, 2004). Another possibility is that 8-oxoG excised from DNA of OGG1-expressing cells may induce apoptosis. In support of the latter possibility, it has been reported that KG-1 human acute leukemia cells that have mutational loss of OGG1 activity and accumulate supraphysiological level of 8-oxoG into DNA like *Ogg1*^{-/-} cells undergo G1-phase cell cycle arrest and apoptotic death after treatment with 8-oxoG (Hyun et al., 2006). However, it appears that increased 8-oxoG levels in the DNA of KG1 and *Ogg1*^{-/-} cells do not interfere with transcription and DNA replication (Larsen et al., 2004). This assumption was supported by Arai et al., 2002, who demonstrated that

KBrO₃-treated *Ogg1*^{-/-} liver cells showing more than a 70-fold increase in oxidative guanine lesions regenerated normally (Arai et al., 2002). In line with these observations, 8-oxoG failed to block RNA polymerase II at the lesion in experiments with purified transcription factors *in vitro* (Larsen et al., 2004). Similarly, 8-oxoG only slightly inhibited T7 RNAPol II-mediated transcription *in vitro* and complete lesion bypass was observed when the 8-oxoG lesion was positioned in the non-transcribed strand (Tornaletti et al., 2004).

In conclusion, we show here that *Ogg1*^{-/-} cultures consist of two subpopulations ^LROS and ^HROS cells. ^HROS subsets of *Ogg1*^{-/-} cells show supraphysiological levels of 8-oxoG in their DNA, mitochondrial dysfunction, decreased ATP synthesis and elimination by apoptosis-like processes, which could be responsible for preventing unlimited accumulation of 8-oxoG in DNA of *Ogg1*^{-/-} cell cultures. The most intriguing question is how the ^HROS subset of *Ogg1*^{-/-} cells arises when the cells are clonal in nature. We propose that in the absence of OGG1, *Ogg1*^{-/-} cells are genetically more unstable than *Nth1*^{-/-} or *Ogg1*^{+/+} cells due to accumulation of oxidized guanines that are considered one of the most pro-mutagenic lesions. These mutations are randomly distributed and their particular combinations may cumulate in altered phenotypes such as ^HROS cells. Altered availability and/or changes in structure and functions of mutated proteins in the respiratory chain result in increased ROS production and thereby more oxidative DNA damage to guanines (and other DNA bases) and additional mutations. Increased ROS production and excessive DNA damage is sufficient to induce processes required for elimination of cells via apoptosis or apoptosis-like processes as described in other cell culture models (Dobson et al., 2000; Ruchko et al., 2005). In *Ogg1* knock out mice, oxidative damage to guanines are accumulating in a tissue dependent manner especially under oxidative stress conditions (Arai et al., 2002; Klungland et al., 1999; Minowa et al., 2000; Nishimura, 2002; Osterod et al., 2001), thus further studies are required to explore whether similar phenomena exist *in vivo* in tissues and organs of these animals.

Acknowledgments

This work was supported by P01 AG 021830 (I.B., D.K., T.H) from the NIH/NIA, P01 AI062885-01 (I.B) from the NIAID, NIH/NCI RO1 CA102271-01A (T.H), and NIEHS Center Grant, EOS 006677.

Abbreviations

3-NPA	3-nitropropionic acid
8-oxoG	8-oxo-7,8-dihydroguanine
AA	antimycin A
BER	base excision repair
Fpg	formamidopyrimidine DNA glycosylase
H₂DCF-DA	2',7'-dichlorodihydro-fluorescein diacetate
H₂Et	dihydroethidium
NTH1	

	thymine glycol-DNA glycosylase
OGG1	8-oxoguanine DNA glycosylase
PDL	population doubling level
SOD	superoxide dismutase
ROS	reactive oxygen species
^LROS	cells showing physiological ROS levels
^HROS	cells showing supraphysiological levels of ROS

