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Cell-Surface-Associated Nucleic Acid in Tumorigenic Cells Made Visible with Platinum–Pyrimidine Complexes by Electron Microscopy

(cancer/cell surface DNA/neuraminic acid)

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ABSTRACT **Platinum-pyrimidine complexes have been used as the sole electron-dense stains for electron microscopy on 18 types of mammalian cells. These recently discovered complexes react avidly with nucleic acids *in vitro*, are antitumor agents, and are highly soluble in water. In cells, they are selective for regions rich in nucleic acid and make visible the cellular chromatin, nucleolus, and ribosomes. In addition, cells that are tumorigenic exhibit electron-dense patches at the plasma membrane; normal cells do not. The results of treatment of ascites sarcoma-180 cells with concanavalin A, nucleases, glycosidases, or a protease suggest that the electron-dense patches are DNA, with neuraminic acid involved in the surface binding.**

Platinum coordination complexes have been shown to be active antitumor agents in animals (1) and man (2). The first of this new class of drugs, *cis*-dichlorodiammineplatinum(II) is presently in phase II clinical trials in humans. Preliminary results indicate activity against a wide spectrum of cancers. The dose-limiting side effect of this drug is kidney toxicity. This interesting degree of success has led to the syntheses of large numbers of analogue structures for screening as potential second generation drugs, with the hope of finding coordination complexes with greater activity, less kidney toxicity, and increased solubility for administration in humans.

Our laboratory has recently developed a new class of coordination complexes formed by reacting *cis*-diaquodiammineplatinum(II) with various pyrimidines and substituted pyrimidines (3). These complexes are, at present, poorly defined chemically, and they are all amorphous. They exhibit, however, potent antitumor activity in a number of tumor-host animal screens; they have a low toxicity [e.g., median lethal dose (LD_{50}) $\approx$ 1000 mg/kg]; kidney toxicity is not the dose-limiting side effect; and solubilities range up to 50% (by weight) as solutions in water. These complexes are highly colored, with hues ranging over green, blue, and purple.

Early evidence from a number of laboratories has implicated the reaction of the platinum coordination complexes with DNA as the primary lesion in the cell responsible for the antitumor activity (4). It is well known, however, that many of the simpler complexes also react with proteins, particularly with the sulfur-containing amino acids and with histidines (5). The platinum–pyrimidine complexes interact avidly with nucleic acids, as has been shown in our laboratory (H. C. Pant and B. Rosenberg, personal communication). For example, the addition of dilute (2.5 mM) solutions of the platinum–pyrimidine complexes to DNA solutions causes rapid precipitation of deep blue stained DNA fibers. The reactions with RNA are somewhat slower, but still lead to the precipitation of blue stained RNA fibers. The stained and precipitated fibers of DNA contain about two tightly bound platinum atoms per phosphate. Rates of reactions of the platinum–pyrimidine complexes with proteins are slower by a factor of at least 100.

Since these complexes are so highly soluble, deeply colored, and specific in their interactions with nucleic acids, it was of some interest to determine if they would show the same selectivity in animal cells. Incubation of such cells with the platinum–pyrimidine complexes does lead to a fairly rapid uptake of the blue stain by the cells, which can be discerned in normal light microscopy, but it is difficult to see more than an intensification of blue color in the nuclear region of the cells. Electron microprobe studies for the presence of platinum in the stained cells also indicated a preferential accumulation in the cell nucleus.

Since these drugs contain the heavy metal platinum, it was probable that their presence in cells could be seen by electron microscopy, without treatment with additional heavy metal atoms. Our first studies indicated that the platinum–pyrimidine complexes are useable cytologic stains for electron microscopy. They further verified the selectivity of interactions of the stains with nucleic acids, since only chromatin, the nucleolus, and ribosomes in the cytoplasm were made visible by the stain (6, 7). When we expanded the number of different types of cells tested, we discovered an interesting new phenomenon, namely, the appearance of large patches of deeply stained material at the cell surfaces of tumorigenic cells. Such patches do not appear in normal cells or in transformed cells that are weakly or nontumorigenic. We report here the evidence showing the correlation between the appearance of cell surface stain using the platinum–pyrimidine complexes and the tumorigenicity of the cell types; and finally, the evidence in at least two instances suggesting that the stained surface sites are DNA. This is a preliminary communication and the full details will be presented elsewhere.

