Chemoprevention of Carcinogen-DNA Adducts and Chronic Degenerative Diseases

Alberto Iozzotti, Francesco D’Agostini, Maria Bagnasco, Leonardo Scatolini, Alessandra Rovida, Roumen M. Balansky,2 Carmelo F. Cesaroni, and Silvio De Flora*


Abstract

Molecular dosimetry techniques were exploited in order to assess the efficacy of experimental chemoprevention assays and to evaluate the involvement of DNA alterations, not only in cancer but also in other chronic degenerative diseases. In agreement with other protective effects previously observed in the same animal models, the thiol N-acetylcysteine (NAC) totally prevented or significantly reduced the formation of carcinogen-DNA adducts in three experimental systems in rats. Thus, as assessed by 32P postlabeling, supplement of the diet with NAC decreased both deoxyguanosine-C2-aminofluorene adducts (butanol enrichment) and deoxyguanosine-N2-acetylamino fluorene adducts (nuclease P1 enrichment) formed in rat liver following dietary administration of 2-aminofluorene for 3 weeks. DNA adducts were detected by synchronous fluorescence spectrophotometry in rat liver, lung, heart, and testis following a daily i.t. instillation of benzo(a)pyrene for 3 consecutive days. The whole-body exposure of rats to mainstream cigarette smoke for 40 consecutive days resulted in the appearance of DNA adducts in heart, lung, and aorta, whereas no adduct was detected by synchronous fluorescence spectrophotometry in liver, brain, and testis. Multiple DNA adducts in the aorta were also measured by 32P postlabeling. Administration of NAC by gavage inhibited the formation of DNA adducts in all organs of rats treated with benzo(a)pyrene or exposed to cigarette smoke. It is of interest that a single chemopreventive agent can display a broad-spectrum protective ability. The selective localization of DNA adducts in different organs depends on pharmacokinetics, metabolic capacity, DNA repair efficiency, and cell proliferation rate. Whereas inhibition by NAC of DNA adducts in testis can be correlated with its demonstrated ability to prevent dominant lethal mutations, we raise the hypothesis that DNA adducts in lung, heart, and aorta may be pathogenetically associated with lung cancer, cardiomyopathies, and arteriosclerosis, respectively. In order to explore the involvement of molecular and biochemical alterations in human arteriosclerosis, we started an extensive collaborative project and report here preliminary data showing the presence of DNA adducts in aorta smooth muscle cells obtained from arteriosclerotic patients.