References

- Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact* 1998;111–112. 1–14.
- Arai T, Kelly VP, Minowa O, Noda T, Nishimura S. High accumulation of oxidative DNA damage, 8-hydroxyguanine, in Mmh/Ogg1 deficient mice by chronic oxidative stress. *Carcinogenesis* 2002;23:2005–2010. [PubMed: 12507922]
- Bacsi A, Kannan S, Lee MS, Hazra TK, Boldogh I. Modulation of DNA-dependent protein kinase activity in chlorambucil-treated cells. *Free Radic Biol Med* 2005;39:1650–1659. [PubMed: 16298690]
- Bacsi A, Woodberry M, Widger W, Papaconstantinou J, Mitra S, Peterson JW, Boldogh I. Localization of superoxide anion production to mitochondrial electron transport chain in 3-NPA-treated cells. *Mitochondrion* 2006;6:235–244. [PubMed: 17011837]
- Banath JP, Wallace SS, Thompson J, Olive PL. Radiation-induced DNA base damage detected in individual aerobic and hypoxic cells with endonuclease III and formamidopyrimidine-glycosylase. *Radiat Res* 1999;151:550–558. [PubMed: 10319728]
- Bespalov IA, Bond JP, Purmal AA, Wallace SS, Melamede RJ. Fabs specific for 8-oxoguanine: control of DNA binding. *J Mol Biol* 1999;293:1085–1095. [PubMed: 10547287]
- Bhakat KK, Mokkapaty SK, Boldogh I, Hazra TK, Mitra S. Acetylation of human 8-oxoguanine-DNA glycosylase by p300 and its role in 8-oxoguanine repair in vivo. *Mol Cell Biol* 2006;26:1654–1665. [PubMed: 16478987]
- Blons H, Radicella JP, Laccourreye O, Brasnu D, Beaune P, Boiteux S, Laurent-Puig P. Frequent allelic loss at chromosome 3p distinct from genetic alterations of the 8-oxoguanine DNA glycosylase 1 gene in head and neck cancer. *Mol Carcinog* 1999;26:254–260. [PubMed: 10569802]
- Boldogh I, Milligan D, Lee MS, Bassett H, Lloyd RS, McCullough AK. hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs. *Nucleic Acids Res* 2001;29:2802–2809. [PubMed: 11433026]
- Boldogh I, Roy G, Lee MS, Bacsi A, Hazra TK, Bhakat KK, Das GC, Mitra S. Reduced DNA double strand breaks in chlorambucil resistant cells are related to high DNA-PKcs activity and low oxidative stress. *Toxicology* 2003;193:137–152. [PubMed: 14599773]
- Bos JL. ras oncogenes in human cancer: a review. *Cancer Res* 1989;49:4682–4689. [PubMed: 2547513]
- Burhans WC, Weinberger M, Marchetti MA, Ramachandran L, D'Urso G, Huberman JA. Apoptosis-like yeast cell death in response to DNA damage and replication defects. *Mutat Res* 2003;532:227–243. [PubMed: 14643439]
- Campisi J. Cellular senescence and apoptosis: how cellular responses might influence aging phenotypes. *Exp Gerontol* 2003;38:5–11. [PubMed: 12543256]

