Evidence for DNA damage in patients with coronary artery disease

N. Botto*, A. Rizza, M.G. Colombo, A.M. Mazzone, S. Manfredi,
S. Masetti, A. Clerico, A. Biagini, M.G. Andreassi

*Laboratory of Cellular Biology, CNR Institute of Clinical Physiology, G. Pasquinucci Hospital,
Via Aurelia Sud-Montepepe, 54100 Massa, Italy*

Abstract

According to the "monoclonal hypothesis" of atherosclerosis, several studies suggest that cancer and atherosclerosis may have several fundamental biological mechanisms in common. Therefore, an increase in the mutation rate may be involved in the pathogenesis of atherosclerotic plaques.

The aim of the study was to verify the presence of chromosomal damage in peripheral blood lymphocytes in patients with coronary artery disease by using micronucleus (MN) test, a reliable biomarker in genetic and cancer risk assessment.

Subjects included 53 patients with documented coronary ischemic heart disease (group I); 10 patients with valvular heart disease in absence of atherosclerotic lesions of the coronary arteries (group II) and 16 healthy subjects, age- and sex-matched (group III) were studied as controls. For each subject, two separate cultures were performed and 1000 binucleated cells were scored for the evaluation of MN frequency.

The mean ($\pm$S.E.M.) of MN frequency were 11.9 ± 1.7, 5.9 ± 1.2 and 3.6 ± 0.7 in groups I, II and III, respectively. The MN frequency of group I was significantly higher than that of group III ($P = 0.02$). In group I, MN frequency increased with the number of affected vessels (6.3 ± 0.7, 13.9 ± 1.6, 14.9 ± 5.3 for one-, two-, and three-vessel disease, respectively). Scheffe's test showed that MN frequency was significantly higher in two-vessel compared with one-vessel disease ($P = 0.0077$). Moreover, a positive relationship was found between MN levels and the severity of the disease, calculated by the Duke scoring system ($R = 0.28$, $P = 0.032$), as well as the systolic blood pressure ($R = 0.34$, $P = 0.009$).

These results suggest that coronary artery disease in humans is a condition characterized by an increase of DNA damage, positively correlated with the severity of the atherosclerotic disease. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Coronary artery disease; Micronucleus; DNA damage; Human lymphocytes

1. Introduction

In recent years, considerable advances have been made in the understanding of molecular alterations and genetic determinants in the cardiovascular disease. Recent studies suggest the presence, in atherosclerotic tissues, of specific molecular DNA alterations which may be involved in the development and/or progression of the disease.

The most prevalent hypothesis concerning the pathophysiological mechanisms of atherosclerotic plaque formation is the "inflammatory response to injury" [1], which states that proliferation of smooth muscle cells is an inflammation-fibroproliferative reaction to different insults to the arterial wall. Indeed, the discovery that the cells of an atherosclerotic

* Corresponding author. Tel.: +39-585-493646;
fax: +39-585-493601.
E-mail address: botto@ifc.pi.cnr.it (N. Botto).

plaque are monotypic, led to the proposal of an alternative hypothesis — the "monoclonal hypothesis" [2]. This theory proposes that atherosclerosis begins as a mutation or viral hit, transforming a single, isolated smooth muscle cell into the progenitor of a proliferative clone in the same manner as a benign tumor.

According to this hypothesis, plaques and tumors may share important pathogenic features including the occurrence of one or more mutational events [3–8]. At present, plaque monoclonality is a confirmed observation [9,10], but evidence of clonality does not necessarily have etiological or pathophysiological significance. Nevertheless, some evidence has been reported that identifies and quantifies the involvement of somatic mutations in ischemic heart disease. Moreover, much discussion has arisen about the carcinogenicity of cardiovascular drugs, including diuretics, beta-blockers, calcium antagonists and ACE-inhibitors [11]. Therefore, the issue is so sensitive that an approach through laboratory endpoints of genotoxicity may be worthwhile.

To make a further leap in knowledge, it is necessary to employ studies of mutations in cells from atherosclerotic tissue to verify the presence of specific genetic changes in cells of patients with documented atherosclerosis.

