Differential crosslinking of histones and non-histones in nuclei by cis-Pt(II)

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When nuclei were treated with the chemotherapeutic agent, cis-Pt(II), they were crosslinked to the extent that their nuclear morphology as assayed by light microscopy was retained even in the presence of SDS. Protein analysis showed that the histones were completely absent from these nuclear structures, while the non-histone proteins, with one possible exception, were completely retained. When the nuclear structures in SDS were treated with thiourea to reverse the crosslinks, the non-histone proteins were liberated and the nuclear structures disappeared. When treated with Proteinase K in SDS, the nuclear structures also disappeared, indicating that protein components were necessary to maintain the structures.

Histone *Non-histone* *Nucleus* *Cis-Pt(II)*

1. INTRODUCTION

Crosslinking reagents comprise an important group of chemotherapeutic agents. Their ability to form DNA inter-strand crosslinks is well documented (see Ludlum [1] for review). In addition, various studies have suggested that protein is bound to the DNA after treatment of nuclei or cells [2–7] with various crosslinking agents. Thomas et al. [8] characterized the proteins crosslinked to DNA by nitrogen mustard treatment. Those proteins were released from the DNA by the same conditions that released alkylated purines, results which indicated that they were originally linked through diethylamino moieties to the DNA. When analyzed by SDS gel electrophoresis, the released proteins were found to be a subset of the non-histone proteins.

In this paper, we investigate the effects of cis-Pt(II) on nuclei. This reagent seems to crosslink the non-histone proteins and the DNA into a lattice-work structure which retains an overall nuclear morphology in the light microscope even after SDS treatment. In contrast the histones were not crosslinked to this structure, and could be removed by SDS extraction.

2. MATERIALS AND METHODS

Mouse L1210 cells were grown, labeled with [^{14}C]leucine (New England Nuclear) and [^{3}H]thymidine (New England Nuclear) as previously described [8]. Nuclei, isolated by a detergent method [9] as modified by Thomas et al. [8], were suspended in HCS buffer (10 mM HEPES, pH 6.4, 1 mM $CaCl_2$, 0.32 M sucrose). Cis- or trans-Pt(II), which had been dissolved in HCS for at least 1 h before use was added to the nuclear suspension, which was incubated at 37°C for various periods of time.

For CsCl centrifugation, the solution containing the treated nuclei was dialyzed against 0.02 M EDTA–0.05 M Tris–HCl (pH 7), then briefly sonicated (5 s, setting 1 of a Branson S125 sonifier using a microtip), adjusted to 0.3% Sarcosyl and layered over 3.0 ml of 3.0 M CsCl solution (36.7% CsCl by weight, 0.02 M EDTA, 0.05 M Tris–HCl, 0.3% Sarcosyl, pH 7) and centrifuged in a SW 56 rotor at 40000 rev./min for 23 h at 4°C.

For the analysis of proteins crosslinked in the nuclear structures, 1.5×10^7 nuclei were treated with 1 mM cis-Pt(II) in HCS buffer for 1 h at

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37°C, then washed with HCS to remove remaining drug. The treated nuclei were extracted with 0.1 ml 1% SDS, washed twice more with 1% SDS, then incubated in 0.1 ml 1 M thiourea for 1 h at 37°C [10]. The SDS and thiourea extracts were loaded onto an SDS gel and the proteins analyzed as described previously [11,12].

3. RESULTS

3.1. *CsCl centrifugation*

When isolated nuclei were dissolved in 0.3% Sarcosyl and centrifuged through 3.0 M CsCl, less than 0.1% of the radioactivity ([^{14}C]leucine) present in nuclei was found in the pellet (bottom 0.7 ml of tube) while DNA recovery in the pellet was over 90%. When the nuclei were incubated first with cis-Pt(II), most of the DNA did not pellet through CsCl as it did in the control, but was distributed in several bands in the gradient (fig.1). Protein was also found coincident with the DNA bands, but most was at the top of the gradient.

