MYC Can Induce DNA Breaks In vivo and In vitro Independent of Reactive Oxygen Species

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Abstract

MYC overexpression is thought to initiate tumorigenesis by inducing cellular proliferation and growth and to be restrained from causing tumorigenesis by inducing cell cycle arrest, cellular senescence, and/or apoptosis. Here we show that MYC can induce DNA breaks both in vitro and in vivo independent of increased production of reactive oxygen species (ROS). We provide an insight into the specific circumstances under which MYC generates ROS in vitro and propose a possible mechanism. We found that MYC induces DNA double-strand breaks (DSBs) independent of ROS production in murine lymphocytes in vivo as well as in normal human foreskin fibroblasts (NHFs) in vitro in normal (10%) serum, as measured by γH2AX staining. However, NHFs cultured in vitro in low serum (0.05%) and/or ambient oxygen saturation resulted in ROS-associated oxidative damage and DNA single-strand breaks (SSBs), as measured by Ape-1 staining. In NHFs cultured in low versus normal serum, MYC induced increased expression of CYP2C9, a gene product well known to be associated with ROS production. Specific inhibition of CYP2C9 by small interfering RNA was shown to partially inhibit MYC-induced ROS production. Hence, MYC overexpression can induce ROS and SSBs under some conditions, but generally induces widespread DSBs in vivo and in vitro independent of ROS production. (Cancer Res 2006; 66(13): 6598-605)

Introduction

The MYC proto-oncogene encodes a transcription factor of which the activity has been implicated in diverse functions including cellular growth and proliferation, differentiation, angiogenesis, cellular adhesion, and apoptosis (1). Overexpression of MYC is thought to contribute to tumorigenesis by enforcing unrestrained cellular growth and proliferation (1, 2). MYC and other oncogenes may also contribute to tumorigenesis through the induction of genomic destabilization (3–14). MYC-induced DNA damage may account for the observed proliferative arrest, senescence, and apoptosis of normal human foreskin fibroblasts (NHF) or mouse embryonic fibroblasts in vitro (9, 15). Several studies have suggested that MYC may induce genomic damage in vivo (6, 16, 17).

MYC overexpression is associated with abnormalities in chromosomal number, chromosomal breaks, and translocations (7). MYC has been proposed to induce chromosomal damage through at least three possible non-mutually exclusive mechanisms. First, MYC may enforce cell cycle transit abrogating mechanisms that preserve genomic integrity or mediate DNA repair by interfering with the repair of DNA double-strand breaks (DSBs; refs. 7, 12, 18). Second, MYC and other oncogenes may cause DNA breaks through the induction of reactive oxygen species (ROS; refs. 19–23). Third, MYC has been shown to result in breakage-bridge-fusion cycles and telomere remodeling (24). The relative contribution of these different mechanisms is not known.

Many studies document that ROS can lead to genomic instability and contribute to tumorigenesis (19, 25). ROS can directly result in DNA single-strand breaks (SSBs) or DSBs; purine, pyrimidine, or deoxyriboside modifications; and DNA cross-links (26, 27). ROS can be produced from both endogenous and exogenous sources. One such potential endogenous source of ROS is cytochrome P450 (CYP450) metabolism. The various mechanisms that regulate production of ROS by CYP450 in different cells and tissues have been widely discussed (28, 29).

To evaluate if MYC can induce DNA breaks in normal cells under physiologic circumstances and if these breaks are mediated by the production of ROS, we have examined the consequences of conditional MYC activation in vitro in NHFs and in vivo in normal murine lymphocytes using the Tet System (30). We found that MYC overexpression can induce DNA breaks in vivo and in vitro in normal cells through mechanisms independent of ROS production.

Materials and Methods

Cell culture. NHFs were grown in DMEM without phenol red and supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. In some experiments, cells were grown in vitro in media containing low serum (0.05% FBS) for 48 hours before MYC induction. Fibroblasts were infected with pBabe-puro retrovirus containing MYCER (7, 31).

Induction of MYC activity. MYC was induced in NHFs with MYCER (NHF-MYCER) using 4-hydroxy tamoxifen (4-OHT; Sigma-Aldrich, St. Louis, MO), added to the medium at a final concentration of 1 μmol/L.