Introduction

The thiol NAC has been extensively used over the past 30 years as a mucolytic and antioxidant drug in the therapy of respiratory diseases and, more recently, as an antidote towards certain acute intoxications. Studies performed in this laboratory and elsewhere have shown that NAC exerts antimutagenic effects against a variety of mutagenic chemicals and complex mixtures (for recent reviews, see Refs. 1-3). Moreover, NAC displayed anticarcinogenic effects in various organs of rodents, including skin, mammary glands, Zymbal glands, trachea, lung, liver, colon, and urinary bladder (4-9) (see Table 1). Based on this experimental background and on its proven lack of toxicity, NAC is at present considered one of the most promising chemopreventive agents. It is currently under Phase I clinical trial in the United States (6) and, alone or in combination with retinol palmitate, is assayed in an European Phase III trial (Euroscan) for the prevention of second primary tumors in patients previously treated for squamous cancer of the oral cavity or larynx or for non-small cell lung cancer (10). The mechanisms responsible for the antimutagenic and anticarcinogenic properties of NAC have been extensively investigated in our laboratory (for recent reviews, see Refs. 1-3). In synthesis, in the extracellular environment NAC works as a nontoxic analogue of cysteine. It detoxifies direct-acting mutagens due to its nucleophilic and antioxidant activity, inhibits the nitrosation products of amin compounds formed in an acidic environment, and enhances thiol concentrations in intestinal bacteria. Inside cells, NAC is readily deacetylated to yield cysteine, which is the rate-limiting amino acid in the intracellular synthesis of GSH. As a result, NAC enhances the detoxification of carcinogens which are entrapped in nontarget cells, such as long-lived transport cells (e.g., erythrocytes) and sweeping cells (e.g., pulmonary alveolar macrophages). In target cells, NAC replenishes GSH stores, which is particularly important in case of depletion due either to toxic stress or to cancer-associated viral infections, such as hepatitis B and acquired immunodeficiency syndrome. The metabolism of xenobiotics is influenced by the modulation of Phase II enzymes, the GSH cycle, and the hexose monophosphate shunt. The intracellular derivatives of NAC block electrophilic compounds and metabolites and efficiently scavenge reactive oxygen species. They protect against the down-regulation of nuclear enzymes produced by carcinogens, decrease the carcinogen-induced DNA damage, and prevent the formation of carcinogen-DNA adducts. All of these mechanisms contribute to inhibit the initiation of cancer and are likely to affect later stages of the carcinogenesis process as well. We describe here the results of experimental studies investigating the ability of NAC to reduce in vivo the formation of DNA adducts in different organs of rats exposed to environmental carcinogens. All data reported are either original or represent the expansion of previously published studies (11-13). In addition, we report here the preliminary results of a study on the detection of DNA adducts in human aorta smooth muscle cells from arteriosclerotic patients, which was stimulated by the results of animal chemoprevention assays. Two different molecular dosimetry methods were used, i.e., the 32P postlabeling technique, which detects the DNA adducts formed by a variety of carcinogenic metabolites (14), and SFS, which detects the DNA adducts formed by metabolites of polycyclic aromatic hydrocarbons, mainly benzo(a)pyrene diolepoxide-DNA adducts (15).

Materials and Methods

Treatment of Rats with 2-Acetylamino fluorene. Male Wistar rats (Morini strain), weighing 80-100 g, were given a standard diet and drinking water ad libitum and divided into three groups, each composed of five animals. The first group received a standard diet, the second group received the same diet supplemented with 0.05% 2AAF (Sigma Chemical Co., St. Louis, MO), and the third group received a diet supplemented with 0.05% 2AAF plus 0.1% NAC (a gift from Zambon Group, Bresso, Milan, Italy). After 21 days, the animals were anesthetized with ethyl ether and killed by cervical dislocation. The livers were removed, washed with 10 mM Tris-0.15 M KCl, pH 7.4, and stored at -80°C.

Exposure of Rats to Mainstream Cigarette Smoke. Three groups of six male Sprague-Dawley rats (Morini strain), weighing 180-200 g, were used in

---

1 Presented at the 4th International Conference on Anticarcinogenesis & Radiation Protection. April 18-23, 1993, Baltimore, MD. This work was supported by the National Research Council (CNR-ENEL Project on “Interaction of Energetic Systems with Human and Environmental Health” and CNR-targeted project “Prevention and Control of Disease Factors—FATMA”). Dr. R. M. Balansky is a recipient of a research fellowship in an European Phase III trial (Euroscan) for the prevention of second primary tumors in patients previously treated for squamous cancer of the oral cavity or larynx or for non-small cell lung cancer (10).

2 Permanent address: National Centre of Oncology, Sofia-1756, Bulgaria.

3 To whom requests for reprints should be addressed.

4 The abbreviations used are: NAC, N-acetylcysteine; GSH, reduced glutathione; SFS, synchronous fluorescence spectrophotometry; 2AAF, 2-acetylamino fluorene; BP, benzo(a)pyrene.
this study as described previously (12). Briefly, a whole-body exposure to cigarette smoke was obtained by placing a group of six animals in a sealed 20-liter glass chamber, which was filled by means of a 50-ml syringe with the mainstream smoke (720 ml) generated by a commercial filter cigarette containing 14 mg tar and 0.4 mg nicotine. After 10 min, the chamber was opened and, after a 2-min interval needed for renewing the air, filled again with fresh smoke 11 times (the first day) or 8 times (all subsequent days). Such an exposure was performed daily for 40 consecutive days, thereby accounting for an overall exposure to the smoke generated by 363 cigarettes. Sham-exposed exposure was performed daily for 40 consecutive days, thereby accounting for an overall exposure to the smoke generated by 363 cigarettes. Sham-exposed rats were kept in the same type of chamber for the same time periods but in the absence of cigarette smoke. Solutions of NAC were prepared daily in 0.2 M phosphate-buffered saline, pH 7.4. Due to their acidity, the final pH was adjusted to 5.0 by adding 0.4 n sodium hydroxide. NAC was administered by gavage in aliquots containing 1 g of the thiol per kg body weight 5 h before and 5 h after each daily exposure to cigarette smoke, starting 2 days before time 1. The experiment of the end, all rats were anesthetized and killed. Liver, lungs, heart, aorta, brain, and tests were removed, washed, and stored at -80°C.

