

Deoxyribonucleic Acid Damage in Human Lymphocytes After Percutaneous Transluminal Coronary Angioplasty

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OBJECTIVES	We investigated the presence of oxidative deoxyribonucleic acid (DNA) damage in the peripheral lymphocytes of patients undergoing percutaneous transluminal coronary angioplasty (PTCA) by using the micronucleus test and comet assay, which are sensitive biomarkers of DNA damage.
BACKGROUND	Although it has recognized that ischemia-reperfusion can induce oxidative DNA damage, its occurrence in patients undergoing PTCA has not yet been demonstrated.
METHODS	Three groups of patients were enrolled: 30 patients with documented coronary heart disease who underwent elective PTCA (group I); 25 patients who underwent elective coronary angiography for diagnostic purpose (group II); and 27 healthy, age- and gender-matched subjects (group III). For each subject, the frequency of micronucleated binucleated (MNBN) cells, DNA single-strand breaks (SSBs), endonuclease III-sensitive sites, and sites sensitive to formamidopyrimidine glycosylase (FPG) were analyzed before and after diagnostic procedures.
RESULTS	The mean basal values of MNBN cells ($p = 0.04$), DNA-SSBs ($p = 0.001$), endonuclease III-sensitive sites ($p = 0.002$), and FPG sites ($p < 0.0001$) were significantly higher in groups I and II than in group III. A high significant increase of MNBN cell frequency was observed in group I after the PTCA procedure (11.0 ± 1.3 vs. 19.8 ± 1.6 , $p < 0.0001$), whereas no significant difference was observed in group II (10.2 ± 1.3 vs. 12.9 ± 1.4 , $p = 0.18$). A significant positive correlation was observed between the increase in the MNBN cell rate and total inflation time during PTCA ($R = 0.549$, $p = 0.0017$). The levels of DNA-SSBs (11.7 ± 1.4 vs. 26.5 ± 3.0 , $p = 0.0003$) and FPG sites (13.8 ± 1.8 vs. 22.5 ± 2.4 , $p = 0.01$) were also higher after PTCA.
CONCLUSIONS	Our results provide evidence for oxidative DNA damage after PTCA, likely related to ischemia-reperfusion injury. (J Am Coll Cardiol 2002;40:862-8) © 2002 by the American College of Cardiology Foundation

Reactive oxygen species, such as superoxide anion, hydrogen peroxide, peroxynitrite, and hydroxyl radical, seem to play a pivotal role in the pathogenesis of ischemia-reperfusion injury (1). Free radicals exert their cytotoxic effects by producing several metabolic dysfunctions, including the peroxidation of polyunsaturated fatty acids in membrane or plasma lipoproteins, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of membrane sodium channels, and other protein modifications (2,3). Moreover, it is well known that deoxyribonucleic acid (DNA) damage frequently occurs in cells exposed to oxidative stress (4).

The presence of free radicals has been demonstrated after percutaneous transluminal coronary angioplasty (PTCA) (5-8), but at the present time, the induction of DNA damage by free radicals in the circulating cells of patients undergoing PTCA has not yet been demonstrated.

Therefore, this study was designed to investigate, by using the micronucleus test and comet assay in the human lymphocytes of patients undergoing PTCA, whether this

procedure, considered to be a model of ischemia-reperfusion injury, may cause genetic damage.

We have to consider that the induction of micronuclei in circulating human lymphocytes is a well-known cytogenetic test widely employed to assess the genetic effects of spontaneously and mutagenically induced DNA damage (9).

The comet assay is considered a sensitive marker of oxidative stress and DNA damage (10). Indeed, the use of lesion-specific enzymes, such as endonuclease III and formamidopyrimidine glycosylase (FPG), which create DNA strand breaks at sites of oxidative damage, is a fast approach for measuring of oxidative DNA damage (11,12).

