Stress-induced senescence predominates in endothelial cells isolated from atherosclerotic chronic smokers

Nada Farhat¹, Nathalie Thorin-Trescases¹, Guillaume Voghel, Louis Villeneuve, Maya Mamarbachi, Louis P. Perrault, Michel Carrier, and Eric Thorin²

Department of Surgery and Research Center, Institut de Cardiologie de Montréal, Université de Montréal, 5000, rue Bélanger, Montréal, QC H1T 1C8, Canada

Abstract

Age-associated telomere shortening leads to replicative senescence of human endothelial cells (EC). Risk factors for cardiovascular disease (CVD) accelerate ageing, while there is a concomitant rise in oxidative stress known to promote stress-induced senescence (SIS) in vitro. Of all risk factors for CVD, smoking is most associated with the development of inflammation and accelerated atherosclerosis due to a prooxidant–antioxidant imbalance. We tested the hypothesis that SIS predominates in EC isolated from chronic smokers with premature atherosclerosis undergoing coronary artery bypass graft surgery (CABG). We isolated and cultured EC from segments of internal mammary arteries from smoker, former smoker, and nonsmoker coronary patients. Senescence of EC was induced by serial passage and quantified by the measurement of telomere length and senescence-associated β -galactosidase activity. Compared with nonsmokers, smoker patients were 10 years younger at the time of CABG, evidence of premature atherosclerosis. Cellular senescence was independent of telomere length and directly related to oxidative damage. EC exhibited higher expression levels of markers of oxidative stress (lipid peroxidation level and caveolin-1 mRNA), inflammation (angiopoietin-like 2 mRNA), hypoxia (vascular endothelial growth factor (VEGF)-A mRNA), and cell damage (p53 mRNA). In conclusion, a high oxidative stress environment in EC isolated from atherosclerotic chronic smokers predisposes to SIS rather than replicative senescence.

Keywords

smoking; atherosclerosis; oxidative stress; caveolin-1; stress-induced senescence; telomere; replicative senescence

Introduction

Free radical-dependent cellular damage remains the basis of the theory of ageing (Harman 1956). Thus, any risk factor for cardiovascular disease (CVD) that magnifies the age-associated rise in free radical production would accelerate the ageing process. At the cellular level, ageing leads to senescence (Ben-Porath and Weinberg 2004, 2005). We previously

²Corresponding author (eric.thorin@umontreal.ca).

¹Both authors contributed equally to this work.

proposed that in cultured endothelial cells (EC) isolated from coronary patients, there was an interplay between the 2 major pathways of senescence (Voghel et al. 2007)—replicative senescence, due to telomere shortening following cell divisions, and stress-induced senescence (SIS)—which together contributed to the premature senescence of diseased EC. The duration of exposure to risk factors for CVD, rather than the biological age of the donor, predicted the onset of senescence (Voghel et al. 2007). We also reported that in EC from coronary patients, oxidative stress is a major contributor of SIS, as chronic antioxidant treatment could temporarily delay the onset of senescence (Voghel et al. 2008).

Of all the risk factors, smoking produces the most free radicals, both acutely and chronically (Nicita-Mauro et al. 2008). Cessation of smoking (Kondo et al. 2004; Wannamethee et al. 2005) and antioxidant therapy (Heitzer et al. 1996) improve endothelial function in smokers, suggesting a role for increased levels of oxygen-derived free radicals (Barua et al. 2003). In coronary artery disease, cigarette smoking causes injury to the vascular endothelium by direct cytotoxicity, leading to premature endothelial dysfunction (Blann and McCollum 1993; Ambrose and Barua 2004). Premature inflammation, atherosclerosis, and cardiovascular death are also reported in smokers (Nicita-Mauro et al. 2008). We therefore hypothesized that in vivo chronic exposure to tobacco, which contains mitogenic, carcinogenic, and oxidative molecules, predominantly leads, in vitro, to SIS of human vascular EC. We found that among patients with severe coronary artery disease, chronic smokers underwent coronary artery bypass graft surgery (CABG) 10 years earlier than nonsmokers. In addition, EC isolated from coronary smokers displayed, early in culture, unique markers of oxidative stress and cell damage that, in turn, predisposed EC to oxidative stress-dependent senescence, rather than to telomere-dependent senescence.