Transgenic mice. For in vivo studies, we used a transgenic mouse line with the β-gal System in which MYC is conditionally expressed in T cells with the help of a tissue-specific promoter (30).

Confocal microscopy for detection of ROS in MYC-induced NHFs. To investigate the production of ROS in MYC-induced NHFs, NRF2-MYCER cells were grown on coverslips in 24-well tissue culture dishes in normal and low serum and MYC was induced for 4 hours with 4-OHT. Cells were then treated with 1 μmol/L of an antioxidant, N-acetyl cysteine (NAC; Sigma-Aldrich), overnight, before MYC activation. Cells were then stained with 5 μmol/L of the fluorescent indicator of oxidative stress, 2′,7′-dichloro-fluorescein diacetate (DCFDA; Sigma-Aldrich), for 20 minutes, washed with...
Flow cytometry analysis for detecting ROS. For in vitro studies, NHF-MYCER cells were grown in normal (10%) and low (0.05%) serum in six-well tissue culture dishes and MYC was induced for 4 hours with 4-OHT. Cells were treated with antioxidant NAC overnight, before MYC activation. Generation of ROS was measured by flow cytometry using DCFDA at a final concentration of 5 μM/L at 37°C for 20 minutes. Cells untreated with 4-OHT were used as a negative control whereas cells treated with 1 μM/L hydrogen peroxide for 15 minutes were used as a positive control.

To study if ambient oxygen conditions in the laboratory influenced the production of ROS, NHF-MYCER cells grown in normal (10%) or low (0.05%) serum were cultured under different oxygen concentrations (2% and 20%) and ROS was measured by fluorescence-activated cell sorting (FACS) after inducing MYC for 4 hours by methods mentioned above.

For in vivo studies to determine ROS, we used the transgenic Eqt/MYC/tTA mice expressing MYC conditionally under the influence of the tetracycline activating protein (30). One-month-old mice in which MYC was either activated since birth or inactivated using doxycycline were sacrificed and thymocytes were isolated by standard methods. Cells were then stained with DCFDA and assessed for ROS as above. Cells were exogenously treated with 1 μM/L hydrogen peroxide for 15 minutes to generate DNA breaks. Cohorts of two to four mice were used for each condition per experiment.

Comet assay for detection of DNA breaks. NHF-MYCER cells were grown asynchronously in normal (10%) or low (0.05%) serum and treated with or without antioxidant NAC, overnight, before MYC induction. MYC was induced with 4-OHT and Comet assay for detection of DNA breaks was done with partial modifications of previously published protocols (34). Cells were embedded in low melting agarose on frosted glass slides precoated with two layers of 1% agarose. Cells were lysed in a lysis buffer (2.5% SDS, 2.5 mol/L NaCl, 0.1 mol/L EDTA, pH 10) to detect both DSBs and SSBS/ oxidative base changes and electrophoresed for 20 minutes at 25 V and 200-mA current from negative to positive direction. Cells were stained with ethidium bromide, wet-mounted with cover-slips, and observed under an epifluorescence microscope (Nikon). DNA breaks were analyzed in 100 cells per sample and quantified using the HCSA (High Capacity Slide Analyzer) program and KOMET software (LAL Inc., Laredo, TX). Mean moment of the comet was measured in arbitrary units.

FACS assays for apoptosis. NHF-MYCER cells were grown in normal (10%) and low (0.05%) serum in six-well tissue culture dishes and MYC was induced for 4 hours with 4-OHT. Staining of the cells with propidium iodide was done according to standard protocols (9). Anti-caspase-3 antibody staining was done using the BD Pharmingen caspase-3 staining kit and cells were analyzed by flow cytometry. Cells treated with 5 μM/L hydrogen peroxide were used as a positive control whereas untreated cells were used as a negative control. A total of 100 cells were imaged for each sample analyzed.

