Cancer Biomarkers in Human Atherosclerotic Lesions: Detection of DNA Adducts

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Abstract
Since somatic mutations are suspected to contribute to the pathogenesis not only of cancer but also of atherosclerotic plaques, we measured DNA adducts in the smooth muscle layer of atherosclerotic lesions in abdominal aorta specimens taken at surgery from seven patients. DNA adducts were evaluated in three laboratories by means of different molecular dosimetry methods, including: (a) HPLC/fluorescence, which specifically identifies the DNA adducts of the anti-benzo(a)pyrene (BPDE) isomer; (b) two- and three-dimensional synchronous fluorescence spectrophotometries, which detect DNA adducts of BPDE and other reactive metabolites of polycyclic aromatic hydrocarbons; and (c) 32P postlabeling, which reveals the presence of a variety of types of DNA adducts. The HPLC/fluorescence method provided for the first time evidence for the presence of BPDE-DNA specific adducts in three of six specimens tested. Synchronous fluorescence spectrophotometry displayed broad areas of fluorescence in all seven specimens, thereby suggesting the occurrence not only of BPDE-DNA but also of other DNA adducts with similar fluorescence characteristics. All specimens were also positive at 32P postlabeling, which revealed multiple spots detectable following enrichment either with nuclease P1, or butanol, indicative of the presence of different aromatic DNA adducts. Thus, the data obtained by applying typical cancer biomarkers provide further support to the hypothesis that there may be similarities between the carcinogenic and the atherogenic processes, and in particular that genetic alterations caused by DNA-binding agents in the artery wall may be detected in atherosclerotic lesions.

Introduction
The hypothesis has been raised that the formation of atherosclerotic plaques may be pathogenetically comparable to the formation of benign tumors. According to this theory, at least a part of atherosclerotic lesions may be initiated by mutational events in arterial smooth muscle cells (1). It has also been postulated that an initiation-promotion two-step process may be involved not only in carcinogenesis but also in the genesis of other chronic degenerative diseases, including atherosclerosis (2). Although this issue is rather controversial and other response-to-injury theories, which involve the participation of a number of growth factors, cytokines, and vasoregulatory molecules, may explain the genesis of the atherogenic process as well (3), several converging lines of evidence support the role of DNA alterations as noxious stimuli contributing to the initiation of this pathological event (1). Consistent with this hypothesis was the demonstration, given more than 20 years ago, that a substantial fraction of human atherosclerotic plaques are of monoclonal origin (4). More recently it was shown that the DNA extracted from human atherosclerotic plaques had transforming ability when transfected into NIH3T3 cells, which became capable of inducing tumors in nude mice (5).

In previous studies we detected DNA adducts in rats exposed to either cigarette smoke (6) or benzo(a)pyrene (7, 8) not only in target organs for carcinogenicity of these agents, such as trachea and lung, but also in the aorta. These findings prompted us to start a study aimed at assessing, in specimens of human aorta, a variety of molecular biomarkers which are traditionally used in cancer research. We report here the results of analyses performed with different methods in three laboratories, which, even in the absence of any demonstration of causality, provide sound evidence that DNA adducts do also occur in human atherosclerotic lesions.

Patients and Methods
Patients. A fragment of abdominal aorta was removed at surgery for therapeutic purposes in atherosclerotic patients undergoing aortic graft due to severe aneurism or stenotic lesions of the subrenal aorta. Seven patients were examined, all males except patient 6. All of them were retired, and their ages ranged between 64 and 81 (mean ± SD, 69.7 ± 5.3) years. Three of them were ex-smokers who had stopped smoking 6–12 years before their operation. The other four patients were currently smoking
20–40 cigarettes/day. A complete questionnaire was completed, including information on anamnestic, clinical, and laboratory data.

