

Extracellular Superoxide Dismutase Is a Major Antioxidant in Human Fibroblasts and Slows Telomere Shortening*

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There is good evidence that telomere shortening acts as a biological clock in human fibroblasts, limiting the number of population doublings a culture can achieve. Oxidative stress also limits the growth potential of human cells, and recent data show that the effect of mild oxidative stress is mediated by a stress-related increased rate of telomere shortening. Thus, fibroblast strains have donor-specific antioxidant defense, telomere shortening rate, and growth potential. We used low-density gene expression array analysis of fibroblast strains with different antioxidant potentials and telomere shortening rates to identify gene products responsible for these differences. Extracellular superoxide dismutase was identified as the strongest candidate, a correlation that was confirmed by Northern blotting. Over-expression of this gene in human fibroblasts with low antioxidant capacity increased total cellular superoxide dismutase activity, decreased the intracellular peroxide content, slowed the telomere shortening rate, and elongated the life span of these cells under normoxia and hyperoxia. These results identify extracellular superoxide dismutase as an important antioxidant gene product in human fibroblasts, confirm the causal role of oxidative stress for telomere shortening, and strongly suggest that the senescence-like arrest under mild oxidative stress is telomere-driven.

There is increasing evidence from a great variety of studies that aging is the result of the limited ability of biological systems to avoid stress, especially oxidative stress, and to cope with the resulting molecular damage (1–3). This might be true even for human cellular replicative senescence, which is the complete and irreversible loss of replicative capacity in telomerase-negative cells after a number of population doublings, a number that is constant under standard cell culture conditions. The ability to maintain telomeres above a critical length appears to be crucial for replicative senescence; mortal cells lose some DNA from the tips of telomeres with every round of DNA replication until they senesce (4), and expression of telomerase in the same cells stabilizes telomere length and allows unlimited growth (5). Telomere shortening in telomerase-negative cells is partly caused by the inability of the DNA replication machinery to fully copy the very end of the lagging strand (the “end replication problem” (6)), which sets a lower limit to the

amount of telomeric sequence lost per generation. In addition, mild oxidative stress accelerates telomere shortening and is actually responsible for most of the shortening seen, for instance, in human fibroblasts under standard culture conditions (7, 8).

Mild oxidative stress is not immediately cytostatic. However, it diminishes the replicative capacity of cells. A good experimental example of mild oxidative stress is chronic hyperoxia, which, depending on the oxygen partial pressure and the antioxidant properties of the cells, increases markers of oxidative stress such as malondialdehydes, protein carbonyls (9), lipofuscin (10, 11), and intracellular peroxides (12) and can seriously limit replicative capacity (8, 13, 14). On the other hand, culture of cells under chronic hypoxia (down to about 3% oxygen partial pressure) results in a slower accumulation of stress markers (10) and an increased replicative life span (13, 15, 16), indicating that the standard cell culture condition in itself already poses a mild oxidative stress.

Interestingly, using different pro- and antioxidant treatments, telomere shortening per cell division could be varied by 1–2 orders of magnitude (for review, see Ref. 17). A highly significant variation of telomere shortening rates was already found under constant environmental conditions comparing human fibroblast strains from different donors, and this variation was correlated to differences in antioxidant defense capacity (18). However, such differences could not be explained completely by expression patterns of CuZn superoxide dismutase (*CuZn-SOD*),¹ manganese superoxide dismutase (*Mn-SOD*), and catalase (19).

It was further shown that oxidative stress-induced single-strand breaks accumulate preferentially in telomeres (7, 20) and that these breaks lead to faster telomere loss during DNA replication (21), possibly by transiently stalling the replication fork (22). These results suggested that a telomere-specific deficiency of single-strand break repair causes the stress-dependence of telomere shortening. However, the gene products responsible for low telomeric repair efficiency are unknown.

To identify possible candidate genes for antioxidant defense and telomere damage repair, we compared the expression patterns of stress-related genes in seven human fibroblast strains with different antioxidant capacity and different telomere shortening rates under conditions of both normoxia and hyperoxia. Of 234 genes, 11 showed a variation in the expression pattern, which correlated to antioxidant capacity and/or te-

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¹ The abbreviations used are: SOD, superoxide dismutase; AOC, antioxidative capacity; DCF, dihydrochlorofluorescein; EC-SOD, extracellular superoxide dismutase; GADD153, growth arrest and DNA damage-inducible protein 153; GST M1, glutathione S-transferase M1; MTH1, 7,8-dihydro-8-oxoguanine triphosphatase; NNMT, nicotinamide-*n*-methyltransferase; PD, population doubling; TOP1, topoisomerase 1; ANOVA, analysis of variance; RT, reverse transcription.