Materials and methods

Clinical profile of the donors

Segments of human distal internal mammary arteries (IMA) that were discarded during CABG ($n = 86$) were harvested with low electrocautery energy and excised with cold scissors. Collection of the samples was blind. Appropriate standards for human experimentation were followed, the study was reviewed and approved by our institution's ethics review committee, and the patients gave informed consent. According to notes in their clinical files, donors were divided into active smokers ($n = 26$), former smokers ($n = 40$), and nonsmokers (never smoked) ($n = 20$). Information on cigarette consumption (packages smoked per year) was not available; however, among the smokers, 27% (7/26) had chronic obstructive pulmonary disease (COPD). The group of former smokers was heterogeneous, since the duration of smoking cessation ranged from 0.1 to 30 years (average, 13.5 ± 1.9 years); among them, 22.5% (9/40) suffered from COPD. Only 1 nonsmoker had COPD.

Culture of EC

Endothelial cells were isolated from human IMA, cultured, and characterized, as described elsewhere (Voghel et al. 2007). Cells were incubated in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 10% calf serum, 1% penicillin–streptomycin, 90 µg/mL sodium heparin salt (Sigma-Aldrich, St. Louis, Mo.), 60 µg/mL EC

growth supplement (Becton Dickinson, Mississauga, Ont.), and 100 U/mL fungizone (Gibco, Burlington, Ont.), at 37 °C in a 95% air and 5% CO₂ incubator. EC were serially passaged until senescence was reached. For some experiments (oxidative stress, intrinsic antioxidant capacities estimation, initial gene expression, and initial telomere length), passage 2 was used.

Cells were collected for senescence-associated β -galactosidase staining (SA- β -Gal) (Dimri et al. 1995; Voghel et al. 2007), DNA testing (Southern blotting), RNA testing (real-time reverse transcription – polymerase chain reaction (RT-PCR)), and reactive oxygen species (ROS) measurement. Some cells were plated on cover slips for immunostaining. Before replating, cells were counted and the population doubling level (PDL) was calculated.

Telomere length measurement

Cells were grown in 75 cm² flasks at early (passage 2) and subsequent passages until replicative senescence was reached. DNA extraction was performed with a phenol–chloroform–isoamyl alcohol technique, precipitated with 95% ethanol, and dissolved in Tris–HCl (10 mmol/L, pH 8.6). Restriction fragment lengths (RFL) were quantified by using a Southern blot technique (Voghel et al. 2007).

Real-time RT-PCR

Total RNA (EC at passage 2) was isolated by using an RNeasy kit (Qiagen, Mississauga, Ont.) and was reverse-transcribed into first-strand complementary DNA with MMLV reverse transcriptase, using random hexamer primers designed by Primer Express (version 2.0). Real-time PCR was carried out on diluted RT products by using the DNA-binding dye SYBR Green I to detect PCR products (Mx3005P system, Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Serial dilutions (100 ng to 1 pg) of total RNA isolated from commercially available human aortic EC (Cambrex, Walkersville, Md.) were used as standards (Table 1).

The mRNA level in each sample was calculated relative to GAPDH. PCR was performed at 95 °C for 10 min, and then for 40 cycles at 95 °C for 30 s, 55 °C for 1 min, and 72°C for 30 s. A final dissociation cycle was performed at 95°C for 1 min, 55 °C for 30 s, and 95 °C for 30 s.

Immunofluorescence

Immunostaining was used (EC at passage 2) to assess the expression and subcellular localization of 4-hydroxynonenal (HNE) (rabbit polyclonal anti-HNE, 1:200; Alpha Diagnostics, San Antonio, Tex.), a marker of lipid peroxidation. DNA counterstaining was performed by incubating EC with 2 μ mol/L ToPro-3 (Molecular Probes, Burlington, Ont.). Negative controls were performed by omitting the primary antibodies during the protocol. Cells were visualized with a confocal microscope (Zeiss LSM 510, Carl Zeiss, Oberkochen, Germany). Semiquantitative analysis was performed by measuring the average total fluorescence intensity from 4 or 5 different pictures of the same cover slip (Voghel et al. 2008). Values are expressed in arbitrary units (a.u.) of fluorescence, corrected by the number of cells.