Treatment of Rats with BP. Male Sprague-Dawley rats (Morni strain), weighing 320-360 g, were divided into three groups, each composed of five animals. Control rats received daily i.t. instillations of 2% Tween 80 (Sigma) in distilled water (2 ml/kg body weight) for 3 consecutive days. The second group was treated i.t. with BP (25 mg/kg body weight; Sigma) in 2% Tween 80 aqueous vehicle, again for 3 consecutive days. The third group received NAC (1 g/kg body weight) by gavage 5 h before each BP instillation. Three days after the last treatment, all animals were anesthetized and killed. Liver, lungs, heart, and tests were removed, washed, and stored at -80°C.

Human Aorta Specimens. Blind-coded specimens of human abdominal aorta, taken at surgery from arteriosclerotic patients, were kindly supplied by Prof. G. L. Petrilli (Department of Vascular Surgery, E. O. Galliera, Genoa, Italy). The specimens were immediately transferred to the laboratory, where the three aorta layers were dissected, washed, and separately stored at -80°C.

DNA Extraction. The rat organs were thawed and homogenized in a Potter-Elvehjem apparatus at 4°C in 250 mM sucrose-50 mM Tris-HCl, pH 7.6. DNA was isolated by solvent extraction using an automatic extractor (Gene- pure 341; Applied Biosystems, Foster City, CA) according to Gupta (16) with some modifications as described previously (12). The specimens of aorta medium layer, mainly containing smooth muscle cells, were thawed and processed like the rat organs except that homogenization was carried out in a Polytron apparatus and, after lysis and digestion with RNases and proteinase K, the homogenate was centrifuged at 800 x g for 30 min in order to remove calcified deposits.

Detection of DNA Adducts by SFS. Aliquots of 50-100 µg DNA were assayed by SFS in duplicate or, when the amounts were sufficient, in triplicate for the presence of DNA adducts. The samples were hydrolyzed in 0.1 M HCl at 90°C for 4 h in sealed glass vials. Synchronous scanning was performed with a fixed Δλ of 34 nm between excitation and emission using a Hitachi F-3000 fluorescence spectrophotometer. Peaks in the 379-385 nm emission range were recorded. The mean levels of DNA adducts from each sample were quantified by using suitable standards (15). The thresholds of positivity corresponded to 1.6 adducts/107 nucleotides in the study with cigarette smoke and to 1.2 adducts/107 nucleotides in the study with BP.

Detection of DNA Adducts by 32P Postlabeling. Aliquots of 5 µg DNA were assayed for the presence of DNA adducts by 32P postlabeling, following enrichment with either nuclease P1 or butanol. Standard procedures (14, 17) were followed, except that the area used as a solvent in thin layer chromatography (D3 and D4) was 6.5 cm rather than 8.5 cm. Autoradiography was performed at -80°C for 24-76 h. The identification of adducts in 2AAF-treated rats was achieved according to Gupta and Earley (18), based on the enrichment procedure and chromatographic localization of spots. The adducts were quantified by calculating relative adduct labeling values (17). Biochemicals were purchased from Sigma and Boehringer Mannheim GmbH (Mannheim, Germany), polyethyleneimine thin layer chromatography sheets from Macherey-Nagel (Doren, Germany), and carrier-free [γ-32P]ATP from ICN Biomedicals (Irvine, CA) having a specific activity ≥ 7,000 Ci/mmol.