Our study was performed in a group of patients who underwent elective PTCA for the presence of coronary stenosis. Moreover, to exclude a hypothetical direct effect of X-ray exposure and contrast media on DNA damage, a second group of patients who underwent diagnostic coronary angiography for the presence of myocardial ischemia on effort was also included.

METHODS

Patients. Two groups of patients characterized by the documentation of myocardial ischemia on a stress echocar-

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Abbreviations and Acronyms

CAD	= coronary artery disease
DNA	= deoxyribonucleic acid
FPG	= formamidopyrimidine glycosylase
MNBN	= micronucleated binucleated
PTCA	= percutaneous transluminal coronary angioplasty
SSB	= single-strand breaks

diogram with dipyridamole, performed according to the standard protocol of use in our institute (13), were consecutively recruited: 30 patients (25 men and 5 women; mean age 61.3 ± 2.0 years) undergoing elective PTCA (group I) and 25 patients (19 men and 6 women; mean age 64.4 ± 2.5 years) undergoing diagnostic coronary angiography (group II). In addition, 27 healthy subjects (19 men and 8 women; mean age 60.5 ± 1.4 years) matched for age and gender with the patients of groups I and II were also included as control subjects (group III).

The severity of coronary artery disease (CAD) was expressed simply by the number of affected vessels (one-, two-, or three-vessel disease). Moreover, the extent and severity of CAD were also evaluated by using the Duke scoring system (14), with a grade scale ranging from 0 to 100 (0 = no disease, 100 = most severe disease).

Patients with episodes of angina within the last 24 h before the procedure and myocardial infarction within the last three months were excluded. All patients in whom the procedure was performed on an urgency or emergency basis were also excluded.

For the procedures, the patients received nonionic contrast media (Ipromid, Ultravist 370, Schering, Germany).

All patients gave written, informed consent to be in-

cluded in the present study, which was approved by our local ethical committee.

Venous blood samples were obtained the day before and three days after the clinical and interventional procedures. This interval between the two samples was chosen to perform the micronucleus test and comet assay in peripheral lymphocytes after a relatively extended period of time from the procedure.

The demographic and clinical data, angiographic findings, and procedural variables of the study patients are reported in Table 1.

Cytokinesis block assay and lymphocyte cultures. This technique employs cytochalasin B to interrupt the division of each cell before cytokinesis. Therefore, cells that have completed only one nuclear division can be recognized by their binucleated appearance (9).

The micronucleus is a small chromatinic particle visible in interphasic cells close to the main nucleus. It can come from an acentric chromosomal fragment or from a whole chromosome not correctly incorporated in one of the two daughter cells at the end of the mitotic division. Thus, micronuclei are a reliable biomarker of exposure to both clastogen and aneuploidogen agents that affect the spindle apparatus.

Two separate cultures from each sample were set up by mixing 0.3 ml of whole blood with 4.7 ml RPMI 1640 medium (ICN Pharmaceuticals Inc., Irvine, California), supplemented with 10% fetal calf serum (ICN), 1.5% phytohemagglutinin (Wellcome, Uxbridge, UK), and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin; Sigma, St. Louis, Missouri). Cultures were maintained for 72 h at 37°C in a humidified atmosphere of 5% carbon dioxide in air, and cells were blocked in cytokinesis by adding 6 µg/ml cytochalasin B (Sigma) after 44-h incuba-

Table 1. Demographic and Clinical Characteristics

	Group I (n = 30)	Group II (n = 25)	Group III (n = 27)	p Value
Age (yrs)	61.3 ± 2.0	64.4 ± 2.5	60.5 ± 1.4	NS
Males	25	19	19	NS
Smokers	21	13	10	0.04
Duke score	36.9 ± 1.7	30.5 ± 6.1		NS
Ejection fraction (%)	53.7 ± 1.4	49.9 ± 2.5		NS
Risk factors				
Hypertension	16	15		NS
Diabetes	7	5		NS
Dyslipidemia	22	15		NS
Medications				
Oral nitrate	21	12		NS
Calcium antagonist	16	8		NS
Beta-blocker	12	10		NS
Aspirin	17	9		NS
Procedural variables				
Total inflation time (s)	115.6 ± 12.0	—		
Fluoroscopy exposure rate (Gy/cm ²)	144.1 ± 18.1	55.5 ± 8.7		0.0001
Fluoroscopy exposure time (min)	18.3 ± 1.9	9.1 ± 1.2		0.0003