TABLE I
Primers for Northern probe production and semiquantitative RT-PCR

| Gene | 5'-Forward primer-3' | 5'-Reverse primer-3' | Product size bp |
|------------------|--|--|--------------------|
| <i>Mn-SOD</i> | TAC GTG AAC AAC CTG AAC GTC ACC GAG G | GGT AGT AAG CGT GCT CCC ACA CAT CAA TCC | 400 |
| <i>EC-SOD I</i> | TCC ATT TGT ACC GAA ACA CCC CGC TCA C | CAA ACA TTC CCC CAA AGG AGC AGC TCT CAG | 330 |
| <i>EC-SOD II</i> | TGG ATC CGA GAC ATG TAC GCC AGG | TTC AGC AGA GGC GAA GGT GAG ACC | 738 |
| <i>GADD153</i> | CTC AGC ATG CCT TGC GAA GAA CC | TGA ATT TGG CCA CAA ACG CTT CC | 443 |
| <i>MTH1</i> | GCC TTT GGG GGC AAA GTG CAA G | CAA TGC CCC CAG GTG AAG ATG G | 448 |
| <i>TOP1</i> | TGC TGA AGA GAC GAA TCA TGC CCG AGG | GAA TTG CAA CAG CTC GAT TGG CAC GG | 771 |
| β -Actin | ATC TGG CAC CAC ACC TTC TAC ATT GAG CTG CG | CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC | 838 |

lomere shortening rate. Although there were four DNA modification/repair genes among them, a correlation of gene expression to telomere shortening rate was confirmed only for topoisomerase 1 (*TOP1*). The expression of three heat shock genes (*HSP47*, *HSP90*, and *HSPB2*) as well as two genes known to be involved in drug metabolism (*NNMT* and *GSTM1*) correlated with differences in antioxidant defense capacity between strains. Most strikingly, among all antioxidant genes, extracellular superoxide dismutase (*EC-SOD*) was the one with the highest expression level, the greatest differences between cell strains, and the clearest correlation to both steady-state peroxide levels and telomere shortening rate. This result was confirmed using Northern blotting in a wider panel of cell lines. Higher *EC-SOD* expression correlated with a higher SOD activity in cell lysates. Over-expression of *EC-SOD* increased cellular SOD activity, elongated the replicative life span under normoxia and hyperoxia, and slowed down telomere shortening. We conclude that *EC-SOD* has a major function in the protection of human fibroblasts against mild oxidative stress and that prevention of telomere damage is a major part of this function.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic lung fibroblasts MRC-5 and WI-38 were obtained from ECACC and ATCC, respectively. Human foreskin fibroblasts (BJ strain) originated from the laboratory of J. R. Smith (Houston, TX). All other strains were dermal fibroblasts from adult donors. All strains were extensively characterized in our laboratory in terms of oxidative stress markers, growth potential and telomere length maintenance (12, 18, 23). Cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum in parallel under either normoxia (control) or 40% normobaric hyperoxia using a three-gas cell culture incubator (Zapf Instruments, Sarstedt, Germany). An *EC-SOD* cDNA clone (24) was obtained from S. L. Marklund (Umea, Sweden). Full-length cDNA was cloned into pBabe-puro and verified by sequencing. MRC-5 fibroblasts at a population doubling (PD) level of 25–30 were transduced with either empty pBabe-puro or pBabe-*EC-SOD* retrovirus.

Antioxidative Defense Capacity—Fibroblasts were grown for at least 1 week in parallel under either normoxia or hyperoxia and then stained with dihydrochlorofluorescein diacetate as described (12). The fluorescence intensity of the intracellularly trapped dihydrochlorofluorescein (DCF) indicates the amount of intracellular peroxides and other oxidizing species. The DCF fluorescence in 2×10^4 cells was measured in a flow cytometer (PARTEC, Munster, Germany) using blue excitation and green emission wavelengths. The steady-state antioxidative capacity (AOC) was defined as the ratio DCF(normoxia)/DCF(hyperoxia), resulting in an AOC of 1 for cell strains that can completely counteract the rise in intracellular peroxides under chronic hyperoxic stress.

Expression Arrays—Atlas Human Stress Arrays (Clontech) were used according to the manufacturer's manual. Total RNA from $1-2 \times 10^7$ cells in logarithmic growth phase was DNase-treated, and poly(A) RNA was enriched by hybridization to biotinylated oligo(dT). Probes were labeled with 50 μ Ci of [α - 32 P]dATP during reverse transcription. cDNA was purified by column chromatography and hybridized to the arrays. Quantification was performed using ImageQuaNT software (Amersham Biosciences). The threshold for a hybridization signal was defined as at least two times background variation (determined as the average of all absolute background measurements). Signals lower than this threshold were excluded from the analysis. β -Actin was used as the