Results

Chemoprevention of DNA Adducts in Rats Receiving 2-Acetylaminofluorene with the Diet. No evident spot was detected by 32P postlabeling in the liver DNA of untreated rats (not shown). Administration of 2AAF with the diet resulted in the formation of adducts in rat liver DNA. As shown in the example reported in Fig. 1, panels on the left), different types of adducts were detected following enrichment with butanol or nuclease P1. In particular, the levels recorded in the 5 rats treated with 2AAF (means ± SD) were 118.2 ± 21.3 dG-C9-AAF adducts/108 nucleotides and 10.9 ± 2.8 dG-N2-AAF adducts/108 nucleotides. An evident and significant (P < 0.001) decrease of both types of DNA adduct levels was observed in the liver of rats cotreated with 2AAF and NAC with the diet (Fig. 1, panels on the right), with mean levels of 63.9 ± 10.7 and 2.6 ± 0.9 dG-C9-AAF and dG-N2-AAF adducts/108 nucleotides, thereby accounting for 46 and 76% decreases, respectively.

Detection of DNA Adducts in the liver of Wistar rats after 3 weeks of dietary administration of 2AAF (0.05%) and the protective effect exerted by the concomitant dietary administration of NAC (0.1%). DNA adducts were detected by 32P postlabeling assay following enrichment with either butanol or nuclease P1.

![Image of Fig. 1: Formation of DNA adducts in the liver of Wistar rats after 3 weeks of dietary administration of 2AAF and NAC with the diet (Fig. 1, panels on the right), with mean levels of 63.9 ± 10.7 and 2.6 ± 0.9 dG-C9-AAF and dG-N2-AAF adducts/108 nucleotides, thereby accounting for 46 and 76% decreases, respectively.](image-url)
Chemoprevention of DNA Adducts in Rats Receiving i.t. Instillations of BP. Decreasing levels of DNA adducts were formed in the liver, lung, and heart of all five rats and in the testis of three of five rats treated i.t. with BP (Fig. 5). Of five rats additionally receiving NAC by gavage, four exhibited DNA adducts in the lung, one in the liver and heart, and none in testis. The decrease by NAC of the mean levels of SFS-positive DNA adducts consequent to BP administration was significant in all monitored organs, i.e., liver ($P < 0.01$), lung ($P < 0.05$), and testis ($P < 0.05$).

Detection of Carcinogen-DNA Adducts in Human Atherosclerotic Aorta—Preliminary Data. The analysis by SFS of 23 samples of smooth muscle cells of human abdominal aorta from atherosclerotic patients showed typical fluorescence peaks in 16 subjects (69.6%), with levels ranging between 2.5 and 15.5 adducts/10^7 nucleotides (data not shown). The ^32^P postlabeling analysis (nuclease P1 enrichment) of four aorta samples, all of them SFS-positive, led to the detection of several DNA adducts (Fig. 6).

Discussion

The data reported in this article provide examples of application of molecular dosimetry techniques in experimental studies on chemoprevention of cancer. In addition, they suggest that the same methodological tools can be exploited for assessing the involvement of molecular lesions, not only in cancer, but also in other chronic degenerative diseases. The findings concerning inhibition of carcinogen-DNA adducts, as observed in three separate studies, are consistent with other protective effects exerted by this thiol in the same experimental models. Thus,
The herein described decrease of both dG-N2-AAF and dG-C8-AF adducts to rat liver DNA occurred after 3 weeks of coadministration of NAC with 2AAF in the diet. When followed by a week of standard NAC had been shown to delay by at least one cycle, i.e., 4 weeks, the onset of significant alterations induced by 2AAF in the liver, including changes in the GSH cycle and other cytosolic activities (20), down-regulation of the nuclear enzyme poly(ADP-ribose) polymerase (21), DNA fragmentation, and formation of γ-glutamyltranspeptidase-positive foci (9). Moreover, both NAC and GSH had been successful in totally preventing the formation of Zymbal gland carcinomas, which were conversely detected in all animals treated with the carcinogen alone 6 weeks after completion of the four-cycle treatment.