Double immunofluorescence staining for detection of SSBS and DSBs. For in vitro studies to characterize the types of breaks observed on MYC induction, NHF-MYCER cells were grown in normal and low serum on coverslips in 24-well tissue culture dishes. MYC-induced and uninduced cells were fixed with cold methanol and stained with a blend of anti-Ape-1 (Santa Cruz Biototechnology, Santa Cruz, CA) and anti-γH2AX antibodies (Upstate, Charlotteville, VA). FITC-conjugated antirabbit (Sigma-Aldrich) and Alexa Fluor 594-conjugated antimouse (Molecular Probes) secondary antibodies were used against the respective primary antibodies. Cells were mounted in Vector Shield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) and viewed under an immunofluorescence microscope (Nikon, Eclipse E800, with FDX-35 camera) with FITC and tetramethylrhodamine isothiocyanate filters to observe staining against Ape-1 and γH2AX antibodies. Nuclear staining was done with DAPI and slides were viewed under a UV filter. Multiple images for each sample were captured and analyzed. Cells irradiated with 2 Gy of γ-radiation from a cesium source and cells treated with 1 μM/L hydrogen peroxide were used as positive controls for generating DSBs and SSBS, respectively. A total of 100 cells from multiple fields were imaged for each sample analyzed.

For in vivo studies to characterize the types of DNA breaks, we used the transgenic EqtTA/tet-o-MYC mice described above. One-month-old mice in which MYC was activated constitutively were sacrificed and thymocytes were isolated by standard methods. Thymocytes were stained with DAPI and slides were viewed under a Fluorescence microscope (Nikon, with FDX-35 camera) with FITC and tetramethylrhodamine isothiocyanate filters to observe staining against Ape-1 and γH2AX antibodies. Nuclear staining was done with DAPI and slides were viewed under a UV filter. Multiple images for each sample were captured and analyzed. Cells irradiated with 2 Gy of γ-radiation from a cesium source and cells treated with 1 μM/L hydrogen peroxide were used as positive controls for generating DSBs and SSBS, respectively. A total of 100 cells from multiple fields were imaged for each sample analyzed.

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cDNA microarray. RNA was extracted after 4 hours of MYC induction in NHFs grown in normal (10%) or low (0.05%) serum using standard protocols. cDNA microarray was done using slide-based microarrays manufactured at the Stanford Functional Genomics Facility. These arrays contained >40,000 spots representing ~25,800 genes as estimated by Unigene Clusters. About 50 μg of total RNA were used and subsequent hybridizations were carried out according to the Brown Lab protocols. The Stratagene Universal Human Reference RNA representing total RNA pooled from 10 different cell lines was used as reference. The hybridized arrays were scanned and the data extracted using Genepix 3.0 Software (Axon Instruments) and stored at the Stanford Microarray Database for analysis. To identify genes that were differentially regulated by MYC in normal and low serum conditions, we first filtered genes for adequate technical measurements, as judged by hybridization intensities of 1.5-fold over the local background in both Cy5 (experimental) and Cy3 (reference) channels. For each serum condition, we subtracted the log 2 value of the MYC-off samples from the MYC-on samples; the resulting value indicated the fold change caused by MYC induction. We selected genes that had at least a 2-fold change in response to MYC in either condition. This subset of genes was organized by hierarchical clustering to group genes based on the similarity in their patterns of regulation by MYC.

Real-time quantitative reverse transcription-PCR. RNA was extracted from MYC-induced and uninduced NHF-MYCER cells grown in normal (10%) or low (0.05%) serum using TRIzol reagent and following standard RNA extraction protocols. cDNA was synthesized with oligo-dT using Superscript first-strand synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen, Inc., Carlsbad, CA). Relative quantitation of mRNA for the 29C isoform of CYP50 (CYP2C9), superoxide dismutase 2 (SOD2), and cytochrome c oxidase, isoform 7C (COX7C) genes was done by real-time quantitative RT-PCR using SYBR Green (Applied Biosystems, Foster City, CA) and the 7900 ABI Prism Instrument. The primers were designed using the PRIMER 3 software and GAPDH was used as the housekeeping gene. PCR primers were CYP2C9: (sense) 5′-GGCGGCTCAGACTACATTCA-3′, (antisense) 5′-CCAGTTCGAAGTGGAGGA-3′; COX7C: (sense) 5′-GGCGGCTCAGACTACATTCA-3′, (antisense) 5′-ATCACGATTGCCACAGGC-3′; SOD2: (sense) 5′-GGCGGCTCAGACTACATTCA-3′, (antisense) 5′-GGCCCTCAGACTACATTCA-3′; and GAPDH: (sense) 5′-TGACCCACACATGCT-TAGC-3′, (antisense) 5′-GCATAGACTTGGTCTGAGA-3′. The results were analyzed graphically using the ΔΔCt method (35).