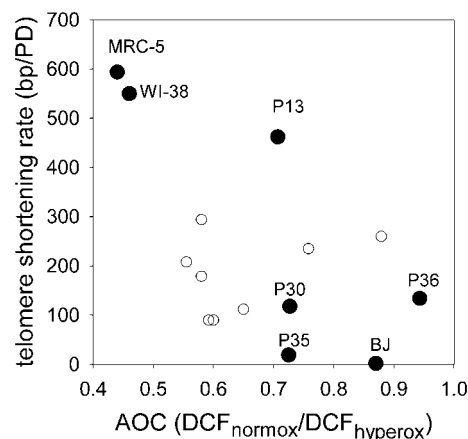


FIG. 1. Correlation between telomere shortening rate under 40% hyperoxia and AOC in human fibroblast strains from 15 different donors. Strains used for expression array analysis are shown as filled circles.

reference signal. Reproducibility was demonstrated in two strains with independent RNA preparations. For each strain, processing and arraying of normoxia and hyperoxia samples were done in parallel. Threshold values were set at 2-fold for the comparison of expression data between normoxia and hyperoxia within the same strain, and at 3-fold for comparison of expression values between cell strains.

RT-PCR and Northern Blotting—RNA was prepared either as described above or using the RNeasy Total RNA kit (Qiagen). Northern blots were probed for *Mn-SOD*, *EC-SOD*, *GADD153*, and β -actin as control. Probes were generated by RT-PCR or by PCR using the *EC-SOD* cDNA (24) as template with the primers given in Table I. Fragments were gel-purified, tested by restriction analysis, and random prime labeled with 32 P-dCTP. ImageQuaNT software (Amersham Biosciences) was used for quantitation.

Genes for *MTH1* and *TOP1* were expressed at low level and thus were analyzed by RT-PCR (SuperScript One-Step RT-PCR, Invitrogen). β -Actin was included as the reference control in each sample. Primers are given in Table I. PCR reactions were run for 30, 33, and 36 cycles to control the linearity of amplification. The expression of the *EC-SOD* transgene was also controlled by RT-PCR. RNA was reverse transcribed using random priming with Truescript RT (Hybaid-AGS), and cDNA equivalent to 50 ng of total RNA was used for a PCR reaction of 30 cycles at 58 $^{\circ}$ C. Two different non-overlapping primer pairs were used (see Table I). The 738-bp PCR band was cloned, and its correspondence to the cDNA sequence of *EC-SOD* was confirmed by sequencing.

SOD Activity— 2×10^6 cells were carefully washed and homogenized in 60 μ l of phosphate buffer (25). After centrifugation the total SOD activity in the supernatant was measured using the Bioxytech SOD-525 Kit (OXIS Health Products).

Telomere Length—Cells at subconfluency were embedded in 0.65% low-melting agarose plugs at a density of 10^7 cells/ml before treatment with proteinase K (7). DNA was digested completely by *HinfI* (60 units/plug, Roche Molecular Biochemicals) at 37 $^{\circ}$ C. Plugs were analyzed in a 1% agarose gel by pulsed field gel electrophoresis (BioRad). Gels were blotted to Hybond N+ membranes (Amersham Biosciences) and hybridized with the telomeric probe (TTAGGG) $_4$ directly conjugated to alkaline phosphatase (Promega, Madison, WI). A chemiluminescence signal was recorded on film within the linear range and analyzed in an imaging densitometer (BioRad). The average telomere length was calculated as the weighted mean of the optical density as