**Extraction of DNA from Atherosclerotic Lesions.** Immediately after removal, the aorta fragments were immersed in sterile physiological saline solution, stored at 4°C, and transferred from the surgery department to the laboratory in Genoa. After careful examination for macroscopic changes, each fragment, the wet weight of which was in the 253–748 mg range, was carefully cleaned of calcifications, fatty deposits, and thrombotic material, and washed with sterile physiological saline in order to remove debris and blood residues. The three artery layers were then dissected and separated as accurately as possible. The adventitial coat was removed, whereas the tunica media and tunica intima were stored separately at -80°C. Due to the severity of alterations of the intima, it was not possible to extract DNA in sufficient amounts for molecular dosimetry analyses. Within 1 month of storage at -80°C, the tunica media, mainly composed of smooth muscle cells, was homogenized in a Polytron apparatus at 4°C in 250 mM sucrose-50 mM Tris-HCl (pH 7.4). DNA was isolated by solutions, fatty deposits, and thrombotic material, and washed in 25-748 mg range, was carefully cleaned of calcification, and then with proteinase K for 5 h at 55°C, followed by two extractions with chloroform only. The material was further washed with water-saturated isooamyb alcohol in one extraction with a phenol-water-chloroform mixture and then with proteinase K for 5 h at 55°C, followed by two extractions with chloroform only. The material was further washed with water-saturated isooamyb alcohol in order to remove noncovalently DNA-bound PAH residues (10) and was precipitated by means of ethyl alcohol. The extracted DNA was quantified by spectrophotometric analysis. The 230:260 and 260:280 ratios of absorbance were as follows: D4, 0.5 ± 0.05, respectively (means ± SD). The amount of DNA recovered from the examined specimens ranged between 10 and 28 μg/100 mg wet tissue, as detected by absorbance at 260 nm.

**Synchronous Fluorescence Spectrophotometry.** A standardized amount of 50 μg DNA for each sample was hydrolyzed in 0.1 N HCl at 90°C for 4 h in sealed glass vials and analyzed by SFS (6, 11) in the Genoa laboratory. Synchronous scanning was performed by using a Hitachi F-3000 fluorescence spectrophotometer with a fixed Δλ of 34 nm between excitation and emission. The intensity of the fluorescence peak was expressed in fluorescence units, which indicate the difference between the intensity of the signal corresponding to the peak and the baseline. This was further subtracted by a fixed value of 10, which was assumed as an arbitrary threshold of sensitivity of the method, in order to distinguish specific fluorescence peaks from aspecific baseline fluctuations. A three-dimensional SFS was additionally performed with the 4 samples for which further 50 μg DNA were available by varying the Δλ from 10 to 106 nm at 4 nm intervals (11). A sample of 50 μg calf thymus DNA (Sigma Chemical Co., St. Louis, MO), with or without the addition of 300 fmol BPT (National Cancer Institute Chemical Carcinogen Repository, Kansas City, MO), was also analyzed as a control.

**HPLC/Fluorescence.** A HPLC/fluorimetric assay was carried out in the Lyon laboratory as described previously (12). Briefly, 100 μg DNA was dissolved in 1 ml purified 0.1 N HCl. The HPLC of the purified HCl (background) showed absence of any fluorescent material. Hydrolysis of DNA was performed for 3 h at 90°C. The products of hydrolysis were separated on reverse-phase HPLC exactly as described by Alexandrov et al. (12) where details on HPLC conditions and monitoring of the fluorescence are reported. The fluorescence signal corresponding to the eluted peak of BP-tetrol was integrated, and the amount of BP-tetrol in the samples was determined from a standard curve generated from the fluorescence peak heights of an authentic BP-tetrol standard each time when analyzing a set of samples. The values thus obtained for BP-tetrol are estimated to correspond to the quantity of the BPDE-DNA adduct detected in the smooth muscle layer of human atherosclerotic lesions. The sensitivity of HPLC/fluorescence is 1 adduct/106 nucleotides (12).

