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Pristane Induced Effects on Chromatin of Rat Lymphoid Cells

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Abstract The effects of pristane on the conformation of chromatin in cells isolated from the lymphoid tissues of pristane-treated Copenhagen rats were examined by flow cytometry, thermal denaturation, sensitivity to enzymatic digestion, and histone protein analyses. Decreases were observed in the fluorescent intensities of propidium iodide (PI) stained nuclei isolated from lymphoid cells of pristane-treated rats when compared with normal rat lymphoid nuclei. Studies to address the possible basis for the pristane-induced changes in the DNA staining characteristics of lymphocytes demonstrated that 1) there were no decreases in the amount of DNA present in the nuclei, 2) nuclei isolated from pristane treated rats were less sensitive to thermal denaturation, as well as DNase I enzymatic digestion, and 3) there were apparent increases in the expression of the H1 histone proteins. Collectively, these results suggest that pristane elicits a conformational change in the chromatin which may be mediated by altered expression of nuclear-associated histone proteins.

Key words: 2,6,10,14-tetramethylpentadecane, histone, DNA conformation, pristane, lymphocyte

Although pristane was initially considered to be relatively inert, recent studies have demonstrated that this isoprenoid induces a myriad of biological effects. For example, studies have demonstrated that pristane induces plasmacytomas in BALB/c mice [1], arthritis in mice [2], formation of a granulomatous substratum [3], immunodepression [4], reduced growth of trout [5], amyloidosis [6], rapid viral induction of plasmacytomas [7], formation of ascites in mice transplanted with hybridoma cells [8], production of growth factors [9], and enhanced growth of various transplanted tumors [10].

Pristane also elicits a promoter-like response in that treatment with 3-methylcholanthrene (3-MC) resulted in few lymphoid malignancies, whereas treatment with 3-MC and pristane resulted in the induction of lymphoid leukemias and lymphomas in > 60% of the rats examined [11–13]. Furthermore, while the above mentioned biological effects of pristane suggest that pristane may act indirectly to elicit the corresponding effects, previous studies in our laboratory indicate that pristane may have a more direct effect on the cellular DNA. Along these lines, pristane induces transient, yet marked changes in the propidium iodide (PI) staining characteristics of lymphoid cells isolated from pristane-treated rats and mice [14–16]. Furthermore, in light of the effects of pristane on the development of lymphoid malignancies in rats treated with 3-MC, as well as its effect on the PI staining characteristics of cells from pristane-treated rodents, it appears that pristane may share similar characteristics with 12-0-tetradecanoylphorbol-13-acetate (TPA). TPA, a known tumor promoter, has also been shown to affect the conformation of chromatin [17]. For example, altered staining characteristics as well as increased histone protein synthesis and phosphorylation of specific histone proteins have been reported [18,19].

The biological relevance of the effects of pristane observed to date pertains to the fact that pristane is a ubiquitous isoprenoid derived from chlorophyll and is present in appreciable amounts in our common foodstuffs [20]. The purpose of the studies presented below was to further examine the effects of pristane on lymphoid cells and more specifically to address the

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possible mechanism whereby pristane elicits a change in chromatin conformation.

METHODS

Animals and In Vivo Treatment

Female Copenhagen rats (~120 g, 6 months old) were used for all studies and were maintained and experimentally treated as previously described [14]. A single intraperitoneal injection of 1 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, MO) was administered 4, 14, or 30 days prior to cell isolation. Appropriate age-matched, normal rats were routinely used throughout this study.

Nuclei Preparation and Analysis

Nuclei were obtained by cellular disruption of peripheral blood lymphocytes with a Stansted cell disrupter (Stansted Fluid Power LTD., Stansted, Essex, UK) followed by differential centrifugation at 500 × *g* to remove any debris or whole cells and 6000 × *g* to pellet the nuclei as described [21]. The nuclei were 1) visualized by light microscopy, 2) diluted to 5×10^5 nuclei/ml, 3) fixed with 1% paraformaldehyde for 1 hour, 4) washed twice with 0.15 M phosphate buffered saline (PBS, pH 7.2), 5) stained with 0.02% propidium iodide (PI; 0.5 ml per ml of nuclei) for 30 minutes and 6) analyzed by flow cytometry as previously described [14]. A modification of the method described by Darzynkiewicz et al. [22] was used for the thermal denaturation studies. Briefly, isolated lymphocytes (unfixed and resuspended in 0.15 M PBS) were preincubated for 10 minutes at 12, 27, 37, 55, 80, and 100°C, immediately fixed by adding an equal volume of 2% paraformaldehyde and incubated at R.T. for 1 hour prior to washing in PBS, PI staining and flow cytometric analyses as described above.