Immunoblotting for CYP2C9. Western blot analysis for CYP2C9 isoforms was done by standard techniques in MYC-induced NHF-MYCER cells grown in normal and low serum. Anti-CYP2C9 antibody (Gentest, San Jose, CA) was used as primary antibody whereas antirabbit horseradish

4 http://genome-www5.stanford.edu/MicroArray/SMD
5 http://cgmp stain.Fluorescence-activated cell sorting (FACS) after inducing MYC for 4 hours by methods mentioned above.
peroxidase (HRP) conjugate was used as secondary antibody. α-Tubulin (Calbiochem, San Diego, CA) was used as a loading control and anti-mouse HRP conjugate (Amersham Biosciences, Piscataway, NJ) was used as secondary antibody.

**RNA interference against CYP2C9.** Small interfering RNA was obtained commercially from Ambion (Austin, TX) and transiently transfected into uninduced or MYC-induced NHFs according to the instructions of the manufacturer using Oligofectamine (Invitrogen). MYC-induced cells that were either untransfected or transfected with an unrelated small interfering RNA (CDK2) were used as controls.

**Inhibition of CYP2C9 using sulfaphenazole.** Sulfaphenazole (Sigma) was added to MYC-induced NHF-MYCER cells grown in normal and low serum and any change in the generation of ROS was analyzed by FACS as mentioned above.

**Statistical analysis.** To determine the statistical significance of our results, we calculated $P$ values for all our experiments by the standard $t$ test method.

**Results**

**MYC induces ROS production in low serum or ambient oxygen saturation.** Recent reports suggest that MYC can induce DNA damage through the production of ROS (10, 23, 36). However, these experiments were done in NHFs that had been adapted to grow in 10% serum and ambient supraphysiologic oxygen saturation (20%). We investigated if MYC induced ROS in NHFs growing in physiologic conditions. We found that MYC overexpression *in vitro* in NHFs causes significant increase in ROS above background when grown in low serum (0.05%) and ambient oxygen saturation ($P = 0.001$), as measured by either confocal microscopy (Fig. 1A and B) or FACS analysis (Fig. 1C). Levels of ROS production by MYC were comparable to NHFs treated *in vitro* with hydrogen peroxide (Fig. 1B and C). However, MYC failed to induce significant levels of ROS in NHFs grown in normal serum (10%; $P = 0.4638$) and/or physiologic oxygen saturation (2%; $P = 0.4638$; Fig. 1D). Notably, MYC also failed to induce ROS in NHFs grown in normal serum but cells were arrested in the cell cycle through contact inhibition, as validated by FACS analysis (data not shown). Hence, MYC induces significant amounts of ROS only in NHFs grown in low serum and ambient oxygen saturation.

**MYC can induce DNA breaks through a ROS-independent mechanism.** Although MYC-induced insignificant amount of ROS in NHFs grown in normal serum and physiologic oxygen saturation, MYC induction within 4 hours induced DNA breaks in >90% of NHFs irrespective of growth conditions as detected by the Comet assay, modified as previously described to detect both DNA damage through the production of ROS.