**32P Postlabeling.** 32P postlabeling analyses were conducted in parallel in the Research Triangle Park and Genoa laboratories. In the former laboratory the postlabeling procedure was as described previously (13). In brief, 2.5–10 μg of aorta DNA were enzymatically digested and treated with nuclease P1 or butanol extracted. The digests enriched for adducted nucleotides were then labeled for 30 min with approximately 50 μCi [γ-32P]ATP (3000 Ci/mmoll) (Amersham, Arlington Heights, IL). The radiolabeled digest was applied to polyethyleneimine-cellulose plates prepared according to the method of Gupta et al. (14) for multidirectional TLC. The solvents were as follows: D1, 1 μl sodium phosphate (pH 6.8), overnight; D3, 4 μl lithium formate, 7 μl urea (pH 3.45), with predevelopment to 1 cm with 2.5 μl ammonium formate (pH 3.5); D4, 0.8 μl lithium chloride, 7 μl urea, 0.5 μl Tris-HCl (pH 8.0), with predevelopment to 1 cm with 0.5 μl Tris-HCl (pH 8.0); D5, same direction as D4 using 1 μl sodium phosphate (pH 6.8). Intensifying screen enhanced autoradiography at -70°C for 4–48 h was used to detect the presence of radiolabeled adducts on the TLC plates. The individual adducts were carefully excised and counted for radioactivity in a β counter (model CA1900; Packard, Downers Grove, IL). A similar procedure was followed in the Genoa laboratory, except that the adducts were enriched only with the nuclease P1 method. Moreover, 160 μCi of [γ-32P]ATP, with a specific activity of 7000 Ci/mmoll (ICN Pharmaceuticals, Irvine, CA) were used. Precast polyethyleneimine-cellulose sheets (Macherey-Nagel, Düren, Germany) were chromatographed without predevelopments, urea concentration was 6.5 μl in D3 and D4, and 1.7 μl sodium phosphate (pH 6.0) was used as a buffer in D5. The average sensitivity of 32P postlabeling is in the 106–107 adduct/nucleotides range, depending on the structure of the DNA adduct.

**Results**

As shown in Fig. 1, all seven tested aorta samples showed evidence for the presence of DNA adducts in at least one of the methods used. The only result falling below the threshold of sensitivity of the methods used was recorded with specimens 5, 6, and 7 at HPLC/fluorescence. There was no
HPLC/fluorescence revealed a peak corresponding to BPT produced by the hydrolysis of the BPDE-DNA adducts, in 3 of 6 tested specimens. BPDE-DNA adducts were calculated to occur at levels between 3.8 and 5.7 adducts/10⁸ nucleotides. All samples, including those in which BP tetrols were not detectable, showed other fluorescent peaks (see Fig. 1), the nature of which is currently unknown. These peaks may correspond to other DNA adducts revealed by ³²P postlabeling and may contribute to the broad areas of fluorescence detected by SFS.

³²P postlabeling detected the presence of a major adduct migrating in the typical zone for many PAH-DNA adducts including BPDE. Additional adducts were also consistently detected. There was a statistically significant correlation ($r = 0.86; P < 0.05$) between the quantitative data regarding ³²P postlabeling with nuclease P₁ enrichment in Research Triangle Park and Genoa laboratories. Three samples, whose amounts were sufficient for further analyses, showed the presence of several DNA adducts with butanol enhancement that were not detected with nuclease P₁. On an average, the total amount of adduct recovered by butanol was 1.6 times higher than by nuclease P₁. No significant correlation was detected between the number of cigarettes currently smoked per day and the amount of DNA adducts, and no significant difference existed in the mean values of adducts by comparing the 4 current smokers with the 3 ex-smokers, irrespective of the method used. Water blanks and calf thymus DNA blanks were consistently negative under our experimental test conditions (data not shown).

SFS detected in all test samples the presence of fluorescent material covalently bound to DNA, at excitation wavelengths in the 344–351-nm range and with a Δλ of 34 nm. In order to have a more complete information on the specificity of the data generated by this technique, we additionally tested DNA samples by means of three-dimensional SFS, which relates the intensity of fluorescence not only to the excitation or emission wavelength at a fixed Δλ (34 nm) but also by varying Δλ intervals. Fig. 2 shows the