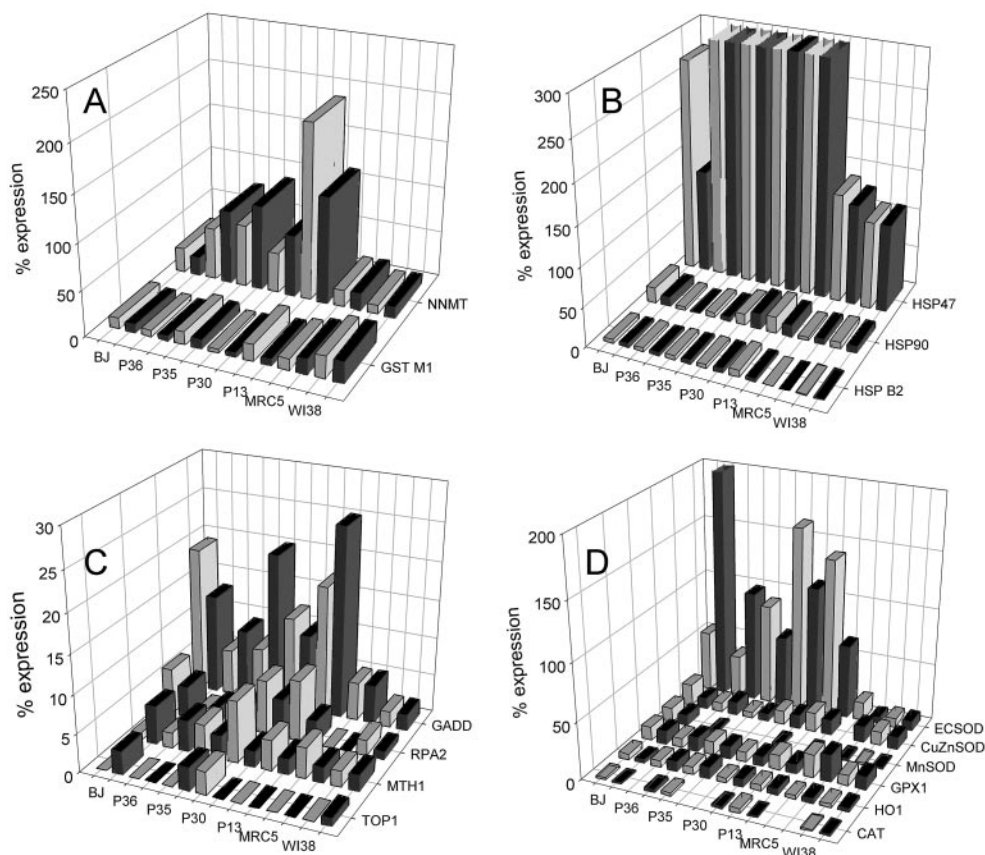


FIG. 2. Expression levels of candidate antioxidant/telomere maintenance genes in seven human fibroblast strains under normoxia (gray bars) and hyperoxia (darker bars) as measured by expression array (in % of cytoplasmic β -actin signal). Candidates are defined by an expression pattern broadly correlated to antioxidant capacity and/or telomere shortening rate of the strains. Where no bars are shown, expression was below the stated significance levels. A, drug-metabolizing enzymes NNMT and GST M1 gene expression levels. B, gene expression levels of heat shock proteins HSPB2, HSP90, and HSP47. C, expression patterns of the DNA modification and repair genes *GADD153*, *RPA2*, *MTH1*, and *TOP1*. D, expression of antioxidant enzymes *EC-SOD*, *CuZn-SOD*, *Mn-SOD*, glutathione peroxidase (*GPX1*), heme oxygenase 1 (*HO1*), and catalase (*CAT*).

described previously (20). The telomere shortening rate per PD was calculated as the slope of the linear regression. To test for nonspecific degradation of DNA, blots were rehybridized with the minisatellite probe (CAC)₈, which was end-labeled with [³²P- γ]ATP (7).

RESULTS

We had shown previously that both the antioxidative capacity (measured as the ability of cells to maintain their peroxidative potential under increased oxygen partial pressure) and the telomere shortening rate varied widely among fibroblast strains derived from different donors (18). Interestingly, both parameters were significantly correlated, suggesting that oxidative stress might be a major cause of telomere shortening in agreement with earlier data (for review, see Ref. 17). To identify genes that might be responsible for the observed differences, we selected seven fibroblast strains, representing the extremes in terms of telomere shortening rate and antioxidative capacity, for array analysis (Fig. 1). Four of the selected strains (BJ, P30, P35, P36) showed low telomere shortening rates and either high (BJ, P36) or moderate (P30, P35) antioxidative capacity, whereas two others (MRC-5, WI-38) displayed a large increase in DCF fluorescence under hyperoxia and high telomere shortening rates. One strain (P13) showed a high telomere shortening rate and a moderate antioxidative capacity.

In these seven cell strains, we measured the expression of 234 genes involved in stress response under both normoxia and hyperoxia using low-density arrays. Of these, 129 genes did not display any significant differences between strains or under increased oxidative stress using the significance levels as de-

scribed under "Experimental Procedures." Differences in the expression levels in most of the remaining 105 genes were clearly not correlated to either the telomere shortening rate or AOC. Only a few genes showed an expression pattern that might suggest correlation to cellular peroxide levels or telomere shortening rates. Among these were none of the kinases, cell cycle-related genes, or structural proteins included in the arrays. Those genes with potentially related patterns fall into four groups, namely drug-metabolizing enzymes, heat shock proteins, DNA modification and repair enzymes, and antioxidant enzymes (Fig. 2).

Among the drug-metabolizing enzymes (Fig. 2A), nicotinamide-*n*-methyltransferase (*NNMT*) was expressed at a significantly lower level in strains with low antioxidant capacity. By catalyzing NAD methylation, *NNMT* might be involved in free radical defense. On the other hand, glutathione *S*-transferase M1 (*GST M1*) tended to be higher in MRC-5 and WI-38. *GST M1* transfers reduced glutathione to a wide variety of exogenous and endogenous substances to detoxify them.

Three heat shock proteins, HSPB2, HSP90, and HSP47, showed lower mRNA levels in those fibroblast strains with low AOC. None of these mRNAs appeared to be regulated by hyperoxia (Fig. 2B). It is possible that a relative deficiency in these gene products contributes to an increased pro-oxidant state in MRC-5 and WI-38 fibroblasts.