**Figure 1.** MYC induces ROS production in NHFs grown in low serum and nonphysiologic oxygen concentrations. MYC was activated for 4 hours with 1 μmol/L 4-OHT in NHF-MYCER cells maintained in low (0.05%) or normal (10%) serum in the presence and/or absence of antioxidant NAC. Cells were stained with DCFDA for detection of ROS and viewed by confocal microscopy (A) and the fluorescence intensity (B) was quantitated using Metamorph software. Statistical significance between MYC-induced ROS production in low versus normal serum was proved by unpaired $t$ test that gave a $P$ value of 0.004. The amount of ROS generated by MYC in normal serum was statistically insignificant ($P = 0.07$). C, mean fluorescence intensity of DCFDA in MYC-induced NHF-MYCER cells was measured by flow cytometry. Uninduced cells stained with DCFDA were used as a negative control to detect background levels of ROS produced in these cells. Uninduced cells treated with 1 μmol/L hydrogen peroxide for 15 minutes were used as a positive control. Cells pretreated with NAC were used to detect any decrease in ROS production. Background level of ROS was normalized to 100%. The amount of ROS generated by MYC induction in low serum as against normal serum was found to be statistically significant, giving a $P$ value of 0.0037. D, NHF-MYCER cells were subject to ambient (20%) and physiologic (2%) oxygen concentrations using respective oxygen incubators. Cells were either grown in normal or low serum and MYC was activated for 4 hours using 4-OHT. Cells were stained with DCFDA and analyzed by flow cytometry for generation of ROS as a measure of mean fluorescence intensity. Representative of three reproducible experiments. Dotted line, background level of ROS, normalized to 100%. Statistical significance of MYC-induced ROS in low serum and 2% O$_2$ was proved by unpaired $t$ test ($P = 0.0037$). MYC-induced ROS in normal serum and 2% O$_2$ was found to be insignificant ($P = 0.4638$) when compared with background levels of ROS in the cells.
DSBs and SSBs (Fig. 2A and Supplementary Fig. S1; ref. 35). Notably, after MYC induction in normal or low serum, >96% of cells exhibited comet moments above background (0-5 units; Supplementary Fig. S1Bi,i). Moreover, treatment with NAC reduced levels of ROS to background (Fig. 2B) but failed to prevent MYC from inducing DNA breaks as measured by the Comet assay (Fig. 2A and Supplementary Fig. S1). Often NAC failed to eliminate ROS completely because we used a NAC concentration that is well tolerated by normal cells. Treatment with 1 μmol/L hydrogen peroxide for 15 minutes resulted in DNA damage comparable to MYC induction by 1 μmol/L tamoxifen for 4 hours. Although the Comet assay generally is considered capable of distinguishing cells with DNA damage from those undergoing apoptosis, we considered that it was possible that the DNA breaks we were detecting by the Comet assay could instead reflect early apoptotic conditions.

To better characterize the mechanism by which MYC induces DNA breaks, we carried out cDNA microarray analysis of MYC-induced NHFs grown in low serum. For cDNA microarray analysis, untreated NHFs grown in low serum were used as a negative control, whereas cells treated with 5 μmol/L hydrogen peroxide for 15 minutes were used as a positive control. Cells were analyzed after 4 hours of MYC induction. In NHFs grown in low serum, MYC gave a 6601 Cancer Res 2006; 66: (13). July 1, 2006

**Figure 2.** MYC induces DNA breaks in vitro both dependent and independent of ROS production. MYC was activated for 4 hours in NHF-MYCER cells grown in low and normal serum. Cells were either subjected to Comet assay (A) for detection of DNA breaks or stained with DCFDA and analyzed by flow cytometry (B) to investigate ROS production. DNA breaks were quantitated using the HCSA program and the KOMET Software. A, for each condition, 100 cells were imaged and mean moment of the comet tails was analyzed in arbitrary units as a measure of DNA breaks. B, for detection of ROS, mean fluorescence intensity of DCFDA-stained cells was acquired by FACS and analyzed graphically. For detection of ROS as well as DNA breaks, uninduced cells were used as a negative control and cells treated exogenously with hydrogen peroxide to induce ROS and/or DNA damage were used as a positive control. Cells pretreated with antioxidant NAC were used to detect any change in production of ROS or DNA breaks induced by ROS. B, dotted line, background level of ROS. Statistical significance was proved by unpaired t test that gave a P value of 0.03 when the amount of ROS generated on MYC induction in low serum was compared with that in normal serum. C, NHFs stained with propidium iodide (i) and anti-caspase-3 monoclonal antibody (ii) were analyzed by FACS for percentage of apoptotic cells after MYC induction in normal and low serum. Cells treated with 5 μmol/L hydrogen peroxide were used as a positive control whereas uninduced cells were used as a negative control.