Data are presented as the mean value ± SEM or number of patients or control subjects.
NS = not significant.

tion. Harvesting of cells, hypotonic treatment, fixation, and slide preparation were performed according to the standard method of use in our laboratory (15). For each sample, 1,000 binucleated cells were scored blindly under an optical microscope (final magnification $\times 400$) for analysis of micronuclei, following the criteria for acceptance of micronuclei (9). We evaluated the micronucleated binucleated (MNBN) cell frequency as the number of micronucleated cells per 1,000 cells.

Comet assay. PRIMARY DNA DAMAGE. The alkaline single-cell gel-electrophoresis assay was performed in 10 patients in group I, 10 patients in group II, and 11 subjects in group III (healthy control subjects). In its alkaline version, the formation of DNA single-strand breaks (SSBs) becomes apparent, and the amount of DNA migration, a "comet-like tail," indicates the amount of DNA damage in the cell.

The assay was performed according to the method previously described (10-12). Briefly, isolated leukocytes were suspended in a solution of 0.5% low-melting agarose and pipetted on a microscope slide precoated with 1% normal-melting agarose. Then, the slides were immersed in a lysis solution containing Triton X-100 and 2.5 mol/l NaCl to lyse the cells and allow DNA unfolding. The resulting nucleoids were electrophoresed for 25 min at 25 V (300 mA) in alkaline buffer (1 mmol/l Na_2EDTA , 300 mmol/l NaOH, pH >13).

OXIDATIVE DNA DAMAGE. The measurement of oxidative DNA damage was performed by using specific repair enzymes that induce breaks in the DNA sites of oxidative damage. Endonuclease III was used to detect oxidized pyrimidines, and formamidopyrimidine glycosylase (FPG) was used to detect damaged purines, including 8-oxoguanine (11,12). After lysis, the slides were incubated at 37°C for 45 min with endonuclease III and 30 min with FPG. Then, the slides were washed in a neutralizing buffer (0.4 mol/l Tris-HCl, pH 7.5) and stained with 50 $\mu\text{g}/\text{ml}$ ethidium bromide and covered with a cover slip.

For each subject, 50 comets were analyzed under a fluorescence microscope. The percentage of DNA in the tail was taken as a measure of DNA break frequency. The net amount of damage represented by endonuclease III-sensitive or FPG-sensitive sites was obtained by subtracting the percent DNA in the tail without enzyme (i.e., strand breaks) from the percent DNA in the tail with enzyme incubation.

Comets in each gel were analyzed in a blinded manner, using the Komet 3.0 image program (Kinetic Imaging Ltd., Liverpool, U.K.).

Statistics. The results are expressed as the mean value \pm SEM. The data for the three independent groups (I, II, and III) were analyzed by analysis of variance, and significant differences between the pairs of mean values were tested by the Scheffé test. The Scheffé test was chosen for multiple comparisons because it is very impermeable to violations of the assumptions typically associated with the multiple com-

parisons procedure. Statistical evaluations of differences before and after PTCA were performed by using the paired Student *t* test. The association between an increase in DNA and the effect of each procedural variable (e.g., total inflation time needed for PTCA, radiation dose, fluoroscopy exposure time) was evaluated by simple regression analysis. Multiple regression analysis was also applied to investigate the effect of each variable (e.g., age, gender, smoking status, atherogenic risk factors, total inflation time, radiation dose, fluoroscopy exposure time) in determining the MNBN cell increase. Statistical analyses were performed using the statistical package Statview, version 5.0 (SAS Institute, Abacus Concepts, Inc., Berkeley, California).