Therefore, in order to investigate the presence of genetic damage in patients with coronary artery disease (CAD), we performed a cytogenetic monitoring study employing the micronucleus (MN) test, a sensitive and reliable system for the evaluation of spontaneous and mutagen-induced DNA damage in human peripheral blood lymphocytes [12].

2. Materials and methods

2.1. Study population

Three groups of patients were prospectively studied. Group I consisted of 53 patients, recruited from our hospital, with clinical evidence of coronary artery disease as defined by clinical presentation (angina pectoris and/or myocardial infarction) and angiographic documentation of CAD. Group II consisted of 10 patients admitted to our hospital with a diagnosis of valvular heart disease in which angiographic examination excluded the presence of CAD. No patients were receiving antioxidant therapy. The medications used by patients included nitrates, oral aspirin, calcium-channel blockers and ACE-inhibitors. Clinical and demographic characteristics of patients and controls are reported in Table 1. Group III included 16 healthy control subjects matched with the patients for age, sex and smoking habits. Each subject was interviewed concerning smoking status, occupational

Table 1
Demographic and clinical characteristics of the patients and controls

	Coronary artery disease ($n = 53$)	Valvular heart disease ($n = 10$)	Healthy control subjects ($n = 16$)
Age (years)	64.9 ± 1.5	58.5 ± 7.2	60.7 ± 2.0
Gender (n)			
Men	45	8	10
Women	8	2	6
Smoking habit (n)			
Smokers	10	2	4
No smokers	5	2	2
Ex-smokers	38	6	10
Hypertensives (n)	50	6	0
Diabetes (n)	11	2	0
Drug therapy (n)			
Nitrates	42	0	0
Aspirin	43	2	0
ACE-inhibitors	22	3	0
Calcium-channel blockers	29	2	0

exposure to potential carcinogens and mutagens, and dietary history. Smokers were classified as individuals who smoked at least three cigarettes per day at the time of analysis; ex-smokers had quit smoking for at least 6 months and non smokers were individuals who had never smoked. Individuals who smoked less than three cigarettes per day were not included in the study. Informed consent was obtained from all patients and control subjects according to our ethical committee. Venous blood samples were taken before angiographic procedure to exclude the effects of X-ray exposure.

2.2. Angiographic study

All patients underwent coronary angiography; coronary stenosis was considered significant in the presence of a luminal diameter narrowing of $\geq 50\%$. The severity of CAD was expressed simply by the number of affected vessels (one-, two-, or three-vessel disease). Moreover, the extent and the severity of CAD was also evaluated by means of the Duke scoring system [13]. This CAD prognostic index considers the number of the diseased major vessels (one-, two-, and three-vessel, as well as left main coronary artery disease), the percent narrowing of the major vessels, and involvement of the left anterior descending coronary artery, particularly when the proximal segment shows a severe stenosis ($\geq 95\%$). The Duke score ranges from 0 to 100 grade scale ($0 =$ no disease, $100 =$ most severe disease).

2.3. Lymphocyte preparation and MN assay

Peripheral blood was collected using heparin as an anticoagulant. Cellular cultures from each subject were set up by mixing 0.3 ml of whole blood with 4.7 ml of RPMI 1640 medium (GIBCO), supplemented with 10% fetal calf serum (GIBCO), 1.5% phytohemagglutin (PHA, GIBCO) and antibiotics (penicillin 100 IU/ml and streptomycin 100 mg/ml, Sigma, St. Louis, MO, USA). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. For evaluation of MN frequency, cells were blocked in cytokinesis by adding, after 44 h, cytochalasin B (6 μg/ml final concentration, Sigma). Cell cultures were then harvested after 72 h. Harvesting of cells, hypotonic treatment, fixation and slide preparation were performed according to

standard methods [14,15]; fixed cells were dropped onto clean microscopic slides, air dried and stained by the Giemsa technique.

2.4. Slide scoring

For each sample, 1000 binucleated cells were scored blindly under the optical microscope (final magnification 400×) for MN analysis, following the criteria for MN acceptance listed by Fenech [16]. MN frequency was expressed as the number of micronucleated binucleated cells (MNBN), containing one or more MN per 1000 cells.