MYC can induce DSBs and SSBs through ROS-independent mechanisms. To quantitatively evaluate whether MYC is inducing oxidative damage/SSBs and/or DSBs, a double immunofluorescent assay was developed by staining for Ape-1, which measures oxidative base damage and SSBs (26, 37, 38), and by γH2AX phosphorylation staining, which measures DSBs (39–41). MYC was found to induce both oxidative base damage/SSBs and DSBs in NHFs in vitro as evidenced by combined staining for both Ape-1 and γH2AX (Fig. 3A). The frequency of DNA damage generated by oxidative stress, as measured by Ape-1 staining (FITC), was higher (76%) in cells in low serum as compared with cells in normal serum (27%; P = 0.0025) and decreased significantly (P = 0.0024) on treatment with NAC (25% in low serum and 10% in normal serum; Fig. 3B and C). In contrast, there was a statistically insignificant difference (P = 0.4915) in DSBs (33%) in MYC-induced NHFs grown in low serum as compared with normal serum (22%). This frequency did not decrease on treatment with NAC (35% in low serum and 22% in normal serum) as measured by γH2AX staining (Alexa Fluor 594; Fig. 3B and C). Therefore, MYC induces DNA breaks through multiple mechanisms depending on physiologic circumstances: (a) oxidative damage/SSBs through ROS production in low serum and (b) DSBs independent of ROS production, irrespective of serum concentrations.

Elevated levels of CYP2C9 correlate with MYC-induced ROS in NHFs in low serum. To better characterize the mechanism by which MYC induces oxidative damage in low serum, we carried out cDNA microarray analysis of MYC-induced NHFs grown in low
versus normal serum (Supplementary Fig. S3). We sought genes that were selectively induced by MYC in low serum and had plausible functions in mediating oxidative stress. Supplementary Table S1 shows a list of genes that are listed in the order presented in Supplementary Fig. S3. “+” indicates increased expression on MYC induction whereas “−” indicates decreased expression on MYC induction; n.c. indicates no change on MYC induction. Indeed, MYC regulation of downstream genes was strikingly different in low versus normal serum (Supplementary Fig. S3; Supplementary Table S1). Our results show that cluster “b” was different in low versus normal serum (Supplementary Fig. S3; serum but induced by MYC in normal serum whereas COX7C expression was repressed in MYC-induced cells in low serum (Fig. 4A)). Next, we directly tested if CYP2C9 mediates ROS production induced by MYC. RNA interference against CYP2C9 inhibited MYC-induced ROS production (Fig. 4C). As a positive control, we validated that RNA interference against CYP2C9 blocked ROS caused by treatment of cells with hydrogen peroxide. MYC-activated cells that were either untransfected or transfected with an unrelated control small interfering RNA (CDK2 in this case) did not show any decrease in ROS, providing evidence of the specificity of the reduction of ROS observed using small interfering RNA against CYP2C9.

Treatment with sulfaphenazole (Fig. 4D), a specific CYP2C9 inhibitor, also inhibited MYC from inducing ROS. We conclude that CYP2C9, at least in part, contributes to ROS production mediated by MYC induction in serum-starved cells.

MYC induces DSBs in vitro independent of ROS. Finally, we evaluated whether MYC overexpression can induce DSBs in vitro. MYC was activated in murine lymphocytes using the Tet system as previously described (30). MYC was found to induce DNA breaks as detected by the Comet assay (Fig. 5A and Supplementary Fig. S5A). Although the amount of DNA breaks in hydrogen peroxide–treated uninduced thymocytes was higher compared with MYC-activated thymocytes in vivo, the pattern of cellular distribution of comet moments in MYC-activated thymocytes was not.