- Candeias LP, Steenken S. Reaction of HO* with guanine derivatives in aqueous solution: formation of two different redox-active OH-adduct radicals and their unimolecular transformation reactions. Properties of G(-H)*. *Chemistry* 2000;6:475–484. [PubMed: 10747414]
- Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of Reactive Oxygen Species by Mitochondria: CENTRAL ROLE OF COMPLEX III. *J Biol Chem* 2003;278:36027–36031. [PubMed: 12840017]
- Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G---- T and A----C substitutions. *J Biol Chem* 1992;267:166–172. [PubMed: 1730583]
- de Souza-Pinto NC, Eide L, Hogue BA, Thybo T, Stevnsner T, Seeberg E, Klungland A, Bohr VA. Repair of 8-oxodeoxyguanosine lesions in mitochondrial dna depends on the oxoguanine dna glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial dna of OGG1-defective mice. *Cancer Res* 2001;61:5378–5381. [PubMed: 11454679]
- Dobson AW, Xu Y, Kelley MR, LeDoux SP, Wilson GL. Enhanced mitochondrial DNA repair and cellular survival after oxidative stress by targeting the human 8-oxoguanine glycosylase repair enzyme to mitochondria. *J Biol Chem* 2000;275:37518–37523. [PubMed: 10982789]
- Dou H, Mitra S, Hazra TK. Repair of oxidized bases in DNA bubble structures by human DNA glycosylases NEIL1 and NEIL2. *J Biol Chem* 2003;278:49679–49684. [PubMed: 14522990]
- Elder RH, Dianov GL. Repair of dihydrouracil supported by base excision repair in mNTH1 knock-out cell extracts. *J Biol Chem* 2002;277:50487–50490. [PubMed: 12401779]
- Hamilton ML, Guo Z, Fuller CD, Van Remmen H, Ward WF, Austad SN, Troyer DA, Thompson I, Richardson A. A reliable assessment of 8-oxo-2-deoxyguanosine levels in nuclear and mitochondrial DNA using the sodium iodide method to isolate DNA. *Nucleic Acids Res* 2001a;29:2117–2126. [PubMed: 11353081]
- Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A. Does oxidative damage to DNA increase with age? *Proc Natl Acad Sci U S A* 2001b;98:10469–10474. [PubMed: 11517304]
- Hazra TK, Izumi T, Maidt L, Floyd RA, Mitra S. The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. *Nucleic Acids Res* 1998;26:5116–5122. [PubMed: 9801308]
- Hazra TK, Kow YW, Hatahet Z, Imhoff B, Boldogh I, Mokkalapati SK, Mitra S, Izumi T. Identification and characterization of a novel human DNA glycosylase for repair of cytosine-derived lesions. *J Biol Chem* 2002;277:30417–30420. [PubMed: 12097317]
- He YH, Xu Y, Kobune M, Wu M, Kelley MR, Martin WJ 2nd. Escherichia coli FPG and human OGG1 reduce DNA damage and cytotoxicity by BCNU in human lung cells. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L50–55. [PubMed: 11741815]
- Hu J, de Souza-Pinto NC, Haraguchi K, Hogue BA, Jaruga P, Greenberg MM, Dizdaroglu M, Bohr VA. Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. *J Biol Chem* 2005;280:40544–40551. [PubMed: 16221681]
- Hyun JW, Yoon SH, Yu Y, Han CS, Park JS, Kim HS, Lee SJ, Lee YS, You HJ, Chung MH. Oh8dG induces G1 arrest in a human acute leukemia cell line by upregulating P21 and blocking the RAS to ERK signaling pathway. *Int J Cancer* 2006;118:302–309. [PubMed: 16052517]
- Ikeda S, Biswas T, Roy R, Izumi T, Boldogh I, Kurosky A, Sarker AH, Seki S, Mitra S. Purification and characterization of human NTH1, a homolog of Escherichia coli endonuclease III. Direct identification of Lys-212 as the active nucleophilic residue. *J Biol Chem* 1998;273:21585–21593. [PubMed: 9705289]
- Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T, Barnes DE. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A* 1999;96:13300–13305. [PubMed: 10557315]
- Larsen E, Kwon K, Coin F, Egly JM, Klungland A. Transcription activities at 8-oxoG lesions in DNA. *DNA Repair (Amst)* 2004;3:1457–1468. [PubMed: 15380101]
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147–157. [PubMed: 8689682]