Enzymatic Degradation and Electrophoresis

Nuclei isolated from the lymphoid cells of normal and pristane-treated rats were digested with DNase I (Sigma Chemical Co., St. Louis, MO) as previously described [23]. Briefly, 2.5×10^7 nuclei, suspended in 10 mM Tris-1 mM $MgCl_2$ buffer (pH 7.8), were digested with 1, 5, 10, 50, or 100 units of pancreatic DNase I for 1–90 minutes at 27°C. Empirical analyses to examine the effects of the amount of DNase I on the rate of digestion demonstrated that the reaction mixture containing 10 units of DNase I was optimal; only data obtained under these conditions are presented below. The digestion was stopped by the addition of 0.5 M EDTA (pH 8.0). The digest was then centrifuged and the supernatant containing the nuclear digest quantified by measuring the absorbance at 260 nm. An equivalent of 10 μg of DNA for each sample was also loaded onto a 0.7% agarose gel and electrophoresed at 45V for 4 hours in a Tris-acetate running buffer.

Histone Protein Isolation

Total histone proteins were extracted from the nuclei of lymphoid cell isolates from normal and pristane-treated rats. Briefly, histones were extracted from 2.5×10^7 isolated nuclei using a 2 M solution of NaCl (pH 6.0) as described by Johns [24]. Concomitant studies using a sulfuric acid extraction, as described by Balmain et al. [25], were also conducted with similar results. Protein content was quantified by measurement of absorbance at 280 nm. Total histone protein (50 μg) was electrophoresed in a 0.1% SDS-15% PAGE for 6 hours at a constant current of 25 mA. The gels were stained with 1% Coomassie brilliant blue and destained by acetic acid/ethanol (7:10 v/v). The gels were then scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc.).

RESULTS

Previous studies demonstrated that lymphoid cells isolated from pristane-treated rats exhibited altered DNA staining characteristics [14], i.e., decreased PI staining intensities. Since subsequent studies indicated the majority of the tissue-associated pristane was localized in the plasma membranes [14] and also appeared to affect membrane fluidity [26], the question as to whether or not changes in the plasma membrane may directly affect the PI-staining characteristics, (e.g., changes in permeability of the membrane to the dye) was addressed. Fig. 1 depicts representative flow cytometric profiles which demonstrate that 1) nuclei from lymphoid cells from pristane treated rats exhibited different PI-staining intensities when compared to nuclei from normal lymphoid cells and 2) these changes were similar to the previously reported PI-staining characteristics of intact lymphoid cells isolated from pristane-treated rats [14]. Furthermore, diphenylamine analyses of DNA content indicated the decreased staining intensities were not due to a decrease in the amount of DNA per unit number of cells (data

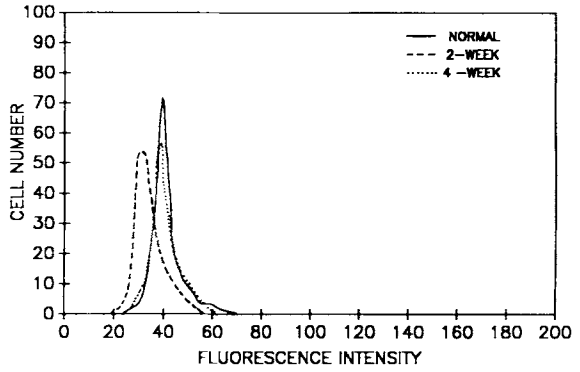

Fig. 1. Flow cytometric profiles of propidium iodide (PI) stained nuclei isolated from peripheral blood lymphocytes from a normal rat or rats previously treated 14 or 30 days with a single i.p. injection of pristane. Nuclei were obtained by Standsted disruption of intact cells, centrifuged, stained with PI, and analyzed by flow cytometry as previously described. Results are representative of ≥ 10 rats for each variable.