We further investigated the potential of CYP2C9 in mediating ROS production by MYC. First by quantitative RT-PCR and second by Western blot analysis, we confirmed that MYC overexpression resulted in a 3-fold increase in CYP2C9 mRNA (Fig. 4A), associated with a 2-fold increase in CYP2C9 protein expression (Fig. 4B), in low serum versus normal serum. In addition, we observed a decrease in SOD2 mRNA levels in MYC-induced cells both in low and normal serum whereas COX7C expression was repressed in MYC-induced cells in low serum (Fig. 4A). Next, we directly tested if CYP2C9 mediates ROS production induced by MYC. RNA interference against CYP2C9 inhibited MYC-induced ROS production (Fig. 4C). As a positive control, we validated that RNA interference against CYP2C9 blocked ROS caused by treatment of cells with hydrogen peroxide. MYC-activated cells that were either untransfected or transfected with an unrelated control small interfering RNA (CDK2 in this case) did not show any decrease in ROS, providing evidence of the specificity of the reduction of ROS observed using small interfering RNA against CYP2C9.

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resembled more closely that of cells treated with hydrogen peroxide than that of uninduced thymocytes (Supplementary Fig. S5B). Thus, MYC overexpression in vivo in lymphocytes can cause DNA damage.

MYC overexpression in vivo in murine lymphocytes was not found to induce a measurable increase in ROS production as measured by FACS analysis of DCFDA staining of the cells (P = 0.78; Fig. 5B). As a positive control, lymphocytes incubated exogenously with hydrogen peroxide exhibited measurable amount of ROS, in turn associated with DNA breaks detected by Comet assay (Fig. 5A and B).

Finally, MYC-activated thymocytes showed a predominance of γH2AX over inactivated ones, indicative of significant generation of DSBs (60%; P = 0.002) but not SSBs (27%; P = 0.614; Fig. 5Cii), proving that MYC-induced DSBs are independent of ROS as observed from Ape-1 staining of hydrogen peroxide–treated and γ-irradiated thymocytes from MYC-inactivated mice. Notably, similar to our in vitro data, MYC activation in vivo did not induce detectable apoptosis by Annexin V staining (data not shown). Thus, MYC induction in lymphocytes in vivo can result in DNA breaks independent of ROS and this is not associated with any apoptosis.

Figure 4. MYC-induced ROS in NHFs in vitro correlate with MYC-induced overexpression of CYP2C9 in low serum. A, fold changes in mRNA expression levels of CYP2C9, SOD2, and COX7C genes by real-time RT-PCR. B, changes in protein levels of CYP2C9 in MYC-induced NHF-MYCER cells in low and normal serum. C, MYC was induced in NHF-MYCER cells and mean fluorescence intensity of DCFDA in these cells was measured by flow cytometry. Graphical quantitation for ROS assay in NHF-MYCER cells (A) transfected with CYP2C9 small interfering RNA (B) treated with 10 μmol sulfaphenazole. Statistical significance was proved by t test.

Figure 5. MYC induction fails to induce ROS but generates DSBs in vivo in murine lymphocytes. MYC was either constitutively active or inactivated (with doxycycline) in Eμ-TAATc-α-MYC mice for 4 weeks since birth. A, thymocytes isolated from these mice were subjected to Comet assay for detection of DNA breaks, which were quantified in arbitrary units, as a measure of the mean moment of the comet tails using the KOMET software. B, thymocytes from MYC-activated or inactivated mice in (A) were stained with DCFDA and analyzed by flow cytometry to investigate ROS production. Dotted line, background levels of ROS, normalized to 100%.

C, i, thymocytes from the above mice were fixed in cold methanol and stained as above (Fig. 3) with anti-Ape-1 and γH2AX antibodies for detection of SSBs and DSBs, respectively. Cells exogenously treated with 1 μmol/L hydrogen peroxide for 15 minutes or subjected to 2 Gy of γ-irradiation to induce DNA breaks were used as control. ii, the amount of oxidative damage/SSBs and DSBs induced due to MYC overexpression in different serum conditions was expressed as a percentage of total cells counted. The amount of DSBs detected in MYC-activated thymocytes was statistically significant (P = 0.002) when compared with the inactivated thymocytes but that of SSBs was not.
Discussion

MYC overexpression is generally presumed to cause tumorigenesis by inducing unrestrained cellular proliferation and to be restrained from causing tumorigenesis by inducing proliferative arrest, senescence, and/or apoptosis (1, 7, 9, 15). Here we show that MYC overexpression both in vitro and in vivo induces frequent DNA breaks. Our results are consistent with a multitude of reports that MYC can induce genomic instability (3–5, 7–13, 45). One caveat to these studies is that many of these experiments were done in vitro. Several reports have suggested that MYC induces genomic instability in vivo (6, 16, 17). Our results are consistent with these findings. We report that MYC overexpression in vivo in normal murine lymphocytes can induce DSBs. Notably, DNA breaks were detected to be not associated with increased ROS.