METHODS

Cells and Treatment. Cells were obtained from continual cell lines, primary cultures, or directly from animals. The cells in monolayer continuous culture were trypsinized at 37° until they detached from the incubation vessel surface. The cells in suspension continuous culture were centrifuged to a pellet and

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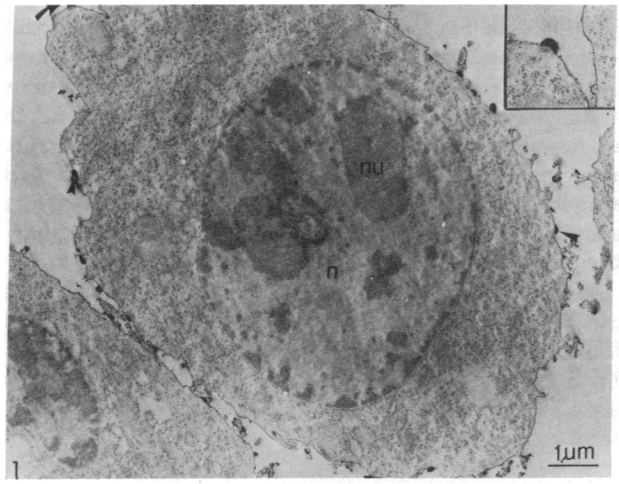

FIG. 1. Ascites sarcoma-180 cell treated with platinum-pyrimidine complex staining only the nucleolus (nu), chromatin material within the nucleus (n), and ribosomes. Note the presence of surface patches at the plasma membrane (*arrows*). One such patch (*curved arrow*) is enlarged (4×) as an inset for clarity.

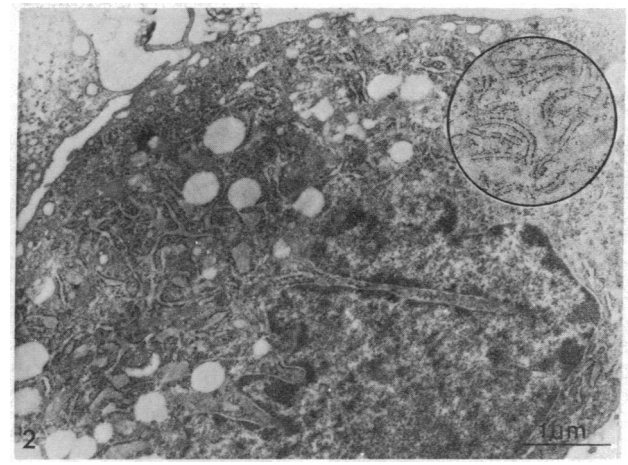

FIG. 2. Normal human fibroblast treated with platinum–pyrimidine complex showing the nuclear staining and the ribosomes. Note the absence of patches at the plasma membrane. Inset shows enlarged view (3×) of the endoplasmic reticulum outlined by the ribosomes.

resuspended in phosphate-buffered saline (pH 7.2) for further treatment. Primary cells were obtained immediately after animals were killed, by trituration and trypsinization. After the primary cell cultures were established, the cells were processed in a similar manner to the continual cell lines. Tissue culture conditions were incubation at 37° in an atmosphere of 5–10% CO_2 in 90–95% air at 100% humidity. Cells taken directly from the animals were obtained either surgically or by aspiration from the peritoneal cavity.

For most experiments, the cells were fixed and stained. However, some experiments involved treatment of the cells with proteins before they were fixed and stained. This pretreatment consisted of incubation of the cells as a suspension in 1% (w/v) solutions of the proteins in phosphate-buffered saline at 37°. Aliquots of the cell suspensions were taken at various times and centrifuged for removal of the protein solutions by decanting. They were washed twice with phosphate-buffered saline. Controls were maintained in phosphate-buffered saline throughout the incubation period.

All cells were fixed as a suspension at 4° for 1 hr in a 1% glutaraldehyde solution in 50 mM cacodylate buffer at pH 7.4. They were then stained for varying periods of time up to 1 hr in a 1% platinum–thymine (or uracil) complex in phosphate-buffered saline solution as a suspension at 4°. The cells were rinsed twice with a cold, equal volume mixture of acetone and water, and then dehydrated through a series of graded acetone/water solutions. After infiltration, the cells were embedded in Araldite 502 and sectioned for viewing directly under the electron microscope (Hitachi HU11E, operated at 75 kV). No other heavy metal postfixation treatment or counterstaining was performed.