2.5. Statistical analysis

The results were expressed as mean ± S.E.M. The data for the three independent groups (I, II and III) were analyzed by ANOVA analysis and the significant differences among a group of means were tested by the Scheffe's test. The Scheffe's test was chosen for multiple comparisons because it is generally considered to be one of the most conservative tests and also because it is very robust to violations of the assumptions typically associated with the multiple comparisons procedure (including heterogeneous variances). Because MN values are not normally distributed, the logarithmic transformation of original data was used for statistical analysis in order to normalize the distribution values. Multiple regression analysis was applied to investigate the effect of each variable (age, gender, smoking status, Duke score, systolic blood pressure, dyslipidemia, drug number) in determining MN frequency. The statistical analyses were performed using the statistical package Statview 5.0.1 (SAS Institute).

3. Results

The mean (±S.E.M.) MN frequencies were 11.9 ± 1.7, 5.9 ± 1.2 and 3.6 ± 0.7 in groups I, II and III, respectively. The mean MN frequency of group I was significantly higher than that of group III ($P = 0.02$). No significant differences were present between group I and group II ($P = 0.29$), or between group II and group III ($P = 0.88$).
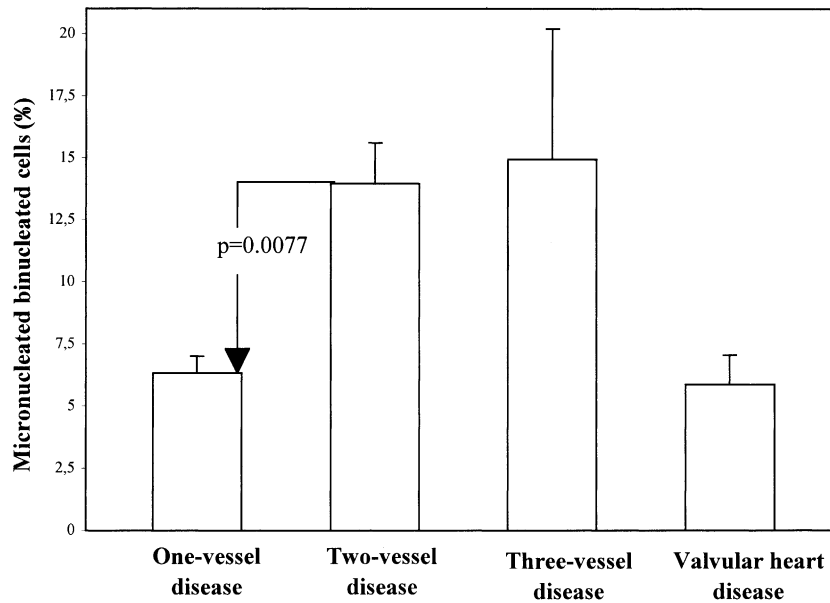
Fig. 1. Mean MN frequencies in patients with one-vessel disease ($n = 16$), two-vessel disease ($n = 22$), three-vessel disease ($n = 15$) and valvular heart disease ($n = 10$).

One-, two- and three-vessel disease were observed in 16, 22, and 15 patients, respectively. As shown in Fig. 1, the mean MN frequency increases with the number of affected vessels (6.3 ± 0.7, 13.9 ± 1.6, 14.9 ± 5.3 for one-, two-, and three-vessel disease, respectively). Scheffe's test showed that the MN frequency was significantly higher in two-vessel compared with one-vessel disease ($P = 0.0077$). For each CAD patient the extent of coronary artery disease, expressed as Duke score, is reported in Table 2, in which the patients are listed according to the number of diseased vessels and the Duke score itself.

Multiple regression analysis showed that Duke score and systolic blood pressure appeared to be the only two independent determinant factors in determining MN frequency in our cardiovascular population (Table 3). The relationship between MN frequency and Duke score as well as systolic blood pressure is shown in Fig. 2.

4. Discussion

To the best of our knowledge, this study is the first cytogenetic monitoring in human lymphocytes of patients with CAD.

The evidence for a monoclonal origin of human atherosclerotic plaques has emphasized the need to consider the development of atherosclerotic disease as a formation of a benign tumor [2]. In this hypothesis the plaques may be a result of a clonal growth of the smooth muscle cells, in response to a mutational event [3–8]. Indeed, epidemiological studies indicate that some mutagenic agents may exert carcinogenic as well as atherogenic effects [17–23].