Intrinsic antioxidant capacities estimation

5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) (Molecular Probes) was used as a nonspecific marker of ROS. Living EC (passage 2) were loaded with 5 µmol/L CM-H₂DCFDA (in physiologic salt solution) for 30 min at 37 °C, washed, and allowed to recover for 10–15 min at 37 °C. The fluorescence was then observed at 488 nm under a confocal microscope. Negative controls consisted of unstained cells (autofluorescence). Human IMA EC used in this study displayed an unstable basal level of CM-H₂DCFDA fluorescence, a level that significantly increased over time (Figs. 4A and 4B). This is not observed in healthy cells, such as porcine or murine aortic EC, unless they are exposed to a superoxide dismutase inhibitor (data not shown), suggesting that EC isolated from coronary patients are more prone to produce ROS. Thus, we adapted the protocol that usually consists of measuring only the intensity of ROS fluorescence. Instead, EC were exposed to the laser light (time series at 488 nm) until the fluorescent signal stabilized. Cells in a different field were then exposed to 100 µmol/L H₂O₂ to obtain maximal oxidative activity, and the fluorescent signal was recorded until saturation. Identical parameters were used for experiments with EC from different donors (objective 63 × 1.4 plan-Apochromat oil, laser Argon 488 nm, 0.1% transmission; HFT 488 dichroic mirror, with filter LP505, pinhole size 1.7 Airy unit). The frame size of the images was 512 pixels × 300 pixels, and the scan speed was 2.56 µs per pixel. The time needed to reach 50% saturation in both control and H₂O₂-stimulated cells was calculated with LSM 510 software. The maximal fluorescence intensity in both control and H₂O₂-stimulated cells was also measured. The parameters of $\text{time } 50\%_{(\text{control} - \text{H}_2\text{O}_2)}$ and $\text{fluorescence } \text{max}_{(\text{H}_2\text{O}_2 - \text{control})}$ reflect the total intrinsic antioxidant capacities of the cells: the shorter the time and (or) the smaller the fluorescence, the lower the resistance to oxidative stimuli, and the lower the antioxidant defences.

Statistical analysis of the data

Continuous data are presented as means ± SE, with *n* indicating the number of patients. Appropriate univariate analysis (*t* test or ANOVA with Fisher's post hoc test) was used (Statview 4.5). A *p* < 0.05 was considered statistically significant.

Results

Patient characteristics

The clinical parameters were evenly distributed among patients, except for the mean age of the donor: smokers (56 ± 2 years of age) underwent CABG surgery 10 years before (*p* < 0.05) nonsmokers (67 ± 2 years) or former smokers (66 ± 2 years) (Table 2).

Telomere-dependent replicative senescence

Initial telomere length was similar among groups (8.9 ± 0.4 kbp, 9.3 ± 0.3 kbp, and 9.0 ± 0.2 kbp in EC from non-smokers, former smokers, and smokers, respectively). However, the telomere shortening rate (the difference between telomere length measured at senescence and that measured initially, corrected by the cumulative replicative potential, $\text{RFL}/\Sigma\text{PDL}$) was significantly reduced in EC from smokers, compared with nonsmokers (*p* = 0.009) or

former smokers ($p = 0.02$) (Fig. 1A). We previously reported that the onset of replicative senescence could be predicted by initial telomere length (Voghel et al. 2007). Accordingly, although short initial RFL predicted the development of early senescence in cultured EC isolated from nonsmokers ($p = 0.04$) (Fig. 1B) and former smokers ($p = 0.004$, data not shown), initial RFL measured in EC from smokers (Fig. 1C) did not correlate with the propensity to develop senescence ($p = 0.47$). Altogether, these results are suggestive of a minor role for replicative senescence in EC from smokers.

Senescence profile

Serial passage induced senescence in EC, whether or not the donor was smoking. Senescence, however, appeared slightly later in EC from smokers than from nonsmokers (Fig. 2); the time needed to reach 50% senescence was 17 days longer in EC from smokers ($p = 0.03$). The PDL reached at 50% senescence was also higher in smokers ($p = 0.03$) (Fig. 2), suggesting a slightly higher replicative potential. The senescence profile of EC from former smokers was intermediate, suggesting some normalization toward a non-smoker phenotype.

Initial EC biomarkers of oxidative stress

Oxidative stress and free radical production were evaluated early in culture—that is, at passage 2 (cumulative PDL = 4.5 ± 1.4 ; SA- β -Gal-positive cells = $5.1 \pm 0.8\%$; $n = 46$)—to reflect the *in vivo* situation rather than the effect of cell culture.

