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Characterization of chromatin modified with reactive oxygen species: Recognition by autoantibodies in cancer

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Abstract

Objectives: To study the binding of chromatin modified with reactive oxygen species (ROS) with circulating autoantibodies present in cancer patients.

Design and methods: Chromatin isolated from goat liver was modified by superoxide radical plus singlet oxygen and hydroxyl radicals. Sera from 47 patients with various types of cancers were tested for binding to native and modified chromatin by direct binding and competition ELISA.

Results: Maximum modification of thymine (54%) was found in case of chromatin modified with hydroxyl radical whereas by the combined action of singlet oxygen and superoxide anion radical, guanine was modified most (72%). Autoantibodies in cancer sera recognized modified chromatin in preference to native chromatin. Band shift assay with affinity-purified IgG from sera of cancer patients reiterated the results obtained with serum samples.

Conclusion: Greater recognition of ROS-modified chromatin with the autoantibodies in cancer sera is indicative of reactive-oxygen-species-induced chromatin damage in cancer patients.

Keywords: Chromatin; Cancer; Hydroxyl radical; Superoxide radical; Singlet oxygen; Autoantibodies

Introduction

All of the human genome is packaged into chromatin. Although chromatin was historically thought of as an inert repressive structure, we now know that it is truly a living vibrant entity [1]. Chromatin consists of DNA and associated proteins. There are two types of proteins in chromatin. Histones are small, well-defined basic proteins, whereas non-histone chromosomal proteins include a large member of widely diverse structural, enzymatic and regulatory proteins, most of which are yet to be characterized.

It is well established that free radicals and other reactive oxygen species (ROS) are continuously produced *in vivo* and can damage most cellular components [2]. Reactive oxygen species, formed exogenously and endogenously, are known to be cytotoxic and may be implicated in the aetiology of a number of diseases including cancer [3–6]. ROS have been known to play an important role in the initiation and promotion of multistage carcinogenesis [7]. There are numerous mechanisms for generation of ROS *in vivo*. It has been proposed that many of the damaging effects could be attributed to chemically reactive species like superoxide, hydrogen peroxide and hydroxyl radical [3]. The role of ROS in the progression stage of carcinogenesis is evident from the fact that a number of different free radical generating compounds enhance the malignant conversion of benign papillomas into carcinomas and their effectiveness may be related to the type of radicals produced into the biological system [8].

DNA appears to be an important target of ROS in tumour biology since it is firmly established that cancer is a genetic disease [9]. An increased production of oxygen derived species within cells frequently leads to DNA damage by a variety of mechanisms and such species can probably initiate and promote cancer [10,11]. The superoxide anion, singlet oxygen and the most reactive of the ROS, the hydroxyl radical, have

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been implicated in mutagenesis, carcinogenesis and aging [4,12–14]. The hydroxyl radical produces a unique and extensive pattern of chemical modifications in DNA and nucleoprotein, including modified bases and DNA protein cross-links [15–17]. It has been postulated that the oxidative DNA base modifications increase genetic instability and thus contribute to tumour progression in laryngeal cancer [18]. Because of their ability to damage DNA, free radicals are thought to be involved in all stages of carcinogenesis [10,19]. Origin of anti-dsDNA autoantibodies in cancer patients and tumour-bearing mice has been demonstrated [20]. Evidence exists that tumour cells have abnormal levels and activities of antioxidant enzymes, such as superoxide dismutase or catalase leading to accumulation of superoxide and hydrogen peroxide that induce damage to DNA [21]. The genotoxicity of reactive oxygen species is well established [5]. The underlying mechanisms involve oxidation of DNA by ROS [22]. However, it has been recently shown that hydrogen peroxide (H_2O_2), the major mediator of oxidative stress, can also cause genomic damage directly. Thus, H_2O_2 at pathologically relevant concentrations rapidly induces higher order chromatin degradation [23].