Among the DNA modification and repair genes, four of them showed expression patterns in the arrays that appeared related to antioxidant capacity and telomere shortening rate (Fig. 2C).

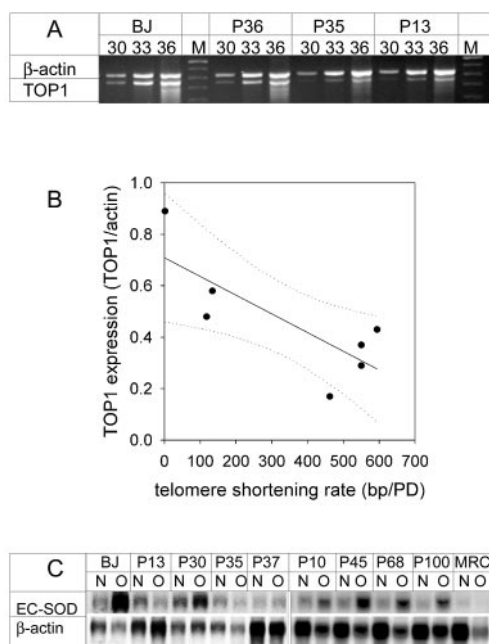


FIG. 3. Confirmation of gene expression patterns. A, semiquantitative RT-PCR for *TOP1* (lower band) and β -actin (upper band) in cell strains BJ, P36, P35, and P13. PCR cycle numbers are given above the lanes. M, 100-bp marker (from 600 to 1000 bp). B, correlation of *TOP1* expression (signal intensity ratio *TOP1*/ β -actin, 33 cycles) with telomere shortening rates in seven fibroblast strains. The correlation is significant with $p = 0.0366$. C, Northern blots for *EC-SOD* and β -actin in 10 fibroblast strains under normoxia (N) and hyperoxia (O).

However, such a correlation remains to be evaluated in the case of replication factor A 2 (*RPA2*), whereas Northern blotting and semiquantitative RT-PCR failed to confirm a significant correlation of either *MTH1* or *GADD153* expression with telomere shortening rate (data not shown).

Topoisomerase 1 (*TOP1*) mRNA expression was at the limit of significance in the array data (Fig. 2C). However, topoisomerases have been implicated in telomere stability in both yeast (26) and human cells (27). Thus, the expression pattern warranted further examination by RT-PCR. Results from seven fibroblast strains showed a significant inverse correlation between *TOP1* levels and telomere shortening rates (Fig. 3, A and B).

The mRNA levels for the antioxidant enzymes CuZn-SOD, glutathione peroxidase, heme oxygenase 1, and catalase were generally low and were hardly different between strains and between normoxia and hyperoxia (Fig. 2D). This result is not unexpected in the case of catalase, which is known to be post-transcriptionally regulated. In fact, Western blotting showed significantly higher catalase immunoreactivity in BJ as compared with MRC-5 or WI-38 (not shown). The array analysis indicated a higher Mn-SOD content in BJ fibroblasts as compared with all other strains (Fig. 2D), a result that has been confirmed by Northern blotting (not shown). However, the most striking result was the considerable difference in *EC-SOD* mRNA levels between cells with low and high peroxide contents (Fig. 2D). Moreover, hyperoxia resulted in a compensatory up-regulation of this mRNA in the two strains with the best antioxidant capacity (BJ and P36).

These results were confirmed by Northern blot (Fig. 3C). The correlation between array and Northern blot results for *EC-SOD* was significant ($p = 0.02$, hyperoxia). To establish the relevance of *EC-SOD* expression more firmly, additional fibroblast strains (including those shown in Fig. 1), which had previously been characterized in terms of DCF fluorescence and telomere shortening rate (18), were included in the analy-

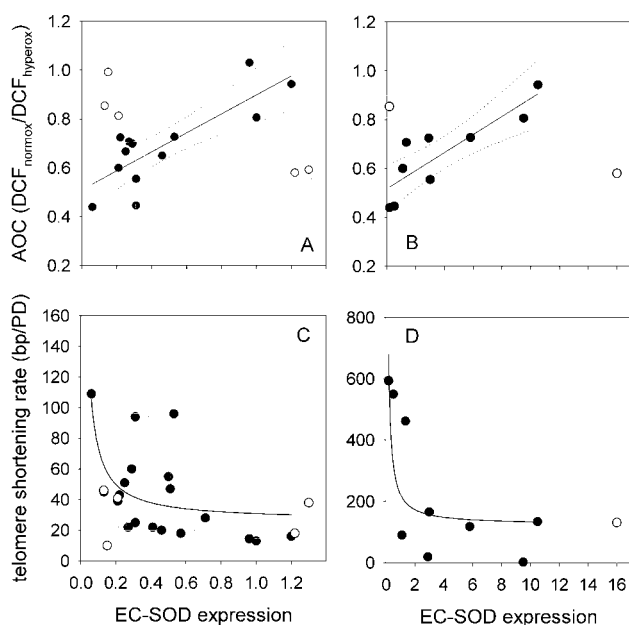


FIG. 4. Correlation of *EC-SOD* expression (related to β -actin signal intensity) with antioxidant capacity AOC (A and B) and with telomere shortening rate (C and D) under either normoxia (A and C) or hyperoxia (B and D). Data are mean \pm S.E. of measurements done in triplicate. Linear (A and B) and inverse (C and D) correlations were calculated as least-squares fits excluding those strains marked by open circles in A. The 95% confidence intervals for the linear correlations are indicated in A and B by dotted lines.