Initial levels of HNE, a marker of lipid peroxidation, were significantly higher in EC from smokers than from non-smokers or former smokers (Fig. 3).

In EC from coronary patients, whether smokers or non-smokers, the level of CM-H₂DCFDA fluorescence increased over time (Figs. 4A and 4B), most likely because of strong generation of ROS by the cells and concomitant oxidation of the dye. In healthy porcine EC, the level of CM-H₂DCFDA fluorescence is approximately 10 500-fold lower than in human IMA EC (unpublished data). Furthermore, in healthy EC, the signal remains stable over time (data not shown). EC from smokers and nonsmokers behaved differently when exogenous H₂O₂ was added at a sublethal concentration. In EC from smokers, the CM-H₂DCFDA signal rose faster than the signal observed in the absence of H₂O₂ stimulus. In contrast, EC from nonsmokers and former smokers developed a slow and weak signal in response to the laser light in the presence of H₂O₂, demonstrating the endogenous ability of the cell to scavenge oxidative stress. Taken together, these data suggest that there is a significantly lower antioxidant capacity in EC from smokers than from non-smokers.

Genomic EC markers of smoking

Gene expression levels were quantified at passage 2, as described above. The initial gene expression of caveolin-1, a protein known to promote oxidative SIS (Galbiati et al. 2001; Voghel et al. 2007, 2008), was 8-fold higher ($p = 0.03$) in EC from smokers (Table 3). In addition, caveolin-1 expression positively correlated with the oxidative stress marker HNE ($p < 0.001$, $r = 0.850$, $n = 10$; data not shown). On the other hand, initial ATM expression, a protein known to function as a transducer of double-strand DNA breaks and telomere

dysfunction (Pandita 2002; Herbig et al. 2004), was 2-fold lower in EC from smokers ($p = 0.02$) or former smokers ($p < 0.05$). Similar data were observed at the ATM protein level (data not shown). Initial angiopoietin-like 2 (ANGPTL2) mRNA levels, a potential marker of inflammation (Sun et al. 2007), were 4 times higher ($p = 0.04$) in EC from smokers than from nonsmokers (Table 3). Similarly, initial gene levels of the angiogenic factor VEGF-A were 3 times higher ($p < 0.001$) in EC from smokers. Damage was evidenced by a 2-fold increase in gene expression of the tumour suppressor gene p53 ($p = 0.049$) and protein p53 (data not shown), a downstream target in EC from smokers. Cessation of smoking was associated with a reduction of caveolin-1, ANGPTL2, p53, and VEGF-A mRNA levels, but not ATM mRNA levels (Table 3).

Discussion

The major finding of the present study is that SIS predominates in EC isolated from active smokers undergoing CABG surgery. Smoking is associated with an increased oxidative stress and a reduced antioxidant defence capacity of the cells. These results are in agreement with the premature atherosclerosis that developed in these patients, a 10-year prematurity in coronary disease that strikingly matches the reduced life expectancy of active smokers (Doll et al. 2005; Nicita-Mauro et al. 2008).

Cellular senescence, estimated by SA- β -Gal, was slightly delayed in EC from smokers compared with nonsmokers (Fig. 2). This small delay is most likely related to the younger age of the smokers. This increase in replicative potential could be attributed to a better integrity of the mitotic clock of the cells. Although EC eventually entered senescence in both groups, the main pathway of senescence differed dramatically between groups. In smokers, replicative senescence appeared minimal, since (i) telomere shortening was smaller (Fig. 1A), (ii) ATM gene expression was lower in EC from smokers, suggesting lower molecular and telomeric instability (Table 3), and (iii) initial telomere length was not correlated with the propensity to develop senescence (Fig. 1B). On the other hand, (i) the massive oxidative stress and damage, evidenced by an increased level of lipid peroxidation (Fig. 3), (ii) the lack of antioxidant defence mechanisms (Fig. 4), and (iii) an increased caveolin-1 and p53 gene and (or) protein expression (Table 3) in EC isolated from smokers are all factors that drive senescence through SIS. This contrasts with the work of Valdes et al. (2005), in which telomere shortening, measured in white blood cells, was greater in women who smoked. The difference in cell types is most likely responsible for this discrepancy. It is possible that an increased inflammatory phenotype promotes leukocyte mobilization, a high turnover rate, and thus excessive telomere attrition, but this remains speculative. We also observed no difference in nuclear or cytosolic human telomerase activity between EC from smokers and nonsmokers (data not shown), suggesting that telomere shortening is not compensated for in EC from smokers by higher telomerase activity. Cessation of smoking was associated with a decrease in the oxidative stress marker HNE (Fig. 3); an improvement in intrinsic antioxidant properties (Fig. 4); a decrease in caveolin-1, p53, VEGF-A, and ANGPTL2 gene expression (Table 3); and a higher telomere shortening rate (Fig. 1). Altogether, these data strongly suggest that EC from chronic smokers are predisposed to SIS, but not to telomere-dependent senescence pathways. The fact that cigarette smoke induces cellular senescence