- Malecki EA. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. *Brain Res Bull* 2001;55:225–228. [PubMed: 11470319]
- Minowa O, Arai T, Hirano M, Monden Y, Nakai S, Fukuda M, Itoh M, Takano H, Hippou Y, Aburatani H, Masumura K, Nohmi T, Nishimura S, Noda T. Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice. *Proc Natl Acad Sci U S A* 2000;97:4156–4161. [PubMed: 10725358]
- Mitra S, Hazra TK, Roy R, Ikeda S, Biswas T, Lock J, Boldogh I, Izumi T. Complexities of DNA base excision repair in mammalian cells. *Mol Cells* 1997;7:305–312. [PubMed: 9264015]
- Nicotera P, Leist M. Mitochondrial signals and energy requirement in cell death. *Cell Death Differ* 1997;4:516. [PubMed: 16465273]
- Nishimura S. Involvement of mammalian OGG1(MMH) in excision of the 8-hydroxyguanine residue in DNA. *Free Radic Biol Med* 2002;32:813–821. [PubMed: 11978483]
- Olive PL, Wlodek D, Banath JP. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res* 1991;51:4671–4676. [PubMed: 1873812]
- Osterod M, Hollenbach S, Hengstler JG, Barnes DE, Lindahl T, Epe B. Age-related and tissue-specific accumulation of oxidative DNA base damage in 7,8-dihydro-8-oxoguanine-DNA glycosylase (Ogg1) deficient mice. *Carcinogenesis* 2001;22:1459–1463. [PubMed: 11532868]
- Reed JC. Mechanisms of apoptosis avoidance in cancer. *Curr Opin Oncol* 1999;11:68–75. [PubMed: 9914881]
- Ruchko M, Gorodnya O, LeDoux SP, Alexeyev MF, Al-Mehdi AB, Gillespie MN. Mitochondrial DNA damage triggers mitochondrial dysfunction and apoptosis in oxidant-challenged lung endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L530–535. [PubMed: 15563690]
- Russo MT, De Luca G, Degan P, Bignami M. Different DNA repair strategies to combat the threat from 8-oxoguanine. *Mutat Res* 2007;614:69–76. [PubMed: 16769088]
- Sager R. Senescence as a mode of tumor suppression. *Environ Health Perspect* 1991;93:59–62. [PubMed: 1663451]
- Sakumi K, Tominaga Y, Furuichi M, Xu P, Tsuzuki T, Sekiguchi M, Nakabeppu Y. Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. *Cancer Res* 2003;63:902–905. [PubMed: 12615700]
- Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A* 1994;91:10771–10778. [PubMed: 7971961]
- Shinmura K, Yokota J. The OGG1 gene encodes a repair enzyme for oxidatively damaged DNA and is involved in human carcinogenesis. *Antioxid Redox Signal* 2001;3:597–609. [PubMed: 11554447]
- Shukla LI, Adhikary A, Pazdro R, Becker D, Sevilla MD. Formation of 8-oxo-7,8-dihydroguanine-radicals in gamma-irradiated DNA by multiple one-electron oxidations. *Nucleic Acids Res* 2004;32:6565–6574. [PubMed: 15601999]
- Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res* 2000;60:6788–6793. [PubMed: 11156366]
- Skulachev VP. Bioenergetic aspects of apoptosis, necrosis and mitoptosis. *Apoptosis* 2006;11:473–485. [PubMed: 16532373]
- Smart DJ, Chipman JK, Hodges NJ. Activity of OGG1 variants in the repair of pro-oxidant-induced 8-oxo-2'-deoxyguanosine. *DNA Repair (Amst)* 2006;5:1337–1345. [PubMed: 16861056]
- Stevnsner T, Nyaga S, de Souza-Pinto NC, van der Horst GT, Gorgels TG, Hogue BA, Thorslund T, Bohr VA. Mitochondrial repair of 8-oxoguanine is deficient in Cockayne syndrome group B. *Oncogene* 2002;21:8675–8682. [PubMed: 12483520]
- Szczesny B, Hazra TK, Papaconstantinou J, Mitra S, Boldogh I. Age-dependent deficiency in import of mitochondrial DNA glycosylases required for repair of oxidatively damaged bases. *Proc Natl Acad Sci U S A* 2003;100:10670–10675. [PubMed: 12960370]
- Takao M, Kanno S, Shiromoto T, Hasegawa R, Ide H, Ikeda S, Sarker AH, Seki S, Xing JZ, Le XC, Weinfeld M, Kobayashi K, Miyazaki J, Muijtjens M, Hoeijmakers JH, van der Horst G, Yasui A. Novel nuclear and mitochondrial glycosylases revealed by disruption of the mouse Nth1 gene encoding an endonuclease III homolog for repair of thymine glycols. *Embo J* 2002;21:3486–3493. [PubMed: 12093749]