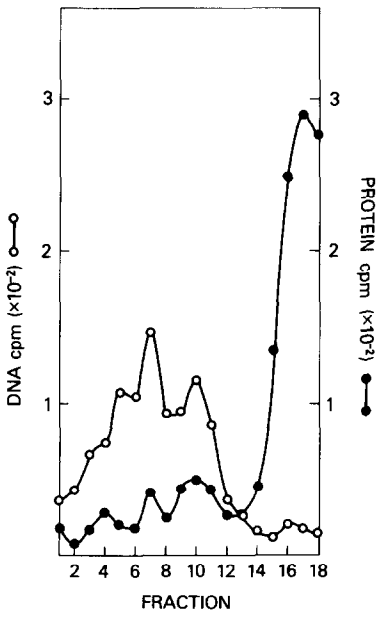

Fig.1. Equilibrium centrifugation of cis-Pt(II)-treated nuclei in 3.0 M CsCl. L1210 nuclei, labeled with [^{3}H]thymidine and [^{14}C]leucine were treated in HCS buffer with 1 mM cis-Pt(II) for 1 h at 20°C. Unreacted compound was removed by washing the nuclei with HCS buffer. Nuclei were then processed through 3.0 M CsCl as described in the text.

More striking was the finding that in the light microscope, structures with nuclear morphology could be seen in fraction 7 and structures with nucleolar morphology could be seen in fraction 10, even though these gradients contained 0.3% Sarcosyl and 3 M CsCl to disrupt all noncovalent bonds. We concluded from this experiment that cis-Pt(II) had at least partially fixed these nuclei, but that at the same time much of the protein had been released to the top of the gradient.

The data presented in fig.1 were obtained with nuclei treated with 1 mM cis-Pt(II) for 1 h. Lower concentrations of cis-Pt(II) were effective but required longer incubations. The trans isomer was also tested and was found to be somewhat more efficient than cis-Pt(II) in this regard; 0.3 mM trans-Pt(II) for 1 h fixed nuclei to approximately the same extent as did 1 mM cis-Pt(II) for 1 h.

3.2. *Protein analysis*

In order to investigate the nature of these nuclear structures, the bound and unbound proteins were compared by gel electrophoresis. Since cis-Pt(II) partially fixes the nuclei, the unlinked proteins could easily be extracted from them with 1% SDS. Centrifugation at 100 × *g* for 5 min peileted the nuclear structures. Figure 2 shows that the SDS extract (bottom trace) contained histones in about the same amount as the control (top trace), but few if any non-histone proteins were present.

The proteins bound in the nuclear structures had to be released before analysis. Filipski et al. [10] had shown that thiourea reverses DNA–DNA crosslinks made by cis and trans-Pt(II). Since the bond between Pt(II) and DNA is presumably the same in a DNA–DNA crosslink and a DNA–protein crosslink, thiourea was used to release proteins from the nuclear structures. When thiourea was added to the nuclear structures in SDS, they rapidly disappeared and the solution became quite viscous, indicating that the DNA had remained intact. The proteins released by thiourea treatment were analyzed by gel electrophoresis and their profile was found to be virtually identical with the non-histone portion (fig.2, middle trace) of the control profile (top trace). No histones were present in this fraction; they seem to have all been removed by the SDS treatment prior to the thiourea treatment.