RESULTS

Relationship between oxidative DNA damage and CAD.

Typical examples of DNA alterations observed in patients are reported in Figure 1.

When analyzing the DNA damage by the micronucleus test, the number of basal MNBN cells was higher in groups I and II than in group III (11.0 ± 1.3 and 10.2 ± 1.3 vs. 7.0 ± 0.9 , $p = 0.04$).

The mean values of DNA-SSBs ($p = 0.001$), endonuclease III-sensitive sites ($p = 0.002$), and FPG sites ($p < 0.0001$) were significantly higher in groups I and II (Fig. 2).

Moreover, the mean of FPG-sensitive sites increased with the number of affected vessels (17.2 ± 2.9 , 13.9 ± 2.4 , and 35.8 ± 0.5 for one-, two-, and three-vessel disease, respectively; $p = 0.004$). A significant relationship was found between FPG-sensitive sites and the extent of CAD evaluated by means of the Duke scoring system ($r = 0.663$, $p = 0.01$).

DNA damage and PTCA. Significant differences were observed in the radiation dose (144.1 ± 18.1 vs. 55.5 ± 8.7 Gy/cm², $p = 0.0001$) and fluoroscopy exposure time (18.3 ± 1.9 vs. 9.1 ± 1.2 min, $p = 0.0003$) between groups I and II.

A high significant MNBN cell frequency increase was observed in group I after PTCA (11.0 ± 1.3 vs. 19.8 ± 1.6 , $p < 0.0001$), whereas no significant difference was observed in group II after coronary angiography (10.2 ± 1.3 vs. 12.9 ± 1.4 , $p = \text{NS}$) (Fig. 3). A significant positive correlation was observed between the increase in the MNBN cell rate and the total inflation time during PTCA ($r = 0.549$, $p = 0.0017$) (Fig. 4), although no significant relationships were found between the MNBN cell increase and radiation dose or fluoroscopy exposure time. Finally, multiple regression analysis showed that the total inflation time appeared to be the only determining factor in the increase of MNBN cell frequency ($p = 0.02$).

Oxidative DNA damage and PTCA. The mean levels of DNA strand breaks, measured as the percentage of DNA in the tail, were significantly higher in group I after PTCA (11.7 ± 1.4 vs. 26.5 ± 3.0 , $p = 0.0003$), although no significant difference was observed in group II after coronary angiography (12.5 ± 2.8 vs. 11.6 ± 1.5 , $p = \text{NS}$) (Fig. 5).