- Telford WG, Bradford J, Godfrey W, Robey RW, Bates SE. Side population analysis using a violet-excited cell-permeable DNA binding dye. *Stem Cells* 2007;25:1029–1036. [PubMed: 17185610]
- Tornaletti S, Maeda LS, Kolodner RD, Hanawalt PC. Effect of 8-oxoguanine on transcription elongation by T7 RNA polymerase and mammalian RNA polymerase II. *DNA Repair (Amst)* 2004;3:483–494. [PubMed: 15084310]
- Trzeciak AR, Nyaga SG, Jaruga P, Lohani A, Dizdaroglu M, Evans MK. Cellular repair of oxidatively induced DNA base lesions is defective in prostate cancer cell lines, PC-3 and DU-145. *Carcinogenesis* 2004;25:1359–1370. [PubMed: 15044326]
- Van Remmen H, Hamilton ML, Richardson A. Oxidative damage to DNA and aging. *Exerc Sport Sci Rev* 2003;31:149–153. [PubMed: 12882482]
- Zabkiewicz J, Clarke AR. DNA damage-induced apoptosis: insights from the mouse. *Biochim Biophys Acta* 2004;1705:17–25. [PubMed: 15585170]
- Zakeri ZF, Ahuja HS. Cell death/apoptosis: normal, chemically induced, and teratogenic effect. *Mutat Res* 1997;396:149–161. [PubMed: 9434866]
- Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 2003;34:1359–1368. [PubMed: 12757846]
- Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 1997;253:162–168. [PubMed: 9367498]

Figure 1. Steady-state levels of 8-oxoG in the DNA of *Ogg1*^{-/-} fibroblasts

A, Increased 8-oxoG levels in the nuclear DNA of *Ogg1*^{-/-} cells. Actively replicating cells on microscope cover-slips were fixed and 8-oxoG lesions were determined immunochemically in four independent experiments. Images were captured using a Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope. Fluorescent intensities mediated by 8-oxoG lesions were determined on individual cells with MetamorphTM Version 6.06r software. For each data point more than one hundred randomly selected cells were evaluated. ****p*<0.001. **Inset**: Absence of OGG1 in *Ogg1*^{-/-} cells. Western blot analysis was carried out as described in Materials and Methods. **B**, Representative images showing 8-oxoG-mediated fluorescence from *Ogg1*^{+/+}, *Ogg1*^{-/-} and *Nth1*^{-/-} cells. Magnification: ×180. FLI: fluorescent intensity. **C**, Oxidized guanine levels in DNA shown by comet assay. Cells were suspended in 0.75% low-melting point agarose, spread on microscope slides and lysed in lysis buffer containing NaI. DNA was mock- or Fpg-treated and electrophorized in alkaline condition. Comet moments of randomly selected images (*n* ≥ 50) were evaluated in each sample. **p*<0.05, ***p*<0.01, ****p*<0.001. **D**, Representative comet moment images of *Ogg1*^{+/+} and *Ogg1*^{-/-} cells. Single arrow head points to comet moment showing 3- to 4-fold, double arrow head shows an example for >7-fold increase in comet moments compared to the average comet moment of *Ogg1*^{+/+} cells. Magnification: ×180. **E**, Distribution of DNA Comet moments of *Ogg1*^{+/+} and *Ogg1*^{-/-} cells. Comet assays were carried out as described in legend to C. DNA was Fpg-treated and electrophoresed in alkaline conditions. Tail moments of cells are shown as individual full squares for *Ogg1*^{+/+} and full circles for *Ogg1*^{-/-} cells. ****p*<0.001.