4.1. Comparison with previous studies

Recent studies suggest that DNA alterations are present in atherosclerotic tissues and can play a fundamental role in the pathogenesis of this disease. Indeed, DNA samples extracted from smooth muscle cells of atherosclerotic lesions possess transforming capability [24,25]. This evidence, together with the discovery of microsatellite instability in human atherosclerotic lesions [26–28], suggests the hypothesis that genomic destabilization can result in the misregulation of the cells harboring these mutations and may play a pivotal role in the formation of atherosclerotic plaque.

In addition, cytogenetic studies of human plaque smooth muscle cells have revealed the presence of a variety of karyotipic abnormalities, primarily

Table 2
Severity of CAD by Duke score [13][a]

Patients	Vessels involved	Duke score
1	1-VD (50%)	19
2	1-VD (50%)	19
3	1-VD (50%)	19
4	1-VD (50%)	19
5	1-VD (75%)	23
6	1-VD (75%)	23
7	1-VD (95%)	32
8	1-VD (95%)	32
9	1-VD (95%)	32
10	1-VD (95%)	32
11	1-VD (95%)	32
12	1-VD (95%)	32
13	1-VD (95%)	32
14	1-VD (95%)	32
15	1-VD (prox LAD 95%)	48
16	1-VD (prox LAD 95%)	48
17	2-VD (75%, 50%)	37
18	2-VD (75%, 50%)	37
19	2-VD (75%, 50%)	37
20	2-VD (75%, 75%)	37
21	2-VD (75%, 75%)	37
22	2-VD (75%, 75%)	37
23	2-VD (75%, 75%)	37
24	2-VD (75%, 75%)	37
25	2-VD (95%, 50%)	37
26	2-VD (95%, 50%)	37
27	2 VD (95%, 75%)	37
28	2-VD (95%, 95%)	42
29	2-VD (95%, 95%)	42
30	2-VD (LAD 95%, 50%)	48
31	2-VD (LAD 95%, 75%)	48
32	2-VD (LAD 95%, 75%)	48
33	2-VD (LAD 95%, 95%)	48
34	2-VD (LAD 95%, 95%)	48
35	2-VD (LAD 95%, 95%)	48
36	2-VD (LAD 95%, 95%)	48
37	2-VD (prox LAD 95%, 95%)	56
38	2-VD (prox LAD 95%, 95%)	56
39	3-VD (75%, 75%, 50%)	56
40	3-VD (95%, 50%, 50%)	63
41	3-VD (95%, 75%, 75%)	63
42	3-VD (95%, 75%, 75%)	63
43	3-VD (95%, 75%, 75%)	63
44	3-VD (95%, 95%, 50%)	63
45	3-VD (95%, 95%, 50%)	63
46	3-VD (95%, 95%, 75%)	63
47	3-VD (95%, 95%, 75%)	63
48	3-VD (prox LAD 50%, 95%, 75%)	67
49	3-VD (prox LAD 50%, 95%, 75%)	67
50	3-VD (prox LAD 50%, 95%, 75%)	67
51	3-VD (prox LAD 95%, 95%, 95%)	74
52	3-VD (prox LAD 95%, 95%, 95%)	74
53	3-VD (left main coronary artery disease, 75%, 95%)	82

[a] 1-, 2-, and 3-VD: one-, two- and three-vessel disease; prox: proximal; LAD: left anterior descending coronary artery.

Table 3
Multiple regression between MN frequency and risk factors

Variable	Regression coefficients	Significance (P)
Gender	−0.122	0.374
Age	0.011	0.934
Smoking	0.034	0.806
Dyslipidemia	0.104	0.419
Duke score	0.265	0.058
Systolic blood pressure	0.308	0.022
Drug therapy	0.005	0.970

involving aneuploidies [29–31]. Moreover, elevated levels of aneuploidy cells were found in aortic endothelial cells of elderly subjects, as well as those of patients with atherosclerosis [32].

In view of this evidence, since MN can contain a fragment or a whole chromosome, it would be of interest to verify the presence of centromeric DNA in MN, by application of fluorescence in situ hybridization (FISH) with alphoid satellite probe [33]. These approaches will permit us to determine whether somatic cells of patients with CAD show a constitutional predisposition to aneuploidy.

