Oxidatively Damaged DNA: A Possible Antigenic Stimulus for Cancer Autoantibodies

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Abstract Reactive oxygen species (ROS) are cytotoxic at higher concentration resulting in cell death, mutations, chromosomal aberrations or carcinogenesis. In this study DNA was modified by singlet oxygen and superoxide anion radicals generated by illumination of riboflavin under 365 nm UV-light. The modified DNA induced high titre antibodies in experimental animals. In enzyme immunoassay, serum antibodies from cancer patients ($n = 34$) showed a higher recognition of the modified DNA, as compared to the native form. This was further confirmed by the gel-shift assay. Immune IgG were used as a probe to detect oxidative lesions in the DNA of cancer patients. DNA isolated from lymphocytes of cancer patients proved to be an appreciable inhibitor of the experimentally induced antibodies against the ROS-DNA. This indicates the presence of oxidative lesions in the DNA obtained from cancer patients. The results show that ROS induced oxidative damage to DNA in cancer patients generate neo-epitopes that are *alien* for the immune system, resulting in autoantibody formation.

Keywords Singlet oxygen species · Superoxide anion radical · DNA · Autoantibodies · Cancer

Introduction

Cancer development in animals/humans is a multistep process involving both endogenous and exogenous stimuli.

One of the endogenous damaging agents are oxygen free radicals that can damage DNA, proteins, lipids etc. [1, 2].

Generation of reactive oxygen species (ROS) through normal cellular metabolism and by exogenous stimulus is a constant problem for which cells have developed multiple defence mechanisms to survive [3–5]. An imbalance between free radical generation and sequestration leads to oxidative stress [6]. Oxygen Free Radical (OFR) related lesions that do not cause cell death can stimulate the development of cancer [7]. The role of ROS in the progression of carcinogenesis is evident from the fact that a number of different free radical generating compounds enhance the malignant conversion of benign papillomas into carcinoma and their effectiveness may be related to the type of radicals produced in the biological system [8].

DNA appears to be the most important target of ROS in cell. In vitro oxidative DNA damage caused by ROS is influenced by a number of factors, especially the concentration and the specific types of ROS produced [9]. The chemistry of DNA damage by several ROS has been elucidated in vitro [10, 11]. Different ROS affect DNA differently; for example, superoxide anion radical and hydrogen peroxide do not react at all with DNA bases, OH generates a multiplicity of products with them [12] while singlet oxygen selectively attacks guanine [13]. When guanine residues oxidized by singlet oxygen are not removed from DNA they are capable of blocking DNA replication which explains biological properties like transforming or transfecting abilities of DNA that are altered by lesions [14]. The unrepaired guanine oxidation products of singlet oxygen can be mutagenic leading G → T transversions [15, 16]. Mutation of DNA is a critical step in carcinogenesis and is linked at the initiation process. [17]. Native DNA *per se* is reported to be non-immunogenic but various insults, e.g. radical damage, cause modifications, both at the

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level of bases and phosphate backbone, generating immunogenic epitopes on DNA that results in antibody response.

In this study, we have discussed the DNA damage by singlet oxygen ($^1\text{O}_2$) and superoxide anion radicals (O_2^-) and its possible role in the development of cancer. Comparative binding of antibodies, present in the sera of various cancer patients, with native and modified DNA has also been studied.

Materials and Methods

Calf thymus DNA, nuclease S1, micrococcal nuclease, anti human IgG alkaline phosphatase conjugate were purchased from Sigma (St. Louis, MO). Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC, Denmark. QIAamp blood midi kit was from Qiagen, USA.

Collection of Sera and Blood Samples

Sera of cancer patients ($n = 34$), with proven clinical diagnosis, were obtained from J.N. Medical College Hospital of A.M.U., Aligarh, India. Normal human sera were obtained from healthy individuals without any disease history in recent past. Informed consent was obtained from all the individuals. All the serum samples were heated at 56°C for 30 min to inactivate complement proteins.