Figure 2. Increased ROS levels in subsets of *Ogg1*^{-/-} cells

A, Microscopic visualization of increased oxidative stress levels in *Ogg1*^{-/-} cultures. Cells in a thermo-controlled microscopic chamber were loaded with H₂DCF-DA and fluorescent images were captured by Photometrix Coolsnap Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope. Representative images from *Ogg1*^{+/+} and *Ogg1*^{-/-} cell cultures. Magnification: ×62. **B**, Percentage of *Ogg1*^{-/-} cells showing increased ROS levels. *Ogg1*^{+/+}, *Nth1*^{-/-}, and *Ogg1*^{-/-} cells at 50% confluence were loaded with H₂DCF-DA, multiple images were taken, and fluorescence intensities of individual cells were determined. Cells exhibiting ≥50% increase in DCF fluorescence (oxidative stress levels) were defined as ^HROS cells. Fluorescence intensities of ≥300 cells were evaluated by Metamorph™ Version 6.06r software for each cell type. **C**, Sorted ^HROS *Ogg1*^{-/-} cells show increased oxidative stress levels. *Ogg1*^{+/+}, *Ogg1*^{-/-} cells were H₂DCF-DA-loaded and sorted into ^LROS and ^HROS cells by FACSaria (Becton Dickinson). DCF fluorescence intensities of ^LROS and ^HROS as well

as the unsorted *OggI*^{+/+}, *OggI*^{-/-} cells were assessed by flow cytometry FACSanto (Becton Dickinson). 15,000 cells were collected and analyzed for each sample (n = 4 or more). **D**, Distribution of cells showing varying DCF fluorescence intensities. *OggI*^{+/+}, *OggI*^{-/-} (upper panel) as well as ^LROS and ^HROS (lower panel) cells were loaded with H₂DCF-DA (5 μM). Oxidative stress levels were assessed by flow cytometry (FACSanto). 15,000 cells were collected and analyzed. Result of one representative experiment out of seven is shown. Upper panel is showing no difference in DCF fluorescence of *OggI*^{+/+} and *OggI*^{-/-} cells (p<0.1). Lower panel: significant (p>0.001) differences in oxidative stress levels between ^LROS and ^HROS cells. **E**, Increase in oxidative stress level in ^HROS cells as a function of PDL. At the indicated PDLs, cells were loaded with H₂DCF-DA and oxidative stress levels were determined by flow cytometry as in legend to **D**. Cells maintained in 5 mM NAC-containing media showed lower levels of ROS. Inset shows the distribution of cells with varying DCF fluorescence. 12,000 cells were analyzed for each sample (n ≥ 5). † indicates that ^HROS cells were lost by PDL-23. **F**, Changes in Comet tail moment as a function of PDLs. Cells were processed as in legend to Fig. 1C and DNA was Fpg-treated and electrophoresed in alkaline condition. Randomly, selected comet moments (n ≥ 50) were evaluated in each sample. **G**, Accumulation of ^HROS in ^LROS cultures. ^LROS cells were serially passaged and at selected PDLs percentage of cells showing increased DCF fluorescence was determined as in legend to Figure 1A,B. A.U: arbitrary units; NAC: N-acetyl-L-cysteine. PDL, population doubling level; *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Increase in mitochondrial ROS generation in a subset of *Ogg1*^{-/-} cells

