

DIFFERENTIAL GENOMIC SUSCEPTIBILITY IN MALIGNANCY
CORRELATES WITH CHANGES IN ATATAT DNA-BINDING PROTEINS¹

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Summary. Accessibility to DNA in the nucleus is important for the regulation of gene expression and for the effect of DNA-modifying drugs. We have now studied differential genome susceptibility in normal melanocytes and the corresponding malignant melanoma. DNA hypersensitivity assays revealed a markedly lesser degradation in melanoma nuclei compared to that in melanocytes. Cross-linking of DNA to nuclear proteins by ultraviolet light showed a cell-type dependent inverse correlation of genomic susceptibility with binding of (dA.dT) (dA.dT) sequences, compared to that shown with (dG.dC) (dG.dC), regardless of methylation in cytosines. Exposure to cholera toxin partly reversed genomic susceptibility and increased DNA/protein cross-linking in melanocytes. In contrast, melanoma cells showed decreased DNA/protein interactions and greater genome susceptibility after exposure to cholera toxin or okadaic acid. Our data suggest that a molecular mechanism for differential genome exposure in cancer cells involves a modified expression of sequence-specific DNA-binding proteins. © 1991 Academic Press, Inc.

Genome regulation in mammalian cells can involve changes in nuclear organization in which transcriptionally competent genes are more exposed, in contrast to repressed genes (1,2).

However, within a specialized cell type, genomic organization may be altered by malignant transformation (2,3). Little is known about the mechanisms involved in differential chromatin structure which is likely to involve changes in DNA/protein interactions. Some of these structural alterations were previously evidenced by assays of differential DNase hypersensitivity (1,2), which showed that tissue-

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specific genes were more sensitive in the corresponding specialized cells. In malignant melanoma, we recently reported (4) that growth of the cells with bromodeoxyuridine which suppresses malignancy (5) and decreases invasiveness (6), is associated with an increased genomic susceptibility (4). These findings were compatible with the effects of cyclic AMP derivatives on Chinese hamster cells, in which partial *in vitro* reversion of transformed properties is also associated with greater genomic susceptibility (7). Although the melanoma hypersensitive DNA was physically isolated and used as a probe to demonstrate an enrichment of *c-myc* sequences (4), no report is available to date on a differential DNA/protein interaction as a mechanism for differential genomic susceptibility in malignancy. As a logical extension to our work with melanoma, we have now investigated some of the factors affecting genomic susceptibility in normal melanocytes that do not form tumors in syngeneic C 57/BL mice (8), for comparison with B16-BL6 melanoma which are highly malignant and invasive in the same host (9).

To detect differential and specific DNA/protein interactions in nuclei from these cells, we have used ultraviolet light induction of cross-linking between nucleic acids and proteins followed by nuclease digestion of unprotected nucleic acids and electrophoretic separation of cross-linked from uncrosslinked proteins under high resolution denaturing conditions (10). These assays when carried out with end-labelled polymers with sequence-specificity, have now allowed the detection of differential nucleoprotein interactions unequally expressed in normal and cancer cells.

MATERIALS AND METHODS

Cells and Tissue Culture.

a) Normal melanocytes: (Melan-A) are diploid pigmented cells derived from normal epidermal melanoblasts from embryos of C57 BL mice (8). These cells do not form tumors, show contact inhibition and a high degree of adhesion to substratum. To promote proliferation these cells require complete Dulbecco's medium and 10% fetal calf serum but have an additional requirement for 160 nM TPA (12-*O*-tetra decanoyl phorbol-13-acetate) (8).

b) Malignant Melanoma: BL6 cells were derived from B16 melanoma by a selection of a highly invasive variant that forms bladder and lung metastasis in C57 BL mice (9). These cells can be induced to undergo pigmentation *in vitro* like their normal counterparts (11), and were grown like melanocytes but without addition of TPA, since this is not required for their growth.

Preparation of Nuclear Proteins. Nuclei from normal melanocytes and melanoma cells were prepared from cells harvested

by detachment with PBS plus 1 mM phenyl methyl sulfonyl fluoride. Cells were then exposed to a hypotonic buffer lysed with non-ionic detergent in the presence of 5% sucrose and purified on a sucrose cushion(4).

Cross-Linking Experiments. Nuclei were extracted with buffer C (20 nM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and centrifuged at 15,000 xg for 10 min at 4 ° (12). The supernatant was diluted 1:4 with H₂O, and equal protein concentrations were used in each case (70 µg) in a 50 µl final volume containing 1 mM MgCl₂, 40 µM spermidine, 6 µg of competitor sonicated calf thymus DNA + 0.5 µg of the ³²P-end labelled polymers. The nucleic acids used for cross-linking in these studies ,were poly (dA.dT) poly (dA.dT), poly (dG.dC) poly (dG.dC), poly (dG.m⁵dC),poly (dG.m⁵dC), poly (dI.dC).poly (dI.dC) ,all from Pharmacia LKB Biotechnology. Aliquots of 0.5 µg of each one of these sequences were end-labelled with gamma-ATP³² and polynucleotide kinase using the NEK-006 Kit from New England Nuclear (Boston, Mass.) for use in cross-linking in the presence of competitor DNA. Nuclear extracts plus end-labelled and competitor DNA were exposed to U.V. light (400 µW/cm²) at 15 cm for 10 min on ice. Then 1 ug/µl of DNase I and 1.25 units/ul of micrococcal nuclease were added for 30 min at 37 ° C. Reactions were stopped adding EDTA to 20 mM and 5X mixture to give a final concentration of 2% SDS.0.1 M 2-mercaptoethanol,0.1 M Tris pH 6.8.for dissociation at 90 ° C for 3 min.