Modification of DNA

Native calf thymus DNA, purified free of proteins and single stranded regions, was modified with singlet oxygen and superoxide anion radical by the method of Waris et al. [18]. A total volume of 3.0 ml contained, 100 $\mu\text{g}/\text{ml}$ DNA, 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.06% Triton X-100 and 40 μM riboflavin. Immediately after mixing, the reaction was carried out in the presence of UV light (365 nm) at room temperature. Samples were extensively dialyzed to remove riboflavin and Triton.

ELISA

An enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96 well, polystyrene immunoplates [19]. Briefly, the plates were coated with 100 μl of the respective antigen (2.5 $\mu\text{g}/\text{ml}$) for 2 h at room temperature and then left overnight at 4°C . After washing three times with buffer (20 mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4 containing 0.05% Tween 20), unoccupied sites were blocked with 1.5% bovine serum albumin in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h at room temperature. The test serum, serially diluted in TBS,

(100 $\mu\text{l}/\text{well}$) was adsorbed for 2 h at room temperature and overnight at 4°C . Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using *p*-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader.

Competition ELISA

Antibody specificity was ascertained by a competitive binding assay [19]. Varying concentrations of inhibitors (0–20 $\mu\text{g}/\text{ml}$) were allowed to interact with a constant amount of antibody (1:100 diluted serum) for 2 h at room temperature and overnight at 4°C . The resulting immune complex was added to antigen-coated plates in place of serum. The remaining steps were same as in direct binding ELISA.

Preparation of Immune IgG

Native and modified DNA (100 μg) were complexed with equal volume (w/w) of methylated BSA and emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly in female rabbits. Subsequent injections were given in incomplete Freund's adjuvant. Each animal received a total of 800 μg of antigen in the course of 8 injections. Blood was collected from marginal ear vein and serum was separated and decomplexed by heating at 56°C for 30 minutes. Pre-immune serum was collected prior to immunization. The sera were stored in small aliquots at -20°C with 0.1% sodium azide as preservative.

Purification of Immunoglobulin G

Immunoglobulin G (IgG) was isolated from sera of cancer patients on a Protein A-Sepharose 4B affinity column [20]. The homogeneity of isolated IgG was checked by performing electrophoresis on 7.5% polyacrylamide gels.

Band Shift Assay

The antigen–antibody interaction was visually detected by 1% agarose gel electrophoresis for 2 h at 30 mA followed by ethidium bromide staining and visualization under UV light [21]. A constant amount of antigen (1 μg) was incubated with increasing concentrations of IgG (0–80 μg) for 2 h at room temperature and overnight at 4°C to facilitate immune complex formation.

Isolation of Human DNA

Blood from human subjects was collected in the presence of an anticoagulant (EDTA). Human DNA was isolated from whole blood using QIAamp Blood Midi Kit supplied

by QIAGEN, U.S.A. The protocol supplied along with the kit was followed

Results and Discussion

ROS are normal by products of aerobic metabolism. They are essential for various defence mechanisms in most cells but can also cause oxidative damage to DNA, proteins, and lipids, resulting in enhanced disease risk [22]. Since DNA plays a central role in information transfer, attention has focused on oxidative damage as a significant source of mutations that lead to cancer and other human pathologies [23]. Native DNA is non immunogenic but various modified forms have been reported to be highly immunogenic because the modified epitopes are recognized as foreign by the immune system. [24]. In the present work we have looked at the presence of antibodies against oxidatively modified DNA in the sera of different type of cancer patients.

Characterization of Modified DNA

Native calf thymus DNA (200 bp fragments) was exposed to $^1\text{O}_2$ and $\text{O}_2^{\cdot -}$ anion radicals generated by irradiation of riboflavin at 365 nm. The structural/conformational alterations in DNA resulted in increased absorption at 260 nm, decrease in melting temperature, low fluorescence intensity following nuclease S1 digestion (data not shown), indicating generation of single strand breaks or partial destruction of secondary structure of DNA making the B-helix highly immunogenic as evident from the induction of high titre antibodies (data not shown).