Chemicals. The platinum–pyrimidine stains were freshly prepared in batches in our laboratory by R. G. Fischer and H. J. Peresie, and stored under reduced pressure. All batches were checked for similar quality by solubility and absorption spectroscopy criteria. All experiments included controls stained with the same batch. The stain solutions were freshly prepared and filtered through Whatman paper just prior to use. It is our experience that these stains are oxidized when exposed to air over long periods of time (weeks).

Culture media for the cells were of the following types: (*i*) KB monolayer: Eagle's minimal essential medium supplemented with 5% calf serum; (*ii*) KB suspension: minimal essential medium (Spinner modified) with 5% calf serum; (*iii*) AV_3 and V-79: minimal essential medium with 5% fetal calf serum; (*iv*) human fibroblasts: minimal essential medium with 10% human serum; (*v*) mouse liver, embryonic skin, chick embryo fibroblasts, and HeLa: minimal essential medium with nonessential amino acids and 10% fetal calf serum; (*vi*) myeloma MOPC-21: minimal essential medium (Dulbecco modified) with 10% horse serum; (*vii*) human lymphoblastoid: minimal essential medium (Chew modified) with 10% fetal calf serum; (*viii*) bovine testes: minimal essential medium with nonessential amino acids, 1 mM sodium pyruvate, 0.5% lactalbumin hydrolysate, and 10% fetal calf serum; (*ix*) rat epithelial skin (RESI): Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate, 0.05% TC yeastolate, and 10% lamb serum; (*x*) sarcoma 180: basal medium (Eagle's) with 2× amino acids and 10% fetal calf serum.

Sources of the proteins were as follows: trypsin (EC 3.4.21.4), Nutritional Biochemical Corp. (Cat. no. 1-300); hyaluronidase (EC 3.2.1.35), Sigma Chemical Co. (Cat. no. H-6254) type I lyophilized; neuraminidase (EC 3.2.1.18), Sigma Chemical Co. (Cat. no. N-2876) purified type V; RNase A (EC 3.1.4.22), Sigma Chemical Co. (Cat. no. R-5500) protease-free type XXII-A, and Nutritional Biochemical Corp. (Cont. no. 5659), crystallized 5×; DNase I (EC 3.1.4.5), Sigma Chemical Co. (Cat. no. DN-100) noncrystalline, RNase-free, or Worthington Biochemical Co. (Cat. no. D-575) crystallized, or Nutritional Biochemical Corp. (Cont. no. 1-111R) crystallized; concanavalin A, Sigma Chemical Co. (Cat. no. C-2010) salt- and carbohydrate-free grade IV.

RESULTS

Examination of cells stained with platinum–pyrimidine complexes under the electron microscope reveals an intense staining of chromatin, nucleolus, and ribosomes (Figs. 1 and 2). The most striking feature of these stains is their selectivity for nucleic acids while the membranous components are not

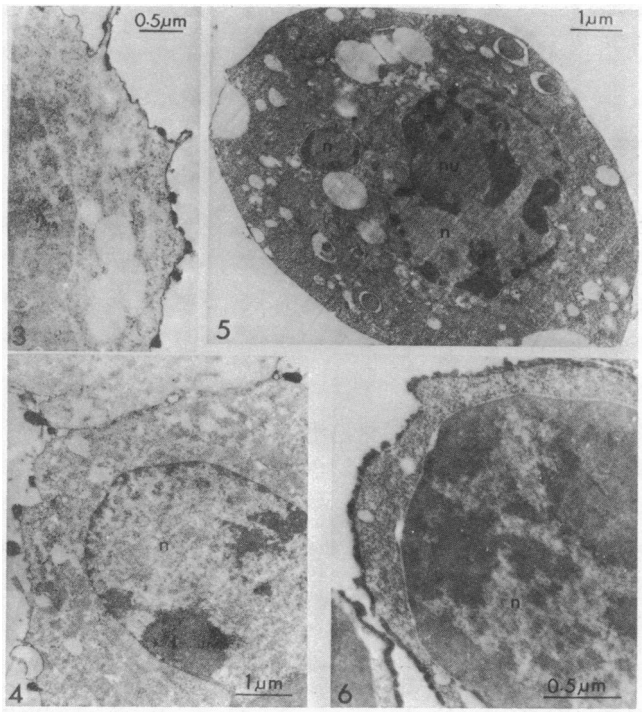

FIG. 3. Section of an L1210 cell showing patches at the plasma membrane. n, nucleus.