The observed ability of NAC to inhibit the formation of SFS-positive DNA adducts in liver, testis, heart, and lung of BP-treated rats fully confirms and broadens the results of a previous study, which had been carried out under identical experimental conditions (13). In that study, NAC had significantly inhibited the formation of DNA adducts in liver and lung and, in addition, had prevented clastogenic damage in pulmonary alveolar macrophages, in spite of the unfavorable pharmacokinetic conditions resulting from the i.t. administration of the carcinogen and administration by gavage of the chemopreventive agent (13). In reference to the protective effect observed in the testis, it is noteworthy that recent studies performed in another laboratory have shown that administration of NAC to the same rat strain used in our experiments enhances GSH concentrations in testis and epididymis and inhibits chemically induced dominant lethal mutations (22).

Previous studies demonstrated that exposure of rats to mainstream cigarette smoke results in severe histological, cytological, cytogenetic, and metabolic alterations (23, 24). NAC efficiently prevented smoke-induced emphysema and inflammatory, hyperplastic, and dysplastic changes of bronchial and bronchiolar mucosa, alterations in the cellularity of bronchoalveolar lavage cells, enhancement of the frequency of micronucleated alveolar macrophages, and toxicity in bone marrow erythroblasts (23). Moreover, NAC hampered the smoke-induced stimulation of the liver activation of several promutagens (24). Evidence was also provided for a selective time-course appearance of SFS-positive DNA adducts in heart and lungs, which was prevented by NAC (12). The present study additionally shows the absence of this type of DNA adduct, not only in the liver, but also in brain and testis, and their presence in appreciable amounts in the aorta of the same smoke-exposed animals. Again, NAC significantly prevented this effect.

The selective localization of DNA adducts in different organs depends on several factors including pharmacokinetics (“first-pass” effects), local metabolism, efficiency and fidelity of DNA repair, and cell proliferation rate. For instance, the liver has a high metabolic capacity but also is efficient at DNA repair and removal of adducts. Both lungs and heart are highly exposed due to “first pass” effects, and a poor local metabolism of xenobiotics, especially in the heart (24), should be compensated by the low efficiency of DNA repair, which will result in a longer persistence of adducts. An important difference between these two organs is that certain bronchopulmonary cells are actively proliferating, which favors their transformation into neoplastic cells. Conversely, heart muscle cells are perennial, which is not compatible with an evolution into a neoplastic mass but may lead to other degenerative diseases, such as cardiomiopathies. A similar situation is envisaged in the aorta, where proliferation of smooth muscle cells only occurs during formation of the atherosclerotic plaque.

Therefore, our working hypothesis is that the formation of smoke-related DNA adducts in the lung may be associated with lung carcinogenesis, in the heart with cardiomiopathies, and in the aorta with arteriosclerosis. Several lines of evidences already exist on the role of somatic mutations in arterial smooth muscle cells as one of the mechanisms contributing to the pathogenesis of atherosclerotic plaques (25). In order to further substantiate this hypothesis, we implemented an extensive project aimed at exploring a number of molecular and biochemical end points in human arteriosclerosis. The results which are reported here show the presence of SFS-positive DNA adducts in the majority of the human aorta samples so far analyzed and the occurrence of multiple spots detectable by 32P postlabeling. These are very preliminary data and will have to be confirmed on a larger scale, compared with several other parameters whose evaluation is now in progress in our laboratory and other laboratories collaborating in this project, and be related to clinical, laboratory, and lifestyle characteristics of the patients under study. In any case, the finding that a chemopreventive agent like NAC is capable of inhibiting the formation of DNA adducts in different organs, where they possibly bear a distinctive pathogenetic meaning, deserves attention and warrants further studies in chronic degenerative diseases other than cancer.

References


Chemoprevention of Carcinogen-DNA Adducts and Chronic Degenerative Diseases

Alberto Izzotti, Francesco D’Agostini, Maria Bagnasco, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/54/7_Supplement/1994s

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.