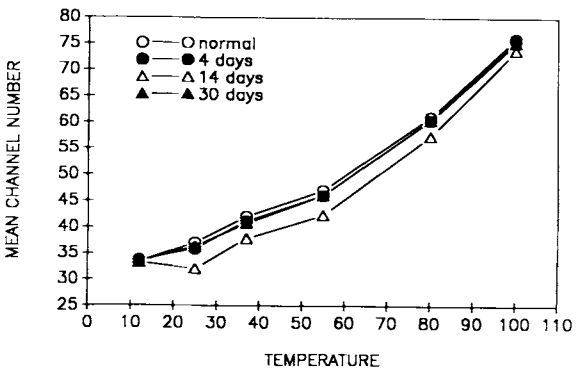

Fig. 2. Sensitivity of lymphoid cells isolated from pristane-treated rats to thermal denaturation. Lymphocytes were isolated from the peripheral blood of normal rats or rats which were treated with a single i.p. injection of pristane (4, 14, or 30 days), incubated at the indicated temperatures, fixed, stained with PI, and analyzed by flow cytometry as described in the Materials and Methods. Results are an average of 3 experiments with n ≥ 3 for each experiment; standard deviations were < 5% of the mean.

not shown). These data clearly demonstrate that the differences in the staining intensities and, by inference, the amount of dye which intercalated with the double-stranded DNA [27] in the nuclei of normal versus pristane-treated rats was not attributable to interference due to possible changes in plasma membranes. These results also indicated that pristane elicited a conformational change in the chromatin of cells isolated from pristane-treated rats such that less dye was intercalated.

One approach used by others to examine altered chromatin conformation has been to assess the effects of thermal denaturation on the intercalation of DNA specific dyes. In order to examine whether or not there were temperature-mediated differences in dye intercalation into chromatin of intact lymphoid cells isolated from normal versus pristane treated rats, a flow cytometric method was employed. The results of these studies are depicted in Fig. 2 and indicated that if the cells were preincubated at 12 or 100°C immediately prior to fixation and PI analyses, there were no detectible differences in the fluorescent intensities between cells isolated from normal and pristane-treated rats. However, with preincubation temperatures ranging from 27 to 70°C differences were observed between the staining intensities of cells isolated 14 days post pristane treatment versus cells from either normal rats or pristane-injected rats, 4 or 30 days after treatment. It should be noted that 14 days post pristane treatment was also the time at which a maximum affect in PI staining characteristics was observed, (Fig. 1). Furthermore, RNase A treatment of the nuclei (5000 units/ml cells for 30 minutes at 37°C) did not alter the results observed.

A second approach to address possible conformational differences was examination of differential sensitivities of nuclei from normal versus pristane-treated rats to enzymatic digestion. For these studies, normal nuclei or nuclei isolated from lymphocytes of pristane-treated rats were digested with DNase I for the indicated times (Figs. 3 and 4). The amount of digested DNA

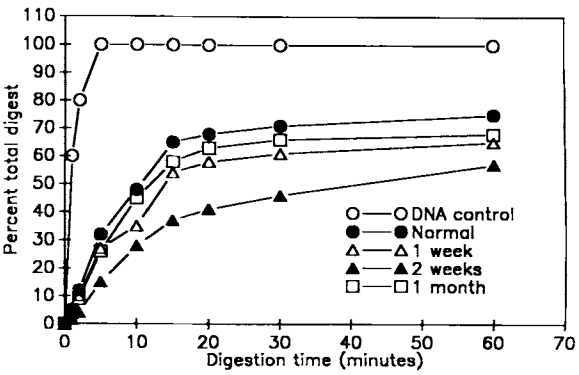

Fig. 3. Sensitivity of nuclei to enzymatic digestion by DNase I. Nuclei isolated from lymphocytes of normal rats or rats treated with 1 ml of pristane, i.p. for 7, 14, or 30 days, were digested with 10 units of DNase I for the indicated times. The amount of DNA in the digested samples was quantified by absorbance readings at 260 nm. Data are presented as percent total digest based on a comparison of the absorbance readings of the experimental samples to the digested amount of purified DNA present after 60 min of DNase treatment, which served as a 100% reference point.

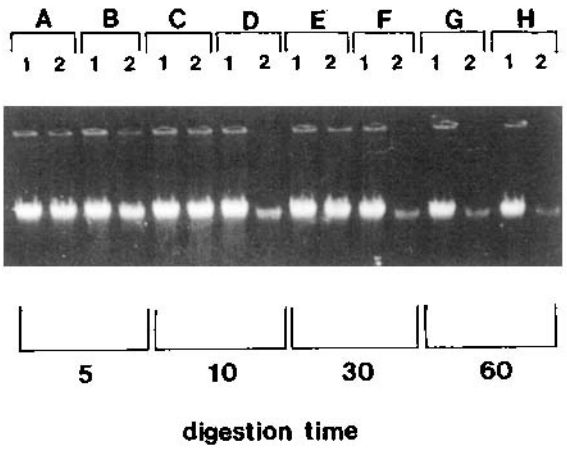

Fig. 4. Electrophoretic patterns of treated and DNase I digested, high molecular weight DNA isolated from lymphocytes of normal versus pristane-treated rats. Equivalent amounts of DNA were electrophoresed through a 0.7% agarose gel and visualized by staining with ethidium bromide. Lane 1 in each case represents DNA without DNase I while lane 2 depicts DNA from nuclei digested with 10 units of DNase I for the indicated minutes. Lanes A, C, E, and G are nuclear digests of samples from a rat 14 days after i.p. injection with 1 ml of pristane. Lanes B, D, F, and H are from a normal rat.