sis. Up-regulation of *EC-SOD* by hyperoxia was observed in a number of cell strains, especially those with high antioxidant capacity (Fig. 3C). In one strain (P30), up-regulation by hyperoxia was seen by Northern, but not in the arrays, possibly because of a higher noise level in the arrays. For 13 of 18 examined fibroblast strains grown under normoxia, *EC-SOD* expression correlated strongly with cellular antioxidative capacity (Fig. 4A). Essentially the same result was found for the correlation between *EC-SOD* expression under hyperoxia and antioxidant capacity (Fig. 4B), although only 11 strains were examined there. The two strains with high *EC-SOD* expression but comparatively low antioxidant capacity (Fig. 4A) displayed conspicuous deregulation of the catalase protein content (data not shown). We have not yet identified a plausible explanation for the deviation from the common trend toward high antioxidant capacity in three strains.

EC-SOD expression is not only a strong statistical predictor of antioxidant defense in most fibroblast strains; it is also significantly inversely correlated to the rate of telomere shortening both under normoxia (Fig. 4C) and under hyperoxia (Fig. 4D). Interestingly, even strains that do not fit the general trend, in Fig. 4, A and B, did not obviously deviate from the correlations shown in Fig. 4, C and D, which might indicate that a moderately good antioxidant capacity suffices to maintain a low telomere shortening rate. Taken together, these correlative data point to *EC-SOD* as the strongest candidate for maintaining low levels of oxidative stress in human fibroblasts under hyperoxia and low rates of telomere shortening.

To discover whether cellular SOD activity is dependent on the level of transcription of *EC-SOD* mRNA, we measured total SOD activity in cell extracts from the most extreme cell strains, BJ and MRC-5. SOD activity in BJ cells was significantly higher than that in MRC-5 (Fig. 5). Retroviral transduction of an *EC-SOD* cDNA (24) resulted in an at least a 10-fold overexpression of *EC-SOD* mRNA in MRC-5 fibroblasts as judged by RT-PCR (not shown). This increased the total cellular SOD

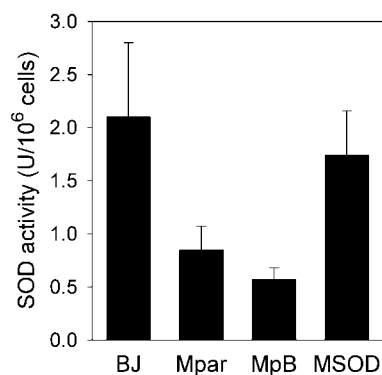


FIG. 5. Total SOD activity in cell extracts (units/million cells) in BJ fibroblasts (BJ), parental MRC-5 (Mpar), MRC-5 transduced with empty virus (MpB), and MRC-5 over-expressing EC-SOD (MSOD). Data are mean \pm S.E. from two to eight experiments. The SOD activity in BJ and EC-SOD-over-expressing MRC-5 cells is significantly higher than in parental or pBabe-transfected MRC-5 fibroblasts (ANOVA, $p < 0.05$).