Detection of Circulating Autoantibodies in Cancer Sera

ROS mediated DNA damage leading to mutations has been strongly implicated in carcinogenesis [25, 26]. Sera from patients suffering from cancer of different organ/organ systems with proven clinical and histopathological diagnosis were used for the study.

Recognition of circulating antibodies by native and modified DNA was studied by direct binding ELISA. Almost all the cancer sera tested showed higher recognition of modified DNA by the circulating autoantibodies (Fig. 1). The antigenic specificity of circulating antibodies was analyzed by competition ELISA. All 5 samples containing sera of lung cancer patients showed greater recognition of modified DNA than native DNA (Fig. 2). Five sera from cancer of urinary bladder showed maximum inhibition in the range of 52.6–68.75% with modified DNA as competitor, while all sera from patients suffering from breast cancer also showed greater inhibition (46–59%) with

Fig. 1 Binding of various cancer sera to native (hatched) and modified DNA (solid). Normal human sera (NHS) serve as negative control. The histograms show mean absorbance values for binding of NHS and sera from patients with cancer of lung (1), urinary bladder (2), breast (3), head and neck (4), oral (5), gall bladder (6), prostate (7), vulva (8), CML (9) and Hodgkin's (10)

Fig. 2 Detection of autoantibodies against native and modified DNA, in the sera of patients with cancer of lung. (a) Cancer sera 1, 2 and 3 by native DNA (open circle, open diamond, open triangle) and modified DNA (filled circle, filled diamond, filled triangle). (b) Cancer sera 4 and 5 by native DNA (open circle, open triangle) and modified DNA (filled circle, filled triangle). The microtitre plates were coated with modified DNA (2.5 µg/ml) and serum dilution was 1:100

modified DNA. The native DNA exhibited below 40% inhibition. These results point to a higher reactivity of antibodies in breast cancer sera towards oxidatively

damaged DNA over the unmodified polymer. Among four sera from head and neck cancer patients two showed higher inhibition (65 and 67.2%) with modified DNA while the other two showed moderate inhibition (45 and 48.5%).

Four sera were collected from patients suffering from oral carcinoma. Inhibition with modified DNA varied from 48 to 69.6% while native DNA showed appreciably less inhibition. However, one sample showed greater inhibition with native DNA (46%) as compared to the modified DNA (40%).

Four sera from gall bladder cancer patients showed inhibition in the range of 39.6–45% with modified DNA and 20.8–28% with native DNA.

Four sera were collected from patients suffering from prostate cancer. Three sera showed maximum inhibition of 25.8, 37, 45.4% with modified DNA and one sera showed almost equal percent inhibition with native and modified DNA (32 and 31%). These results, summarized in Table 1, explain that antibodies in cancer patients are dominantly directed against the oxidatively modified epitopes in the DNA molecule. The findings implicate the role of reactive oxygen species in DNA damage in cancer patients. The modifications/damage renders the DNA molecule immunogenic resulting in the induction of immune response and consequent antibody production.

Band shift assay was performed for the visual detection of antigen-antibody interaction. The data showed difference in the mobility of the antigen due to the formation of high molecular weight immune complexes. Figure 3a shows the formation of immune complex by native DNA and lung cancer IgG. As clearly evident, on increasing the concentration of cancer IgG, keeping the amount of antigen constant, high molecular weight immune complex formation was observed. Similarly, the recognition of modified DNA by cancer IgG was demonstrated by a shift in electrophoretic mobility and corresponding decrease in the

Fig. 3 Band shift assay of cancer IgG binding to (a) native DNA and (b) modified DNA. Native DNA and modified DNA (1 μ g) each were incubated with 10, 20, 40 and 80 μ g of IgG for 2 h at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 h at 30 mA. Lane 1 contains native or modified DNA, while lanes 2, 3, 4 and 5 contain native or modified DNA with increasing concentrations of lung cancer IgG