present in the supernatants of the DNase I treated nuclei from normal versus pristane-treated rats was quantified and plotted versus length of DNase I digestion (Fig. 3). Isolated DNA used as a control was rapidly and completely digested in less than 7.5 minutes, whereas isolated nuclei were not as susceptible to enzymatic digestion. It is interesting to note that normal nuclei and nuclei isolated from cells 4 days and 30 days following treatment with pristane exhibited similar sensitivities to DNase digestion. However, there was a clear difference in the rates of digestion of nuclei from normal rats and from pristane-treated rats at 14 days post injection, i.e., a period of optimal changes in PI staining characteristics. Furthermore, each of the experimental samples exhibited two phases of digestion: a fast, initial rate of digestion during the first 10 minutes followed by a slower rate that appeared to plateau. It should be noted that after prolonged digestion with DNase I the amounts of digested material in digests of each sample were similar (Fig. 3). Differences in the sensitivity to DNase I digestion of nuclei isolated from cells of normal and pristane-treated rats were also apparent by electrophoretic analyses. Fig. 4 depicts representative electrophoretic patterns of high molecular weight DNA of undigested versus DNase I digested samples of nuclei from lymphocytes of normal versus pristane-treated rats. Again, it was clear that the chromatin isolated from lymphocytes of pristane treated rats was less sensitive to enzymatic digestion. It should be noted that the data depicted in Figs. 3 and 4 were representative of 1 of 3 rats per control or experimental group and were reproducible in three independent studies. Speculatively, the chromatin of cells isolated from pristane-treated rats was more protected by chromosomal proteins than chromatin from normal rat lymphocytes.

To determine if the differences in enzymatic sensitivity were due to altered (presumably increased) histone protein expression which reportedly mediates chromatin conformation [28], total histone proteins were isolated from lymphoid cells from normal and pristane-treated rats. Fig. 5 depicts densitometric plots of electrophoretic profiles of histone proteins isolated from peripheral blood lymphocytes of normal rats versus rats after 14 days of exposure to pristane. These data are representative of 4 independent analyses, each exhibiting similar results. Again it should be noted that 14 days was the optimal time point at which 1) a maximum decrease was observed in the PI staining intensities (previously described above) and 2) the nuclei from pristane-treated rat cells were the least sensitive to enzymatic digestion (see above). There was an apparent increase in the amount of H1 protein present, identified based on its characteristic

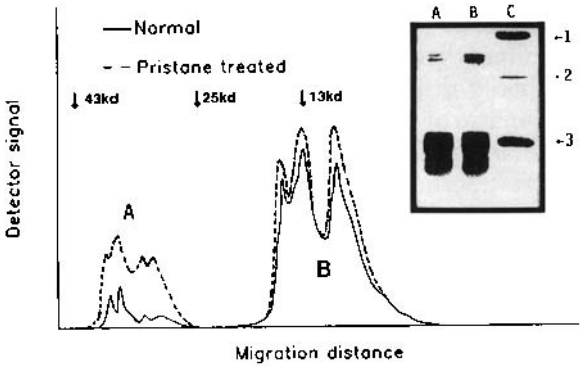

Fig. 5. Densitometric plots of histone proteins isolated from lymphocytes of normal or pristane treated rats (14 *days post i.p.* injection). Total histone proteins were isolated from an equivalent number of cells and separated by SDS-PAGE; electrophoretic profiles are depicted in the insert. H1 and core histones are denoted *A* and *B*, respectively. Insert depicts Coomassie stained electrophoretic profiles: lane A, normal; lane B, pristane treated; lane C, molecular weight markers. Molecular weight markers are ovalbumin (43 kd), chymotrypsinogen A (25 kd) and ribonuclease A (13 kd), denoted 1, 2, and 3, respectively, on the insert.

electrophoretic mobility, in the cells from pristane-treated rats when compared to normal cells. There were also variations in the amount of the core histones present, albeit to a lesser extent than the H1 protein.