Our results seem to be in discordance with reports that MYC induces DNA breaks through ROS (10, 36). To account for this difference, we considered that these experiments had been done at ambient oxygen saturation and/or low serum concentration, both circumstances known to influence ROS production (46, 47). Indeed, MYC activation in vivo or in vitro under physiologic oxygen saturation does not result in the induction of increased ROS production but does result in the formation of DNA breaks. However, MYC activation in vitro in NIHs cultured in ambient oxygen saturation and/or low serum exhibited increased ROS production, which in turn was associated with the formation of oxidative damage of DNA and SSBs. Most importantly, MYC-induced DNA breaks yet failed to induce ROS in vivo in normal murine lymphocytes, a cellular subcompartment in which MYC activation is frequently associated with tumorigenesis. Thus, MYC seems to induce ROS only under specific growth circumstances that do not seem to approximate what is observed in vivo in hematopoietic compartments. Hence, MYC can cause DNA damage in lymphoid cells even independent of ROS. However, our results are consistent with the possibility that MYC can induce ROS under some growth circumstances. Indeed, it has been observed that MYC induces ROS production in vivo in the liver (6, 48).

We were in part able to account for why MYC-induced ROS in low serum by showing that MYC induces CYP2C9, which has previously been shown to mediate ROS production (44). The inhibition of CYP2C9 with RNA interference or a small-molecule inhibitor was sufficient to reduce MYC-associated ROS production. The mechanism(s) by which MYC induces CYP2C9, however, remains to be determined. MYC overexpression also resulted in decreased expression of SOD2, consistent with what has recently been described (36). However, MYC-induced changes in SOD2 did not correlate with changes in serum concentration and thus are unlikely to account for the changes in ROS production that we observed. Most likely, MYC induces ROS production through effects on multiple gene products depending on cell type and growth conditions. We recognize that an alternative explanation for our results is that in low serum, cells are arrested in the cell cycle, and that under these circumstances MYC may induce ROS. Although we cannot rule out this possibility, we did not observe that MYC induces ROS in cells that have been arrested in the cell cycle through contact inhibition (data not shown).

Therefore, MYC overexpression results in DNA breaks through different mechanisms depending on growth conditions. MYC overexpression in cells cultured in low serum and/or ambient oxygen results in increased ROS production associated with predominantly SSBs. However, MYC overexpression in cells cultured in normal serum and physiologic oxygen saturation results in frequent DSBs with very modest amount of ROS production. The mechanism by which MYC induces DSBs is not clear. MYC does not block activation of H2AX; thus, initial recognition of DNA damage is unlikely to be affected. DNA damage, in turn, activates a DNA damage response and results in cell cycle arrest, senescence, or apoptosis (7, 15). MYC could interfere with DNA repair, possibly indirectly, by preventing cell cycle arrest. The loss of p19ARF/p53 function would cooperate with MYC to induce tumorigenesis by abrogating some of these checkpoint mechanisms (9, 45, 49). Alternatively, MYC may disrupt formation of DNA repair complexes and/or disrupt chromatin structure, preventing efficient DNA repair. In this regard, it is provocative that loss of H2AX is frequently associated with MYC activation, suggesting that they could cooperate to induce tumorigenesis (50).

Our results support the notion that MYC overexpression can induce widespread DNA damage in normal cells in vitro and in vivo through mechanisms independent of ROS. DNA damage induced by MYC could provide a potent signal to induce proliferative arrest, senescence, and/or apoptosis (1, 7, 9, 15).

Acknowledgments

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References


MYC Can Induce DNA Breaks In vivo and In vitro
Independent of Reactive Oxygen Species