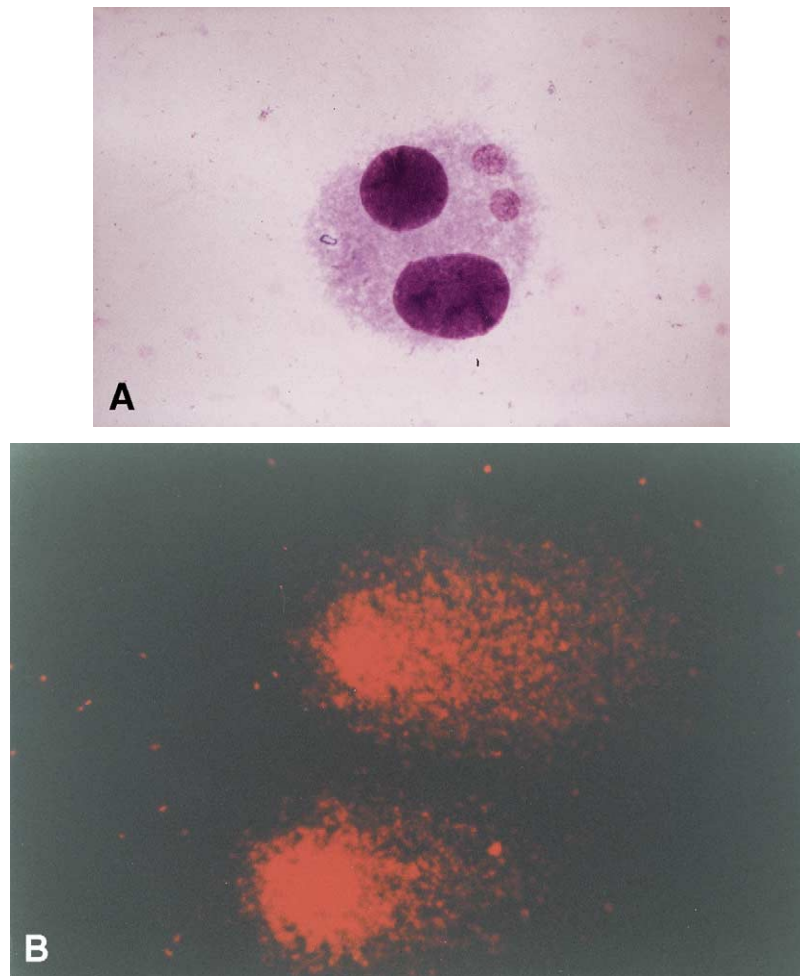


Figure 1. An example of micronucleated binucleated cells with two micronuclei (A) and cell migration patterns produced by the comet assay (B).

Moreover, the FPG-sensitive sites were strongly increased after PTCA (13.8 ± 1.8 vs. 22.5 ± 2.4 , $p = 0.01$), and a trend toward an increase, although not significant, was observed for the levels of endonuclease III (18.7 ± 3.4 vs. 27.1 ± 3.1 , $p = 0.08$). No significant differences were observed in the levels of FPG-sensitive sites and endonuclease III-sensitive sites in group II after coronary angiography.

DISCUSSION

This study shows that patients with CAD, in the basal condition, have a higher level of DNA damage, as compared with normal subjects, according to our previous observation (15). Moreover, to the best of our knowledge, this study provides the first evidence of possible oxidative DNA damage in the peripheral lymphocytes of patients undergoing PTCA.

Comparison with other studies. A number of previous studies have shown an increase in cardiac oxidative stress induced by an injury incurred during primary angioplasty (5-8). Recently, it has also been demonstrated that lipid peroxidation and F2-isoprostane—stable end products of oxygen free radical-mediated lipid peroxidation—are signif-

icantly increased during the reperfusion after PTCA (8,16). Although DNA represents a potential target for free radical attack, no evidence is yet available demonstrating DNA damage as a result of ischemia-reperfusion injury after PTCA.

Indeed, it has been shown that oxidation is a major cause of DNA damage in humans, and the number of oxidative hits on DNA may be $>10^3$ /cell per day (17). Reactive oxygen species cause extensive DNA damage, including SSBs, the formation of modified bases, chromosomal damage, and mutations in mammalian cells (4,18). Several experimental models of ischemic injury have also shown the influence of reperfusion-associated oxygen radicals on DNA damage. In fact, some studies have shown that ischemia-reperfusion induces DNA damage in animal studies and isolated tissue (19-21).

Recent studies have reported that the induction of apoptosis is an initial cellular event induced by balloon angioplasty (22,23). Indeed, it is well known that apoptosis is an effective mechanism for eliminating cells with genetic lesions, thus reducing the potential for genetic instability and cellular dysfunction (24). In addition, the induction of chromosomal and DNA damage has been demonstrated in