FIG. 4. KB cells showing surface patches at the plasma membrane. n, nucleus.

FIG. 5. Ascites sarcoma-180 cell pretreated with DNase I (3 hr). Note the absence of patches at the plasma membrane. n, nucleus; nu, nucleolus.

FIG. 6. (C57/Black × C3H) mouse thymocyte showing continuous binding of platinum–pyrimidine complex at the plasma membrane. n, nucleus.

revealed. This is best illustrated for the endoplasmic reticulum, where the RNA-containing ribosomes are aligned in double rows while the membranous structures cannot be discerned (Fig. 2, inset). These membranes, however, can be brought out when the sections are counterstained with membranous stains such as lead citrate. This contrast is further enhanced if double staining with uranyl acetate and lead citrate is used. These staining properties of the platinum–pyrimidine complex are reproducible in every animal cell examined to date.

Since these complexes exhibit antitumor activity, we stained a number of different tumorigenic cells to investigate any staining features unique to tumor cells. We discovered that tumorigenic cells displayed extremely prominent electron-dense patches at the cell surface. It was quite easy to note these patches on any random thin section examined. One such stained cell type, the ascites sarcoma 180, is shown in Fig. 1. Cells from the normal tissues tested did not display these patches, as is illustrated in Fig. 2. In Table 1, we list all of the different cell designations tested and compare the tumorigenicity of these cells with the appearance of densely stained surface patches in the electron micrographs. These cells were derived from a variety of origins and were maintained either *in vivo* in appropriate host animals or in tissue cultures. The cell designations fall into three major categories: (*i*) cells that are tumorigenic and display electron-dense surface patches, (*ii*) cells that are in continuous cultures and do not exhibit electron-dense patches, and (*iii*) cells derived from normal tissue that also exhibit no surface binding of the platinum–pyrimidine stain. The second category contains cell types that are nontumorigenic or are, at best, weakly tumorigenic.¶ It is apparent that cell surface binding of the platinum–pyrimidine complex occurs only on tumorigenic cells, regardless of the maintenance conditions, whether in tissue culture or derived from host animals.

We have also found that the morphology and pattern of the cell surface patches of electron-dense stains differ in each tumorigenic designation.This is illustrated in Figs. 3 and 4. Each tumorigenic cell designation that we have examined appears to have its own, subtly different, characteristic pattern.

The existence of surface areas in tumorigenic cell types that tend to bind the platinum–pyrimidine complex prompted us to attempt a characterization of these sites. We have subjected ascites sarcoma-180 cells to concanavalin A and various enzymatic pretreatments. The results of these pretreatments are summarized in Table 2. Of the enzymes used, only DNase I and neuraminidase pretreatment caused the almost complete disappearance of the electron-dense patches in all cells examined.

Concanavalin A pretreatment gave conflicting results: many cells had as many patches as the control group, while other cells had little or no patches in the same thin section.

RNase did not remove the surface binding areas of the platinum–pyrimidine complex after 4 hr of pretreatment. DNase I does remove these areas (Fig. 5). These results have been duplicated with RNase from two different suppliers and DNase I from three different suppliers (see *Methods*). The effects of these enzyme pretreatments, coupled with the nucleic acid selectivity of the stain, suggest that the patches are DNA bound to the cellular plasma membrane and that neuraminic (sialic) acid is involved in this binding.

Treatment of the cells with RNase also causes a diminution in the intensity of staining of the ribosomes and nucleolus. The chromatin staining is unaffected by this treatment as far as we have been able to determine. Conversely, DNase I treatment causes an intensity diminution of the chromatin relative to the nucleolus and ribosomes, as well as the eradication of the surface patches.

We have also done some preliminary pretreatments of myeloma (MOPC), rat epithelial skin (RESI) (8), and KB cells with DNase I and RNase. The RNase pretreatment had no effect on the electron-dense surface patches, consistent with our observations on the ascites sarcoma 180. The DNase I pretreatment markedly reduced these patches on the MOPC, but did not in the RESI or KB cells. Therefore, at this stage we cannot conclude that all tumorigenic cells are equally susceptible to removal of the patches by DNase I.