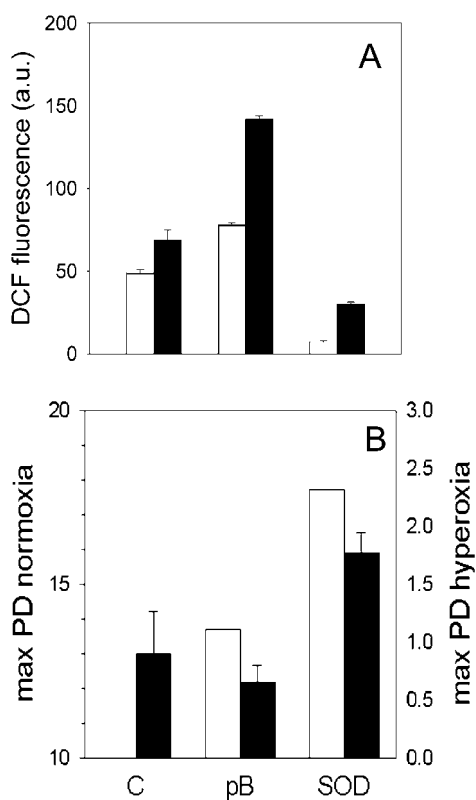


FIG. 6. EC-SOD expression diminishes oxidative stress and increases growth capacity. A, median DCF fluorescence in arbitrary units (a.u.) of parental MRC-5 (C), MRC-5 transduced with empty virus (pB), and MRC-5 over-expressing EC-SOD (SOD) under normoxia (open bars) and 40% hyperoxia (filled bars). Data are mean \pm S.E. from triplicate measurements. DCF fluorescence intensities in EC-SOD-expressing cells are significantly lower than in either parental cells or vector controls under both normoxia and hyperoxia (ANOVA, $p < 0.05$). B, growth capacity (population doublings achieved) under normoxia (open bars, left scale) and under 40% hyperoxia (filled bars, right scale). Data are mean \pm S.E. from five independent experiments (hyperoxia) and from one set of parallel experiments (normoxia). The growth capacity of EC-SOD-expressing cells under hyperoxia is significantly higher than that of either parental cells or vector controls (ANOVA, $p < 0.05$). The difference between control and vector-transduced cells is not significant.

activity in MRC-5 mass cultures up to a level similar to that in BJ fibroblasts (Fig. 5). Thus, we conclude that expression of EC-SOD contributes significantly to cellular SOD activity.

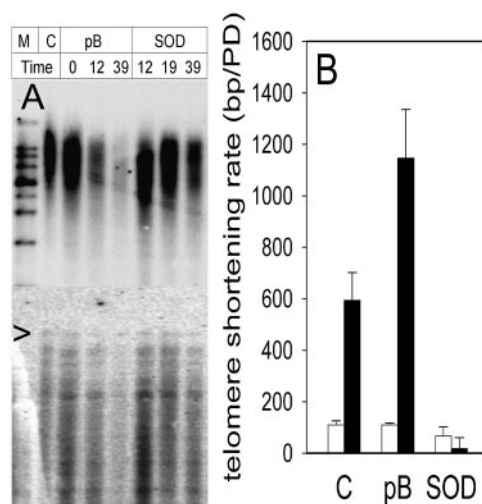


FIG. 7. Telomere shortening is dependent on EC-SOD expression. A, top, telomere Southern blot of MRC-5 fibroblasts transduced with empty virus (pB) and MRC-5 over-expressing EC-SOD (SOD). Culture time under 40% hyperoxia (in days) is indicated at the top of the figure; C denotes untransfected control under normoxia. Bottom, the same gel was rehybridized with an interstitial minisatellite probe. The band at about 20 kb (arrowhead) is seen in all lanes, indicating that there was no unspecific degradation of DNA. B, telomere shortening rates under normoxia (open bars) and hyperoxia (filled bars) in parental MRC-5 (C), MRC-5 transduced with empty virus (pB), and MRC-5 over-expressing EC-SOD (SOD). Data are mean \pm S.E. of triplicate or quadruplicate measurements from two independent mass transduction experiments. Telomere shortening rates in EC-SOD-expressing cells are significantly lower than in either parental cells or vector controls under both normoxia and hyperoxia (ANOVA, $p < 0.05$). Differences between normoxia and hyperoxia in the SOD group are not significant.

Over-expression of EC-SOD markedly decreased the concentration of intracellular peroxides as measured by cellular median DCF fluorescence intensity, both under normoxia and under hyperoxia (Fig. 6A). In accordance with their lower antioxidant capacity, MRC-5 fibroblasts achieve significantly less population doublings than BJ fibroblasts, both under normoxia and, even more so, under hyperoxia (7, 12). The replicative life span of MRC-5 fibroblasts under normoxia increased by about 25% after transfection with EC-SOD as compared with parallel vector only-transfected controls (Fig. 6B). This is comparable with the magnitude of effect of typical antioxidants and free radical scavengers (7, 8, 16). When parental MRC-5 and vector-transfected cells were transferred to hyperoxia at a PD of about 30, they became senescent after about 1 PD in accordance with earlier results (7, 8). EC-SOD over-expression sustained growth of MRC-5 cells under hyperoxia more than twice as long (Fig. 6B).

To examine whether EC-SOD expression would influence the telomere shortening rate of MRC-5 cells, we measured telomere lengths in both vector-transfected and EC-SOD-over-expressing MRC-5 fibroblasts. Cells transduced with empty virus showed an even faster rate of telomere shortening under hyperoxia than parental ones. This was not because of unspecific DNA degradation, as shown by rehybridization of the blots with an interstitial minisatellite probe (Fig. 7A). Mitochondrial uncoupling results in an even more exaggerated telomere shortening within a single cell cycle in mouse zygotes (28). Over-expression of EC-SOD resulted in a significantly lower shortening rate under both normoxia and hyperoxia (Fig. 7). Telomere shortening in EC-SOD-over-expressing fibroblasts under hyperoxia was even slower than in control cells under normoxia. Thus, increased expression of EC-SOD both slows down telomere shortening and postpones replicative senes-

cence in MRC-5 fibroblasts under normoxic and hyperoxic culture conditions.