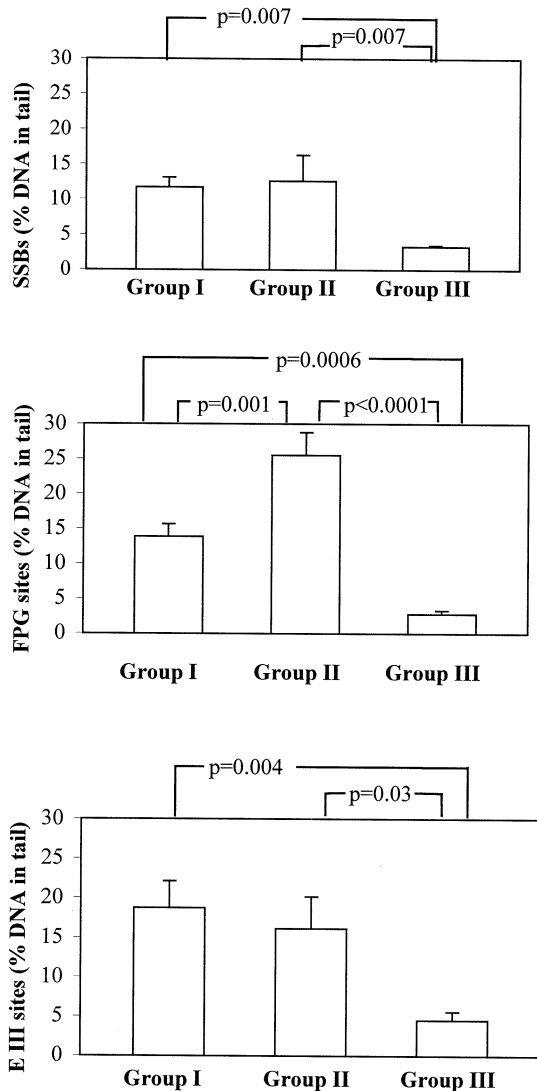


Figure 2. Deoxyribonucleic acid (DNA) damage, as demonstrated by single strand breaks (SSBs), formamidopyrimidine glycosylase (FPG) sites, and endonuclease III (E III) sites, in the lymphocytes of patients classified into three study groups, represented by the mean percent DNA in the tail for 50 comets from each patient.

human peripheral leukocytes after ischemia-reperfusion injury (25,26).

Value and limitations of the study. Our study demonstrates the presence of DNA damage in the lymphocytes of patients with CAD, who represent an ideal situation of increased production of oxygen free radicals. These results are in agreement with another study reporting higher DNA adduct levels in the cardiac tissue of patients with severe CAD (27), as well as with other recent observations suggesting that alterations at the DNA level may contribute significantly to the development of atherosclerotic disease (28-30).

Our results show that PTCA induces an increase in oxidative DNA damage in the circulating cells of patients. These effects were seen in human lymphocytes of the systemic circulation. Peripheral lymphocytes are advanta-

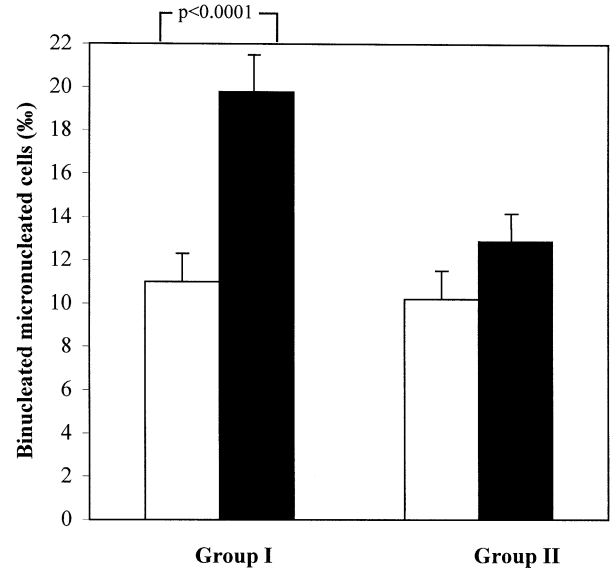


Figure 3. Frequency of micronucleated binucleated cells in patients undergoing percutaneous transluminal coronary angiography (group I) and coronary angiography (group II). **Open bars** = before procedure; **solid bars** = after procedure.

geous for exposure analysis because they circulate throughout the body and thus provide an estimate of average whole-body exposure. In fact, the conceptual basis for this approach is the hypothesis that the extent of genetic damage in peripheral blood lymphocytes reflects critical events for mutagenic and carcinogenic processes in target tissues.