has been reported in alveolar epithelial cells (Tsuji et al. 2006) and in fibroblasts (Nyunoya et al. 2006), but SIS has not been clearly demonstrated.

The fluorescent probe CM-H₂DCFDA is broadly used to nonspecifically estimate the sources of oxidative stress. While our approach to quantify the intrinsic antioxidant capacities does not provide a descriptive assessment of sources of antioxidant defences, it globally estimates the capacities of EC to react to H₂O₂ stimulus. This lack of antioxidant defence is in line with our finding that caveolin-1 gene expression was significantly increased in EC from smokers and positively correlated with HNE levels. The level of caveolin-1 is strictly regulated to maintain cellular integrity, leading to cellular transformation if depleted and to a phenotype of premature senescence if overexpressed (Cho and Park 2005; Galbiati et al. 2001). Our data, therefore, strongly support the concept of the presence of high oxidative stress in EC from smokers. The fact that smoking is associated with increased oxidative stress has been reported in other sources (Nicita-Mauro et al. 2008): in serum and plasma from smokers, high levels of malondialdehydes (Sharma et al. 2005) and F₂-isoprostane (Dietrich et al. 2002) have been observed. Products of oxidative DNA damage were measured in urinary samples (Chen et al. 2004) and lung tissues (Asami et al. 1997) from smokers. Altogether, these data indicate that chronic exposure to tobacco induces potent chronic oxidative stress that could lead to EC damage, SIS, and the premature atherosclerosis observed in our population of smoking patients.

Compared with coronary arteries, IMA rarely develop macroscopic atherosclerotic plaques. Although not exposed to the same mechanical stress imposed on the coronary circulation, they are nevertheless exposed to similar levels of atherogenic agents (Puga 1995). It has been reported that in IMA tissue from smokers, antioxidant capacities were significantly lower than in tissues from nonsmokers, and that the severity of coronary atherosclerotic lesions was inversely correlated to the IMA levels of vitamin E (Mezzetti et al. 1995). On the other hand, it has been reported that vascular smooth muscle cells (VSMC) from IMA, compared with VSMC from coronary arteries, have enhanced intrinsic anti-oxidant enzyme capacities (Mahadevan et al. 2006). To our knowledge, there are no data concerning intrinsic prooxidant or antioxidant capacities in EC isolated from IMA.

EC from smokers display higher initial VEGF-A gene expression (Table 3). Nicotine has been shown to increase VEGF expression (Conklin et al. 2002). Hypoxia is also a strong inducer of both VEGF and VEGF receptor genes, and VEGF is a trophic factor required for EC survival (Kasahara et al. 2001). Combined with the younger age of the donors, these abnormally high levels of VEGF could also contribute to the higher replicative potential of EC from smokers observed in culture. In addition, we observed higher initial ANGPTL2 gene expression in EC from smokers (Table 3). If we consider the impact of COPD, a state of chronic inflammation, on the smoker subgroup, ANGPTL2 mRNA levels were even higher in smokers with COPD than in those without (11.68 ± 5.44 versus 2.29 ± 0.97 , $p < 0.05$; data not shown). ANGPTL2 is a novel growth factor (Morisada et al. 2006) that could be involved in the development of the abnormal vasculature in diabetes and in endothelial inflammation (Sun et al. 2007). ANGPTL2 gene expression was positively correlated with VEGF-A gene expression and replicative potential (data not shown). Thus, both VEGF and ANGPTL2 could promote cell growth in response to the chronic inflammatory stress

associated with smoking. Finally, EC isolated from smokers expressed higher mRNA and higher protein (data not shown) levels of p53. The tumour suppressor p53 is involved in multiple cellular functions, including senescence (Ben-Porath and Weinberg 2005), and is an index of cell damage, since its function is to restrict stress-exposed cells before damage to DNA become irreversible. There is therefore a striking combination of expression of prosurvival and damage biomarkers in EC isolated from smokers. This suggests that in EC isolated from active smokers, p53-driven senescence can occur before reaching the Hayflick limit (that is, independently of telomere shortening), and that oxidative stress triggers senescence upstream of DNA damage.