A, H₂Et/superoxide-mediated fluorescence. Cells were loaded with H₂Et and H₂Et/superoxide reaction product-mediated fluorescence was captured using a Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope (upper left panel). MitoTracker Red (upper right panel) and H₂Et/superoxide-mediated fluorescence is co-localized (lower left panel). Lower right panel is the DAPI-stained image of the nucleus. **B**, HROS cells release significantly more H₂O₂ than LROS cells do. Mitochondria (100 µg/ml) were suspended in 50 µl reaction buffer for 30 min and then 0.25 U/ml Amplex® Red and 1 U/ml of horseradish peroxidase were added. The change in fluorescence (excitation 563 nm; emission 587 nm) was measured on a SpectraMass M2 microplate reader (n = 4). H₂Et, dihydroethidium; MT Red, Mitotracker Red; 3-NPA, 3-nitropropionic acid; AA, antimycin A.

Figure 4. ^HROS cells are eliminated from *Ogg1*^{-/-} cultures by apoptosis-like processes

A, Percentage of annexin V-binding cells in *Ogg1*^{-/-} and control cultures. Columns in **A**, a show percentage of annexin V-binding cells in unsorted *Ogg1*^{-/-}, *Ogg1*^{+/+} and *Nth1*^{-/-} cell cultures (n = 5). Accumulation of annexin V positive cells in ^LROS cultures (**A**,b). ^LROS cells were separated from ^HROS subset, serially passaged and percentage of annexin V-binding cells was determined. As control, *Ogg1*^{+/+} and *Nth1*^{-/-} cells were used. 12,000 cells were evaluated per sample on a FACScanto flow cytometer. **B**, Caspase-3 activity increases in ^HROS cells as a function of PDL. Cell lysates were incubated with 20 μ M Ac-DEVD-AMC in reaction buffer at 37°C for 1 h. Changes in fluorescence intensities were determined at 380 nm (excitation) and 435 nm (emission) wavelengths in an FLx800 microplate reader (n = 4). Caspase-3 activities were expressed as pmoles of 7-amino-4-methyl coumarin released per mg of protein per 60 min. **C**, Morphological changes in subsets of *Ogg1*^{-/-} cells. Cover-slips cultures of cells were photographed by Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200. Left panel: Phase contrast image of *Ogg1*^{-/-} cells. Magnification: $\times 32$. Middle panel: apoptotic-like cells in *Ogg1*^{-/-} cell monolayer (magnification: $\times 126$). Right panel: Morphology of *Ogg1*^{+/+} cells (magnification: $\times 32$). **D**, ATP levels are decreased in ^HROS cells at PDL-17. Annexin V-binding cells were discarded after sorting cells on FACSaria flow cytometer. ATP was extracted from 3×10^6 cells (*Ogg1*^{-/-}, at PDL-6, -10, -17 and *Ogg1*^{+/+}) by adding 100 μ l of boiling distilled water (Materials and Methods). ATP concentration-dependent luminescence intensities were analyzed in a Veritas Microplate Luminometer (**p=0.014).

Figure 5. Expression of OGG1 lowers 8-oxoG levels in *Ogg1*^{-/-} cells

A, Expression of OGG1 in transfected cells. Transfected cells on microscope cover-slips were fixed and stained with OGG1-specific rabbit antibody (OGG1-2672). Binding of primary antibody was visualized by secondary anti-rabbit antibody labeled with FITC (left hand side upper and lower panels). The right upper and lower panels are showing DAPI-stained nuclei of same cells. **B**, Decreased 8-oxoG levels in DNA of *Ogg1*^{-/-} cells. Cells on cover-slips were incubated with 8-oxoG-specific monoclonal antibody, and the binding was visualized with TexasRed-conjugated secondary antibody. Fluorescence images were captured and fluorescent intensities were determined as described in the legend to Fig. 1. FLI, fluorescence intensity; FITC, fluorescein isothiocyanate, DAPI, 4'6-diamidino-2-phenylindole.