Similarly, specific genetic changes may occur in smooth muscle cells of the wall, leading to restenosis, as suggested by an increased mutation rate of microsatellites in both primary and secondary restenotic atherosclerotic plaques (31). It is also possible that the observed DNA damage may increase apoptosis (22,23). However, a recent study suggested that restenotic intimal cells may be resistant to apoptosis (32), and increased DNA synthesis has been observed after balloon angioplasty in an atherosclerotic rabbit model (33). This evidence suggests that intimal hyperplasia of smooth muscle cells may be a result of their clonal growth in response to a mutagenic event, underscor-

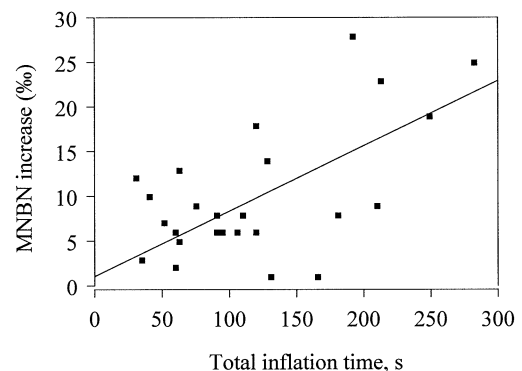


Figure 4. Relationship between micronucleated binucleated (MNBN) cell increase after percutaneous transluminal coronary angiography and total inflation time. $y = 1.123 + 0.072x$; $r = 0.549$, $p = 0.002$.

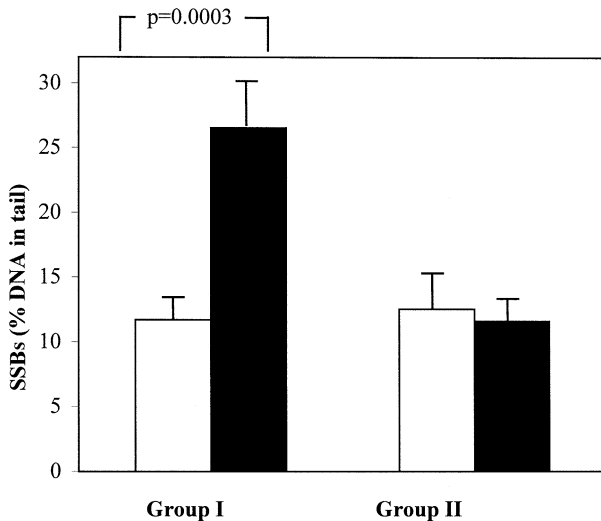


Figure 5. Deoxyribonucleic acid (DNA)-single strand breaks (SSBs) in patients undergoing percutaneous transluminal coronary angiography (group I) and coronary angiography (group II). **Open bars** = before procedure; **solid bars** = after procedure.

ing the similarity between atherosclerotic and carcinogenic processes (28–30).

The hypothesis that oxygen free radicals may be responsible for these alterations is plausible (4,34,35); however, some limitations of this study should also be considered. First and foremost, the possibility that X-ray exposure and contrast media could be responsible for increased DNA damage should be considered (36), even if the observed increase in the DNA alteration rate is not significantly related to radiation exposure.

No direct correlation between oxidative DNA damage and oxidative stress, as biochemical markers (e.g., malonaldehyde), was examined in this study. However, these limitations do not deprecate the main observation of our study, and additional work should be done in this area. Our results provide clear evidence that oxidative DNA damage, particularly the level of 8-oxo-guanine, in the peripheral lymphocytes of patients with CAD is significantly enhanced as compared with that of normal subjects. Further oxidative DNA damage is produced after PTCA, likely related to ischemia-reperfusion injury. Larger studies are indicated to determine whether the increased DNA damage may be an additional prognostic factor in CAD, as well as to investigate the association between oxidative DNA damage and restenotic coronary lesions.

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