4.2. Value and limitation of the study

Our study provides evidence for the existence of an association between micronucleus frequency and the severity of coronary artery disease. The biological plausibility suggested by several authors [34–36] and the good agreement with the study reporting higher DNA adduct levels in cardiac tissue of patients with severe coronary artery disease [22] further strengthen our findings. It is worth noting that the relationship between DNA damage and increased systolic blood pressure generally agrees with several studies documenting the association between cancer mortality and hypertension [37,38], thus strongly suggesting the presence of possible common pathophysiological mechanisms between cancer and hypertension [35].

On the other hand, it is well known that DNA damage frequently occurs in cells exposed to oxidative stress [39], and ischemic heart disease is an ideal example of increased production of oxygen free radicals [40]. In addition, recent studies showed an elevated micronucleus frequency positively correlated with plasma homocysteine [41,42], which is considered to
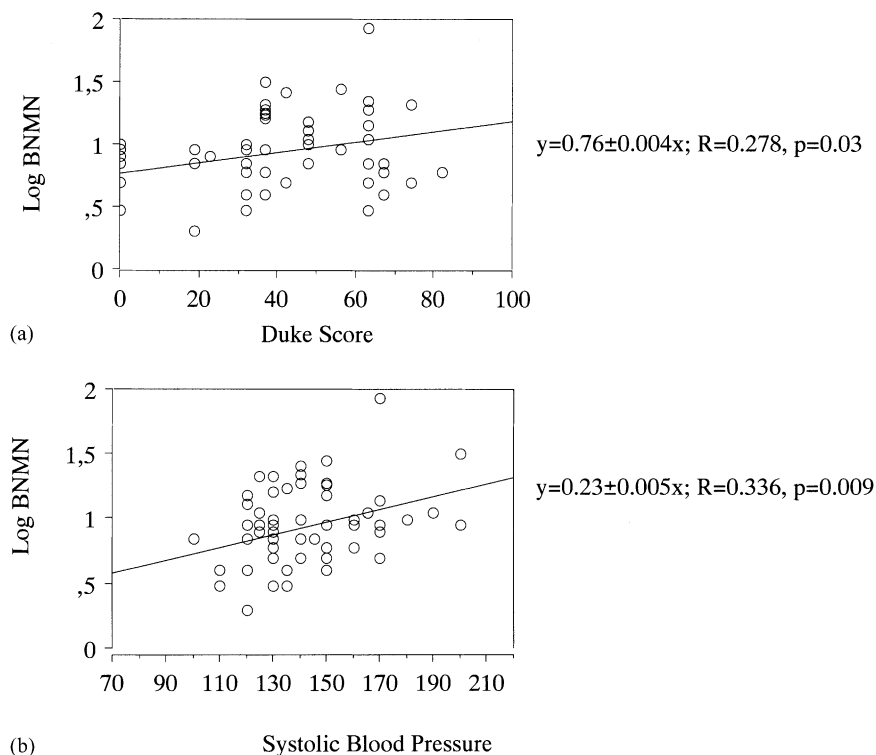
Fig. 2. Simple regression analysis between MN frequency: (a) Duke score; (b) Systolic blood pressure.

be an important risk factor for cardiovascular disease [43].

However, some limitations of this study should also be considered. First and foremost, the possibility that drug therapies could be responsible for the increased micronucleus rate should be considered, even though we have previously observed that long-term calcium antagonist therapy is not associated with an increased incidence of chromosome aberrations in human lymphocytes [44]. On the contrary, it has been recently suggested that nitric oxide and nitrate therapy could show a potential genotoxic activity [45,46]. These limitations encourage additional work in this area. We observed a clear relationship between increased MN frequencies and the severity of coronary artery disease; however, more direct experimental data are needed at this point to evidence DNA damage accumulation in ischemic heart disease and, particularly, to investigate whether the drugs given to treat CAD are potentially mutagenic. Moreover, further studies might include measures of folate, B12 and homocysteine which are

risk factors for cardiovascular disease as well as micronucleus frequency. Finally, it will be interesting to determine whether somatic mutations or genetic changes are present in the smooth muscle cells of the atherosclerotic plaque leading to the development of the lesion or plaque instability.

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