It is possible that the surface patches are either viruses or mycoplasma. Our observations of many cells lead us to believe

¶ We realize that it is difficult to characterize a cell line as tumorigenic since this requires that an immunologically competent host has been found in which a tumor will grow. To our knowledge, such a host has not been found for these four transformed cell lines. The American Type Culture Collection catalogue characterizes the KB line as used in tumorigenic studies, but does not so characterize the HeLa and the AV_3 lines.

TABLE 1. *Cells tested for the appearance of surface-associated stained patches in electron microscopy*

Cell designation	Animal origin	Maintenance conditions	Tumorigenicity	Stained surface patches
KB monolayer[a]	Human	Continuous monolayer	+	+
KB suspension[b]	Human	Continuous suspension	+	+
Sarcoma-180	Mouse	Continuous monolayer	+	+
Myeloma (MOPC-21)[c]	Mouse	Continuous suspension	+	+
Ascites sarcoma-180	Mouse	Mouse	+	+
Ehrlich ascites[d]	Mouse	Mouse	+	+
Leukemia (L1210)[d]	Mouse	Mouse	+	+
Embryonic skin (RESI)[e]	Rat	Continuous monolayer	+	+
Cervix HeLa[e]	Human	Continuous monolayer	− (?)	−
Amnion (AV_3)[f]	Human	Continuous monolayer	− (?)	−
Lymphoblastoid[f]	Human	Continuous suspension	− (?)	−
Lung (V-79)[f]	Hamster	Continuous monolayer	− (?)	−
Fibroblasts (normal)[b]	Human	Monolayer	−	−
Liver (normal)	Mouse	Monolayer	−	−
Kidney (normal)	Mouse	Monolayer	−	−
Thymus (normal)	Mouse	Monolayer	−	−
Embryonic skin (normal)	Mouse	Monolayer	−	−
Testes (normal)[e]	Bovine	Monolayer	−	−
Embryonic fibroblasts	Chicken	Monolayer	−	−

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that neither of these suggestions can account for the existence of such platinum–pyrimidine complex binding sites. The size, shape, and location of the patches do not resemble either microorganism. The patches do not occur in clusters outside the cell surface, as could be expected for mycoplasma. They often appear to be short segments forming an integral part of the external plasma membrane. The patches do not exhibit any fine structure. Classical viral type budding has never been observed. The electron-dense patches are of varied morphology on the same cell. In addition, mycoplasma checks, with either broth or [^{3}H]thymidine, have been performed on the myeloma (MOPC), RESI, and KB lines with negative results.

To our knowledge, the only other report of endogenous plasma membrane bound DNA is for a culture line derived from diploid human lymphocytes (9). We have done platinum–pyrimidine complex binding experiments with thymocytes extracted from the excised thymus of normal Swiss Webster or (C57/Black × C3H) mice with conflicting results. The (C57/Black × C3H) mouse derived thymocytes exhibited a thick, continuous electron-dense layer totally around the periphery of the cell (Fig. 6). Thymocytes from the Swiss Webster mouse were totally devoid of any cell surface electron-dense binding.

CONCLUSIONS

The evidence presented here leads us to the following conclusions: (*i*) Platinum–thymine and platinum–uracil complexes are useable stains for electron microscopy, and are selective for nucleic acids. (*ii*) With these stains, tumorigenic cells display electron-dense patches at the plasma membrane surface. Normal and weakly or nontumorigenic transformed cells do not display these patches. (*iii*) In at least two cases the platinum–pyrimidine complex binding sites at the surfaces of tumorigenic cells have been shown to be DNA.

At present, the evidence from the neuraminidase experiments suggests to us that neuramic acid is involved in the binding of the DNA at the surface.

Apart from the obvious questions raised by this work, if it is confirmed, we would feel remiss if we did not point to its potential applications, both in diagnosis and in providing a

TABLE 2. *Effect of protein pretreatment on the appearance of electron-dense patches on the cell surface of the ascites sarcoma-180*

	Protein pretreatment						
	None	Trypsin	Hyaluronidase	Neuraminidase	RNase	DNase I	Con A
Existence of surface patches	+	+	+	−	+	−	?

Cells were incubated at 37° in 1% protein in phosphate-buffered saline with the exception of trypsin, which was in a 0.25% solution. The incubation times were 1 hr for hyaluronidase, 1 hr for neuraminidase, 3 hr for RNase, 3 hr for DNase I, 1 hr for concanavalin A (Con A), and 0.5 hr for trypsin.

new rationale for chemotherapy in cancer. Indeed, it already has been reported that DNase is effective against lymphatic leukemia in mice (10).

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