Materials and methods

Anti-human IgG-alkaline phosphate conjugate, *p*-nitrophenyl phosphate, Tween-20, Triton X-100 and riboflavin were purchased from Sigma Chemical Company, USA. Protein A-Sepharose CL-4B was purchased from Genei, India. Polystyrene microtiter flat bottom ELISA plates were purchased from NUNC, Denmark.

Serum samples

Serum samples of various cancer patients (of age 35–50 years) proven with histopathological diagnosis were obtained from J. N. Medical college hospital, Aligarh, India. These samples were routine determinations and were not specifically obtained for the study. Fifteen normal human sera (used as negative control) were obtained from non-smoking healthy volunteers of same age group who gave prior informed consent. All serum samples were decomplemented at 56 °C for 30 min before use.

Isolation and modification of chromatin

Chromatin was isolated as described by Bonner et al. [24] with slight modifications. Briefly, 10 g of fresh goat liver was homogenized with 200 mL of saline–EDTA (0.075 mol/L NaCl and 0.024 mol/L EDTA, pH 8.0). The homogenate was strained through 6–8 layers of cheese cloth. The filtrate was centrifuged at 1500×*g* for 15 min. The pellet was homogenized in 40 mL of Tris buffer (0.05 mol/L, pH 8.0) and sedimented at 10,000 g for 15 min. This step was repeated once. The final pellet was suspended in 30 mL of Tris buffer (0.0514 mol/L, pH 8.0). Five-milliliter aliquots of the above suspension were layered on 25 mL portions of 1.7 mol/L sucrose (in 0.01 mol/L Tris buffer, pH 8.0) contained in centrifuge tubes. The upper two thirds of each tube were gently mixed and the tubes were then centrifuged at 21,000 rpm for 3 h in F0650 rotor at 4 °C. The pellets were suspended in 0.01 mol/L Tris buffer, pH 8.0, and dialyzed overnight against the same buffer. The dialyzed suspension was sheared in Virtis homogenizer for 90 s, stirred for 30 min and then centrifuged at 10,000×*g* for 30 min. The supernatant of the above is referred to as sheared liver chromatin.

Modification of chromatin by hydroxyl radical

Aqueous solution of chromatin in PBS, pH 7.4, was irradiated under 254 nm light for 1 h at room temperature in the presence of hydrogen peroxide (15.1 mmol/L). Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4.

Modification of chromatin by superoxide anion radical and singlet oxygen

Chromatin was modified by superoxide anion radical and singlet oxygen generated by the method described earlier [25]. A total volume of 3.0 mL of reaction mixture contained chromatin, $A_{260}=2.0$, 50 mmol/L potassium phosphate buffer, pH 7.8, 1.1 mmol/L EDTA, 0.06% Triton X-100 and 40 μmol/L riboflavin. The reaction mixture was irradiated at 365 nm at room temperature followed by extensive dialysis to remove riboflavin and Triton X-100.

Quantifying base modification

The separation and quantification of bases in DNA separated from modified and unmodified chromatin was performed according to the method described earlier [26]. DNA samples were hydrolyzed in 70% perchloric acid, neutralized and bases separated on DEAE-Sephadex A-25 matrix. Elution was carried out with a linear gradient of 1–20 mmol/L Tris–HCl, pH 7.6, at a flow rate of 40 mL/h. Fractions of 3 mL were collected, absorbance was monitored at 260 nm and elution profile of bases plotted. Individual bases were run as controls. Percent modification in DNA separated from modified chromatin was calculated by measuring peak areas of modified and unmodified bases. Elution profiles of individual bases and hydrolyzed DNA separated from native chromatin served as standards.

Nuclease S1 treatment

Modified samples were characterized by nuclease S1 digestion followed by agarose gel electrophoresis [27]. One microgram each of native and modified chromatin was incubated with nuclease S1 (20 U/μg of DNA) in acetate buffer, pH 5.0, for 30 min at 37 °C. The reaction was stopped by one-tenth volume of 0.2 mol/L EDTA, pH 8.0. The digested samples were analyzed on 1% agarose gel and electrophoresed at 30 mA for 2 h. The gel stained with ethidium bromide was visualized under UV light.