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### Table

<table>
<thead>
<tr>
<th>SFS (Fluorescence Units)</th>
<th>HPLC/Fl (BPDE-DNA adducts/10⁸ nucleotides)</th>
<th>³²P postlabeling (Adducts/10⁸ nucleotides)</th>
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<tbody>
<tr>
<td>#1 48.3</td>
<td>4.8</td>
<td>6.7</td>
</tr>
<tr>
<td>#2 33.3</td>
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<td>9.7</td>
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<tr>
<td>#3 16.7</td>
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<td>#4 26.7</td>
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<td>#5 23.3</td>
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<td>#6 20.8</td>
<td>&lt;1</td>
<td>12.1</td>
</tr>
<tr>
<td>#7 17.0</td>
<td>&lt;1</td>
<td>9.5</td>
</tr>
</tbody>
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**Fig. 1.** Detection by different methods of DNA adducts in tunica media cells of abdominal aorta atherosclerotic lesions from seven patients. The numbers reported at the bottom of each box refer to quantification of DNA adducts and are expressed either as fluorescence units when using SFS or adducts/10⁸ nucleotides when using HPLC/fluorescence (BPDE-DNA adduct) or ³²P postlabeling, detecting a variety of adducts following enrichment with nuclease P₁ or butanol extraction.
modest background of fluorescence yielded by purified calf thymus DNA, and the sharp peak yielded by the addition of small amounts of standard BPT. Also shown are the fluorescence patterns yielded by 4 human aorta DNA samples, displaying a broad area of fluorescence, which reflects the presence of aromatic DNA adducts other than BPDE-DNA. As already indicated by bidimensional SFS (Fig. 1), #1 appears to be the aorta specimen producing the most intense signals, and #5 is characterized by different coordinates of the maximum peak, which are shifted toward longer wavelengths (354 nm). Note that this specimen was negative at HPLC/fluorescence. In addition, $\Delta \lambda$ of the maximum fluorescence peak was greater in all aorta samples than in the BPT-DNA control. This is likely to be attributable to the presence of some residue of DNA-bound PAH metabolites, unconverted to the corresponding tetrols by the

Fig. 2. Three-dimensional synchronous fluorescence spectra recorded with one sample of calf thymus DNA, either with or without addition of 300 fmol BPT or with DNA of tunica media cells of abdominal aorta atherosclerotic lesions from four patients. The X-axis indicates the intervals ($\Delta \lambda$) scanned between excitation and emission wavelengths (nm). The Z-axis indicates the excitation wavelength ($\lambda$ (nm)). The intensity of fluorescence is given on the Y-axis and presented as ranges of fluorescence units.
The herein presented results provide clear evidence that DNA adducts can be detected in the smooth muscle layer of human atherosclerotic lesions. This conclusion is substantiated by the convergent data generated in three laboratories and using different molecular dosimetry methodologies. With the exception of three specimens at HPLC/fluorescence, all analyses gave positive results. Apart from possible interlaboratory variations, the lack of significant correlations between the quantitative data obtained with different methods are likely to be ascribed to the different end points monitored as well as to the small number of patients. In fact, HPLC/fluorescence analyses were designed in order to specifically measure BPDE-DNA adducts, and in particular the highly carcinogenic (+)-anti-BPDE isomer, which after acid hydrolysis is converted into BPT (12, 15, 16). SFS was also developed with the aim of detecting the fluorescent BPT resulting from hydrolysis of BPDE (11). Actually, it has been demonstrated that this technique is not strictly specific for BPDE but additionally reveals other fluorescent derivatives of PAH epoxides (17). This lack of specificity was confirmed by our three-dimensional SFS analyses, which showed that, compared to a standard BPDE-DNA adduct, the fluorescence spectra yielded by aorta DNA samples were distributed over broader λ and Δ λ areas.