DISCUSSION

In light of the results of our previous studies which demonstrated that pristane treatment elicited altered DNA staining characteristics without concomitant changes in DNA content [14–16], the purpose of the studies presented above was to further examine the basis for this apparent effect of pristane on chromatin conformation. Our initial analyses demonstrated that nuclei and intact lymphoid cells isolated from pristane-treated rats exhibited similar DNA staining characteristics, i.e., a decrease in staining intensities when compared with normal nuclei or cells. This indicated that 1) the changes in chromatin conformation were stable within isolated nuclei and 2) the plasma membranes of the lymphoid cells from the pristane-treated rats did not affect dye intercalation.

To further address the mechanism(s) whereby pristane may mediate the observed changes in chromatin conformation, two approaches were employed. First, thermal denaturation studies were conducted to assess the influence of temperature on the pristane induced PI staining characteristics of lymphoid cells. These studies were based on the premise that thermal effects on chromatin conformation may be detected by PI analyses of cells which were paraformaldehyde-fixed immediately after a brief equilibration of the cells at different temperatures. The results indicated that the chromatin in lymphoid cells isolated 14 days after pristane treatment was less susceptible to thermal denaturation. One possible interpretation of these data is that there were differences in the DNA-protein complexes within the nuclei of cells from the pristane-treated rats which affected chromatin conformation, the degree of fixation, and subsequently the amount of intercalated PI (and hence the fluorescent intensity). Speculatively, such an effect was manifested even at temperatures at > 37°C which are known to affect the transition from a native, coiled state of the DNA to one which is less ordered [29]. However, with preincubations at 12 or 100°C it appears that the chromatin was condensed or denatured, respectively, in a similar fashion, i.e., cells from normal and pristane-treated rats exhibited similar staining characteristics at these temperatures. Second, as previously described by others, the extent to which chromatin in condensed affects its sensitivity to DNase digestion [30]. The changes in sensitivity of isolated nuclei to DNase digestion observed herein suggested that either more chromosomal proteins were present (resulting in more tightly condensed chromatin and therefore protection from DNase digestion) or the existing proteins were more tightly complexed to the DNA.

The data obtained from the thermal denaturation and enzymatic studies support the hypothesis that pristane elicits a change in the superstructure of lymphoid DNA. Along these lines, it should be noted that the importance of histones in the assembly and stabilization of cellular DNA are well documented [28,31]. The data presented indicated apparent increases in the amount of H1 histone protein in the nuclei of lymphoid cells from pristane-treated rats when compared to cells from normal rats. Collectively, these data demonstrate that not only was more histone protein present in the cells, but also that resistance to DNase digestion, as well as to heat denaturation, was likely due to the complexity of the interaction of these proteins with the DNA.

Based on studies by others to examine the effects of TPA on histone expression, several other factors may also be important. First, changes in the turnover and/or exchange rates of H1 may result in an altered expression of H1 proteins. Second, several variants of H1 reportedly may affect the condensation of chromatin [32,33]. Third, post-translational modifications of histones may be specifically induced. For example, TPA induces specific phosphorylation of core histones [18,19,25]. Finally, changes in histone protein expression may be attributable to changes in transcriptional events of H1 mRNA [28]. In addition to TPA, other compounds, such as DMSO and sodium butyrate which are chemical inducers of cellular differentiation, also elicit conformational changes in the chromatin and DNA of treated cells [34]. The possibility that pristane elicits one or more responses which are similar to the biological effects of these compounds on histone expression and chromatin conformation remains to be addressed. However, it is interesting to note that pristane and TPA exhibit similar biological effects; i.e., membrane association, altered membrane fluidity, and changes in chromatin conformation even

though the timing and specificity of these effects may vary. Furthermore, the results of a recent study to directly compare the effects of pristane versus TPA on various cell lines transfected with plasmids containing a reporter gene under the regulatory control of viral promoter/enhancer elements clearly indicate that both pristane and TPA can activate gene expression [manuscript submitted]. Whether or not similar events occur within lymphoid cells remains to be determined. Also of interest is the possible effect of pristane on second messenger systems. Along these lines, TPA activates protein kinase C and the phosphorylation of protein substrates, including histone H1 [35]. As a final note, the contribution of non-histone, nuclear-associated proteins was not examined in this study and their contribution to the effects on chromatin conformation cannot be ruled out at this time. Nevertheless, our results clearly demonstrate that pristane, a ubiquitous isoprenoid with tumor enhancing potential, does affect chromatin conformation, as well as histone expression.

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