We need to consider some potential limitations of our study. The patients used in this study were atherosclerotic and hypertensive, obese, and (or) diabetic; the combination of these multiple risk factors for cardiovascular disease makes the analysis of the effect of smoking on senescence complex. We found, however, clear differences between atherosclerotic smokers and atherosclerotic nonsmokers, namely, younger age, lower EC antioxidant capacities, and telomere-independent senescence in the former group. The second possible limitation is the lack of information on the patients' daily cigarette consumption. This may not be critical, however, since it has been reported that alteration of endothelial vasoregulatory activity is independent of the number of cigarettes smoked (Barua et al. 2002). This activity was similar in subjects who smoked 1 pack of cigarettes per week, in those who smoked 1 pack per day, or in those who smoked less.

In conclusion, EC from severely atherosclerotic smokers displayed evidence of long-term in vivo exposure to oxidative stress and inflammation that most likely hastened the need for CABG by a decade. The severe cellular damage induced by chronic smoking drives EC toward a senescence pathway that is telomere-independent and mainly triggered by oxidative stress.

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Fig. 1.

Lower telomere shortening in EC from smokers. (A) Telomere length (RFL, bp) was measured by Southern blot, initially (passage 2) and when cells reached senescence. Telomere shortening was then calculated (ΔRFL) and corrected by the number of cell divisions performed (ΣPDL) in EC from nonsmokers ($n = 16$), former smokers ($n = 30$), and smokers ($n = 21$). *, Significant at $p < 0.05$ compared with nonsmokers. (B, C) Correlation between the marker of propensity to develop senescence (time to reach 50% senescent EC, in days) and initial telomere length (bp) in EC from nonsmokers ($r = 0.526$, $p = 0.04$, $n = 15$) and smokers ($r = 0.164$, $p = 0.47$, $n = 21$). EC, endothelial cells; RFL, restriction fragment length; bp, base pair; PDL, population doubling level; SA- β -Gal, senescence-associated β -galactosidase staining.

	50% of senescent cells: Σ Days	Σ PDL
□ Non smokers	87±4	10±1
Δ Former smokers	103±5 *	12±1
■ Smokers	104±5 *	13±1 *

Fig. 2.

Passage-induced senescence in EC from smokers. Time course of senescence, quantified by SA- β -Gal and induced by serial passage in culture, in EC isolated from nonsmokers (□, $n = 16$), former smokers (Δ, $n = 34$), and smokers (■, $n = 25$). From the profiles of senescence, we calculated the time (Σ days) and the number of cell divisions (Σ PDL) reached when 50% of the cells were senescent. *, Significant at $p < 0.05$ compared with nonsmokers. EC, endothelial cells; SA- β -Gal, senescence-associated β -galactosidase; PDL, population doubling level.

Fig. 3.

Increase in lipid peroxidation in EC isolated from smokers. (A, B) Total levels of HNE were estimated by immunofluorescence staining (red) at passage 2. DNA counterstaining (blue) was performed with ToPro-3. Examples of immunostaining of EC isolated from nonsmoker and smoker patients are shown. (C) Average initial values of total HNE, corrected by the number of cells, in EC from nonsmokers ($n = 8$), former smokers ($n = 9$), and smokers ($n = 5$). *, Significant at $p < 0.05$ compared with nonsmokers. EC, endothelial cells; HNE, 4-hydroxynonenal.

Fig. 4.