32P postlabeling with nuclease P1, enrichment of adducts generally yielded comparable results between the two laboratories with respect to the DNA adduct patterns. The autoradiograms showed the presence of several types of DNA adducts, one of which had similar chromatographic mobility as the BPDE-DNA adduct. Additional adducts were detected following extraction with butanol, suggesting the presence of nuclease P1 sensitive DNA adducts such as the C8-guanine adduct formed following exposures to aromatic amines, nitroarines, and nitrogen heterocycles (18). The detection of DNA adducts in the tunica media cells from atherosclerotic lesions of the abdominal aorta obtained at surgery is consistent with autopsy studies where DNA adducts were detected by 32P postlabeling in the ascending aorta from two smokers (19). As assessed by analyzing heart autopsy samples by means of the same technique, total DNA adducts were 5.1-fold higher in four smokers than in two nonsmokers (13). In our study, irrespective of the method used, we could not detect any significant difference in aorta DNA adducts levels between smokers and ex-smokers, and within current smokers no correlation existed between the number of cigarettes smoked per day and amounts of adducts. Additional studies from both nonsmokers and smokers are needed to determine if tobacco smoke exposure correlates with the formation of DNA adducts in atherosclerotic lesions. Aorta cells are expected to be exposed to DNA-binding metabolites, either migrating from the liver as proximate metabolites or formed in situ. In fact, a series of metabolic findings (1) indicate that aorta smooth muscle cells have the capability of metabolizing chemical mutagens and carcinogens, as shown for instance by the presence of a complete set of activating and deactivating enzymes, and by their ability to convert polycyclic aromatic hydrocarbons into DNA-binding metabolites, or to produce a variety of genetic effects in cocultivated mammalian cells. Interestingly, aorta S9 fractions from atherosclerosis-susceptible pigeons appear to have increased arylhydrocarbon hydroxylase levels (20), and 3-methylcholanthrene-induced atherosclerotic lesions were increased in Ah-responsive mice (21), which is reminiscent of the well known interstrain variations occurring in the metabolism of xenobiotics and in susceptibility to carcinogens. Benzo(a)pyrene also caused overexpression of the c-Ha-ras gene in rat aortic smooth muscle cell cultures (22).

Experimental and epidemiological studies have provided several examples of biological, physical, or chemical agents which may at the same time act as mutagens, carcinogens, and atherogens, although the causality of the association between genotoxic events and atherosclerosis remains to be established. For instance, it is well recognized that large doses of genotoxic and carcinogenic ionizing radiation give rise to atherosclerotic plaques in humans (23), and PAHs can act as initiators and/or accelerators of plaque development in animal models (24, 25). Like in tumors, there is a structure–activity selectivity in these effects. For instance, benzo(a)pyrene is mutagenic, carcinogenic and atherogenic, while its isomer benzo(e)pyrene does not possess any one of these properties (26). Furthermore, dietary heterocyclic amines, which are mutagenic and carcinogenic, induced in mice hemangioendothelial sarcomas (27) as well as calcification and angiproliferation of the epicardium (28). p-Hydrazinobenzoic acid, contained in the mushroom Agaricus bisporus, induced leio-myosarcomas of the aorta when given to mice in drinking water (29). It is also noteworthy that cholesterol, which is accumulated in the matrix surrounding the atherosclerotic lesion and the associated cells (3), may act as a tumor promoter (30), and cholesterol metabolites are mutagenic (1). Oxidants play an important role both in carcinogenesis and in the genesis of cardiovascular diseases, and antioxidant agents have an antagonistic role in both types of processes (31, 32). The thiol N-acetylcysteine prevented the formation of DNA adducts in different organs of rats exposed to cigarette smoke (6) or treated with benzo(a)pyrene (7, 8), including lung, heart and aorta where, due to the different proliferation rate and other factors (8), these molecular biomarkers may be tentatively associated with different diseases, such as cancer, cardiomyopathies and atherosclerosis, respectively. Therefore, the possible involvement of common mechanisms in different chronic degenerative diseases, such as cancer and atherosclerosis, is supported by coincidences concerning not only their risk factors but also the corresponding protective factors.

Due to the small number of samples tested thus far, we cannot draw any conclusion on the source of the observed DNA adducts. The present study will be extended in the future to examine a much larger number of samples from atherosclerotic lesions and from non-atherosclerotic patients. DNA adducts as well as other molecular and biochemical end points will be examined and the results will be related to exposure to known atherogenic risk factors, as inferred from information on lifestyle (including smoking and dietary habits), anamnestic, clinical, and laboratory data.
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References


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