Table 1 Inhibition of the binding of cancer autoantibodies to native and modified DNA

Types of cancer	No of sera tested	Mean percent inhibition at 20 g/ml	
		Native DNA	Modified DNA
Lung	5	24.70	64.98
Urinary bladder	5	36.98	54.51
Breast	4	30.73	52.00
Head and neck	4	39.83	56.00
Oral	4	40.28	55.00
Gall bladder	4	25.35	42.00
Prostrate	4	29.05	34.00

The microtiter plates were coated with modified DNA (2.5 μ g/ml) and the serum dilution was 1:100

fluorescence intensity of the unbound antigen (Fig. 3b). The retardation was more pronounced in case of modified DNA as compared to the native form establishing a higher recognition of cancer IgG by the modified DNA as compared to the native form. These results reiterate the findings of enzyme immunoassay. The recognition of modified DNA by autoantibodies in the sera of different cancer patients coupled with the enhanced inhibition of these autoantibodies by modified DNA, as compared to the native polymer, showed that the oxidatively damaged DNA is a better antigen for cancer autoantibodies. This clearly suggests a possible role for ROS modified DNA in the production of autoantibodies in cancer.

Detection of Oxidative Lesions in DNA

DNA was isolated from lymphocytes of various cancer patients. DNA isolated from lymphocytes of normal healthy individuals were used as controls.

Anti ROS-DNA antibodies were used as an immunochemical probe to detect oxidative lesions in the DNA from cancer patients. Immune complexes, formed by incubating a fixed amount (60 μg) of immune IgG with increasing concentrations on microtitre plates already coated with modified DNA.

Two DNA samples isolated from patients with lung cancer showed high 64 and 68.6% inhibition of immune IgG binding to the immunogen. These patients had a history of smoking and support for the strong oxidative stress in vivo caused by tobacco smoke [27]. Another two samples of DNA from oral cancer patients, showed maximum inhibition of 61.1 and 60% in antibody binding. Two DNA

Fig. 4 **a** Inhibition of binding of anti-ROS-DNA IgG by genomic DNA isolated from lymphocytes of patients with cancer of lung (*open triangle, filled triangle*) oral (*open circle, filled circle*) and prostate (*open square, filled square*). The microtitre plates were coated with modified DNA (2.5 $\mu\text{g/ml}$). **b** Inhibition of binding of anti-ROS-DNA IgG by genomic DNA isolated from lymphocytes of normal individuals (*open triangle, filled triangle*). The microtitre plates were coated with modified DNA (2.5 $\mu\text{g/ml}$)

Table 2 Binding of anti-ROS-DNA IgG to DNA isolated from lymphocytes of cancer patients

Normal/Cancer	No. of samples tested	Maximum percent inhibition at 20 $\mu\text{g/ml}$ (mean)
Normal DNA	2	22.50
Lung	2	66.40
Prostate	2	60.55
Oral	2	39.90

The microtiter plates were coated with modified DNA (2.5 $\mu\text{g/ml}$)

samples isolated from patients with prostate cancer showed 42.7 and 37% inhibition of antibody activity (Fig. 4a). On the contrary, DNA isolated from two normal healthy individuals caused negligible inhibition of 20% and 25% in immune IgG binding to the immunogen (Fig. 4b). These results have been summarized in Table 2. All these results indicate effective binding of immune IgG to DNA isolated from various cancer patients and confirm the presence of ROS induced oxidative lesions in their DNA. Because of less number of samples no definitive conclusions can be drawn. However, larger sample size could help us reach a definitive conclusion. Our preliminary findings may provide some clues for the role of oxidative DNA damage in cancer patients, which might play an active part in the progression of disease. Our results also demonstrate the presence of anti-ROS-DNA antibodies in cancer patients, which could perhaps have a prognostic significance.

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