DISCUSSION

We used low-density arrays of stress-related genes to identify possible candidates involved in the control of telomere shortening rate and antioxidant capacity in human fibroblasts under mild oxidative stress. This resulted in the identification of only one of the DNA modification and repair enzymes; high *TOP1* expression was correlated significantly to low telomere shortening rate. A number of data link the action of topoisomerases to telomeres. Loss of topoisomerase III leads to telomere shortening in yeast (26). Topoisomerase II was shown to cleave telomeres preferentially (29). Topoisomerases interact with members of the RecQ helicase family (30), which are known to bind telomeres. Telomerase activity modifies the sensitivity of human cells to topoisomerase inhibitors (27). *TOP1* is involved in the resolution and repair of single-strand breaks, and we have shown that oxidant-induced single-strand breaks in telomeres are a major determinant of the telomere shortening rate (7, 21). It will be interesting, therefore, to examine the possible role of *TOP1* in telomere repair and length maintenance more closely.

Our array studies revealed that the expression of only one superoxide dismutase, EC-SOD, correlated strongly with antioxidant defense capacity in human fibroblast strains. Steady-state levels of *EC-SOD* mRNA in antioxidatively competent strains were between 5 and 20 times higher than levels of *CuZn-SOD*. They were also 5–20 times higher than *EC-SOD* levels in incompetent strains. This is in agreement with earlier data showing up to 100-fold variation in *EC-SOD* expression between human fibroblast strains (25). *EC-SOD* expression was found to be inducible by mild chronic hyperoxia in some (generally antioxidatively competent) strains but not in others. Such noninducibility has been reported previously (31). Strains with high expression of *EC-SOD* (either endogenously or from a transgene) showed high total SOD activity in cellular lysates, suggesting that EC-SOD contributes significantly to the cellular superoxide dismutase activity. It has previously been shown that between 5 and 25% of EC-SOD protein is not secreted but is retained in the cell lysates (25). This retained fraction is not bound to extracellular heparin sulfate proteoglycan, but little more is known about its distribution.

The two most obvious locations for cell-bound EC-SOD protein are at the plasma membrane and within the endolysosomal system. In both locations, the enzyme might contribute significantly to cellular antioxidant defenses; NADH oxidation by the plasma membrane oxidoreductase system has recently been suggested as a likely source of superoxide (32). SOD activity at the plasma membrane might thus block the generation of reactive oxygen species at source. Alternatively, lysosomes are a major site for cellular iron storage and are thus highly susceptible to damage by hydrogen peroxide (33). The presence of EC-SOD in the endolysosomal system could inhibit superoxide-dependent recycling of catalytically active iron.

In line with these hypotheses, over-expression of *EC-SOD* was found to decrease the amount of cellular peroxides and to slow down telomere shortening, under both standard conditions and increased oxidative load, *i.e.* hyperoxia. This result confirms that telomere shortening under conditions of low to mild oxidative stress is to a large extent caused by telomeric DNA damage (7, 17).

The senescence-like growth arrest of human fibroblasts exposed to mild oxidative stress is undistinguishable from “Hayflick senescence” under standard culture conditions, in terms of telomere length (8, 23) and phenotype (34, 35). The fact that over-expression of an antioxidant, EC-SOD, slows down te-

lomere shortening and extends the replicative life span under both normoxia and hyperoxia in parallel strongly suggests that senescence under such mild oxidative stress conditions is telomere-driven.

In mice, *EC-SOD* over-expression or knockout phenotypes are comparatively mild. However, *EC-SOD* knockout mice are more sensitive to hyperoxia (36), and this increased sensitivity does not seem to be due to modification of the inflammatory response in the lungs (37). More interestingly, EC-SOD plays an important role in brain function, as both young *EC-SOD*-over-expressing and knockout mice are impaired in spatial learning and memory, which has been attributed to a deregulation of nitric oxide catabolism (38, 39). Conversely, knockout mice are more sensitive to focal cerebral ischemia (40), and *EC-SOD* over-expression increases the resistance of mice to global ischemia (41). Furthermore, improved learning and memory performance in aged mice over-expressing *EC-SOD* has been demonstrated recently (42). Together, these data suggest a protective role of EC-SOD in the brain, possibly related to its antioxidant function rather than to its role in nitric oxide turnover. In humans, we found short telomeres in patients with stroke-related (vascular) dementia. We assumed that short telomeres might indicate low antioxidant capacity and thus higher risk of stroke-induced brain damage *in vivo* (18). Data demonstrating a protective role for EC-SOD against ischemic brain damage (40, 41) and age-related deterioration (42) together with the demonstration of a role for EC-SOD in telomere maintenance in human cells (this study) are in agreement with this interpretation.

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