Examples of saturation profiles in EC from a nonsmoker (A) and a smoker (B). Lower intrinsic antioxidant capacities in living EC from smokers was shown by loading EC with the nonspecific fluorescent dye CM-H₂DCFDA at passage 2 (C). EC were stimulated with the laser light in the absence and presence of 100 μmol/L H₂O₂ until the fluorescent signal stabilized. The time needed to reach 50% saturation in both control and H₂O₂-stimulated cells was measured, and the maximal fluorescence intensity in control and H₂O₂-stimulated cells was recorded. $\Delta \text{Time}_{50\%(\text{control} - \text{H}_2\text{O}_2)}$ and $\text{fluorescencemax}_{(\text{H}_2\text{O}_2 - \text{control})}$ were calculated: the shorter time and the smaller fluorescence, the lower the antioxidant capacities of the cells. (D) Average $\Delta \text{Time}_{50\%}$ and (E) fluorescence in EC from nonsmokers ($n = 6$), former smokers ($n = 9$), and smokers ($n = 4$). *, Significant at $p < 0.05$ compared with nonsmokers.

Table 1

Primers used to quantify gene expression of markers of oxidative stress (caveolin-1), DNA breakage and telomere dysfunction (ATM), inflammation (ANGPTL2), hypoxia (VEGF-A), and cell damage (p53). The mRNA level in each sample was calculated relative to GAPDH.

Primers	Forward (5'–3')	Reverse (5'–3')
Caveolin-1	GCTGAGCGAGAAGCAAGTGT	TGGTGAAGCTGGCCTTCCAA
ATM	GGCAGCTGATATTCGGAGGA	CATCTTGGTCACGACGATAC
ANGPTL2	GCAATGCGGGTGACTCCTT	TACCAGGACGGAGTCTA
VEGF-A	GAGGGCAGAATCATCACGAA	AGGAGTCCAACATCACCATG
p53	TGAGGTTGGCTCTGACTGTA	TTCTCTCCTCTGTGCGCCG
GAPDH	TGAAGGTCGGAGTCAACGGA	CATTGATGACAAGCTTCCCG

Note: ATM, ataxia-telangiectasia mutated; ANGPTL2, angiopoietin-like 2; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2

Clinical profiles of patients undergoing coronary artery bypass graft surgery.

	Smokers, <i>n</i> = 26	Former smokers, <i>n</i> = 40	Nonsmokers, <i>n</i> = 20
Sex, male/female	22/4	30/10	15/5
Age, years (range)	56.2±2.4* (25–79)	66.1±1.6 (47–84)	66.5±2.1 (50–80)
BMI, kg/m ²	27.0±0.9	28.7±0.9	28.8±1.1
Glucose, mmol/L	6.5±0.3	6.5±0.2	6.7±0.4
LVEF, %	50.0±2.4	51.8±2.1	52.6±2.7
History of disease, years (range)	8.2±2.6 (0.1–47)	7.0±1.2 (0.1–29)	6.3±1.9 (0.1–23)
Dyslipidemia, %	92 (23/25)	85 (34/40)	89.5 (17/19)
Hypertension, %	58 (14/24)	75 (30/40)	75 (15/20)
Diabetes, %	24 (6/25)	30 (12/40)	45 (9/20)
Family history of CAD, %	94 (16/17)	85 (17/20)	63.6 (7/11)
Renal failure, %	8 (2/25)	17.5 (7/40)	5 (1/20)
COPD, %	27 (7/26)	23 (9/40)	5 (1/20)

Note: Patients were treated with similar medications, including aspirin, angiotensin-converting enzyme (ACE) inhibitors, β -blockers, calcium channels blockers, statins, and nitrates.

*, Significant at $p < 0.05$ versus nonsmokers (ANOVA).

BMI, body mass index; LVEF, left ventricular ejection fraction; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease.

Table 3

Initial gene expression in endothelial cells isolated from coronary patients.

mRNA	Nonsmokers (n)	Former smokers (n)	Smokers (n)
Caveolin-1	0.446±0.091 (8)	0.691±0.131 (19)	3.666±1.436 (14) *
ATM	1.295±0.393 (6)	0.692±0.098 (18) *	0.689±0.089 (14) *
ANGPTL2	1.354±0.461 (12)	1.550±0.306 (25)	5.053±1.944 (17) *
VEGF-A	2.555±0.452 (12)	3.160±0.646 (25)	7.321±1.083 (17) *
p53	1.224±0.092 (5)	1.368±0.143 (15)	2.414±0.533 (12) *

Note:

*, Significant at $p < 0.05$ versus nonsmokers (ANOVA).