These nuclear structures, stable in SDS, seem to

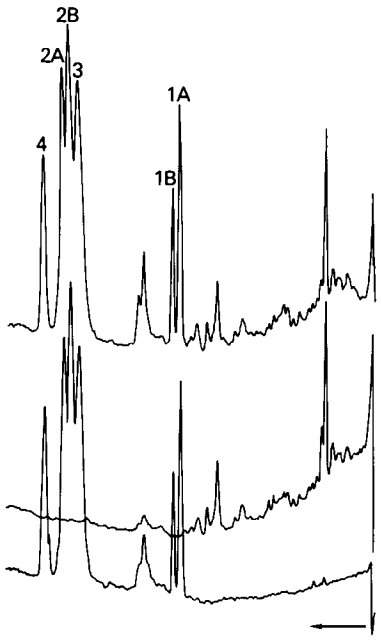

Fig.2. SDS gel analysis of proteins released from cis-Pt(II) treated nuclei. Unlabeled nuclei were treated as in fig.1. After washing to remove unreacted compound, the fixed nuclei were extracted three times with 1% SDS to remove any proteins that were not covalently bound. The pellet from the third SDS wash was suspended in 1 M thiourea and incubated for 1 h at 37°C. During this incubation the fixed nuclei dissolved and the solution became quite viscous. The traces record the density of protein stain. (*Top trace*) Control. Histones are denoted on this trace. (*Middle trace*) Proteins released from SDS pellet after thiourea treatment. (*Bottom trace*) Proteins in SDS extract before thiourea treatment.

have retained virtually all the non-histone proteins but virtually none of the histones. Only two components seemed to be common to both the bound and unbound fractions. Both migrated between H1B and H3, and were found predominantly but not totally in the histone fraction. The faster peak comigrated with the ubiquitin adduct of histone 2A. Ubiquitin is a protein with M_r ~8000 and is attached through its carboxy terminal to lysine 119 in H2A. The equilibrium between ubiquitin and H2A is rapidly reversible [13], suggesting that the ubiquitin is not tightly associated with the DNA. This protein is therefore a hybrid, part histone, part non-histone. Ubiquitinated H2A could have been crosslinked through its ubiquitin moiety and for that reason found partly in the non-histone fraction. Any crosslinking would probably have been through the ubiquitin moiety since H2A itself was not found in the nonhistone fraction. The other protein found in this region seemed to comigrate with HMG 1. Also we cannot exclude the possibility that other minor non-histone proteins, not detected by mass, might be present in both the bound and unbound fractions.

The results from the CsCl gradient show that protein and DNA were both present in the nuclear structures resulting from cis-Pt(II) treatment. The disappearance of these structures after thiourea treatment indicated that cis-Pt(II) crosslinks were involved in maintaining the structures in the presence of SDS or Sarcosyl. Because cis-Pt(II) can cause DNA–DNA, DNA–protein, and protein–protein crosslinks, we tested whether the protein molecules were essential in maintaining the nuclear structures. Proteinase K, a proteolytic enzyme that can digest protein in the presence of SDS, was added to the cis-Pt(II) treated nuclei in SDS. The nuclear structures rapidly disappeared, a result which indicated that at least a fraction of the non-histone protein was necessary for the maintenance of such structures.

4. DISCUSSION

This report shows that cis-Pt(II) can crosslink non-histone nuclear components so that nuclear structures are maintained in the presence of SDS. The finding that the histones can be quantitatively extracted indicates that the non-histone proteins are not just trapped in a gel network, but are covalently bound either to the DNA or to each other. To make a crosslinked structure that still maintains a nuclear morphology in SDS, it is necessary that at least some of the components be crosslinked at several sites.

Lippard and Hoeschele [14] studied the binding of cis and trans-Pt(II) to nucleosome cores which contained no non-histone proteins. They found that the cis isomer made a small amount of histone–histone and histone–DNA crosslinks, but only with incubation times considerably longer than the ones used in this report. We saw no indication of any histone crosslinking with cis-Pt(II) in nuclei under our experimental conditions, even though at the same time virtually all the non-

histone proteins were crosslinked into the nuclear structures.

What could be the molecular basis of the differential crosslinking of histones and non-histones? One possibility concerns the presence or absence of cysteine residues. Among the histones, only H3 has cysteine residues but these are buried and therefore non-reactive [15]. The possible presence of cysteine residues in the non-histones may be one factor leading to much faster Pt binding to these proteins. If most of the non-histone proteins are already involved in tubules, filaments, nuclear matrix, chromosome scaffold, nuclear membrane structures or other very large multisubunit complexes they may be very rapidly crosslinked by cis-Pt(II) to structures stable in denaturing solvents. There is some evidence that many if not most nucleoplasmic proteins may be weakly bound to nuclear sites, even though these may not be the ultimately functional ones [16]. Factors such as these could lead to the striking differences in reactivity between histones and non-histones described in this report.

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