RESULTS

Differential Genome Susceptibility in Nuclei from Normal and Malignant Cells.

Nuclei from normal melanocytes and malignant melanoma were examined after exposure to 0.25 µg/ml of DNase I for 20 min..for comparison of their DNA with that prepared from the same cells embedded in agarose 0.5 % prior to lysis and deproteinization (4). Whereas the latter assay showed undegraded DNA at the top of the gel in both cell types, assays with nuclei exposed to DNase I showed a clearly greater degradation in genomic DNA from normal melanocytes (Fig1).

Okadaic Acid and Cholera Toxin Regulate Genome Susceptibility.

To learn about agents that influence genome susceptibility, subconfluent cultures were exposed for 2 hours to okadaic acid,a tumor promoter (13),which prevents dephosphorylation of proteins in serine/threonine residues(14). We also investigated the effect of cholera toxin,known to stimulate cyclic AMP levels in both

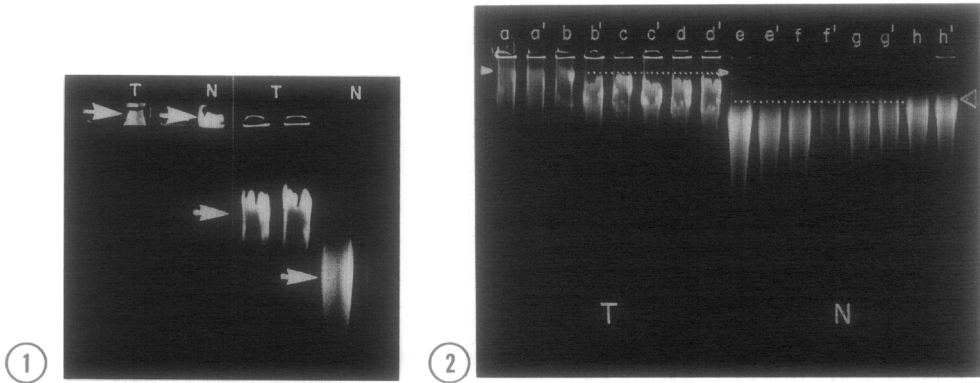


Fig. 1. Differential Nuclease Susceptibility in Nuclei from Melanocyte and Melanoma Cells. DNA from normal melanocytes (N) and malignant melanoma (T) were analyzed after deproteinization (4) of cells embedded in 0.5% agarose plugs or after exposure of nuclei to 0.25 $\mu\text{g}/\text{ml}$ DNase I for 20 min at 20° C. Arrows show that the embedded DNA remained at the top of the 0.8% agarose gel, in contrast to the unequal migration of DNA from nuclease-treated nuclei from melanocytes and melanoma cells.

Fig. 2. Okadaic Acid and Cholera Toxin Regulate Genome Susceptibility. Nuclei were prepared from: melanoma cells (T, a-d) or melanocytes (N, e-h). Lanes (a,a' and e,e') represent DNA from control nuclei; (b,b'; and f,f') after a 2 hour exposure to 10 ng/ml okadaic acid; (c,c' and g,g') after 1 hour with okadaic acid and an additional hour together with 100 ng/ml cholera toxin; (d,d' and h,h') after 1 hour with cholera toxin. Even lanes (') correspond to nuclei exposed to 0.1 $\mu\text{g}/\text{ml}$ DNase I for 10 min at 20° C.

melanoma and melanocytes (15). No degradation of DNA was detected in control melanoma nuclei compared to nuclei from cells exposed to okadaic acid or cholera toxin. However, in the latter case or when cells were jointly exposed to okadaic acid and cholera toxin, we detected a significant genomic susceptibility even in the absence of exogenous nuclease treatment.

A comparable assay with melanocyte nuclei showed an even greater endogenous DNA fragmentation activity (17,18), which presumably is stimulated by okadaic acid and cholera toxin in melanoma but appears constitutive in nuclear preparations from normal melanocytes. Nevertheless, nuclear DNA degradation was less evident in melanocytes exposed for 1 hour to cholera toxin, as shown by the slower mobility of its DNA compared to that of other melanocyte cultures (Fig 2, e-f).

Genome Susceptibility Correlates with Changes in specific DNA-Binding Proteins.

To investigate whether specific DNA-binding proteins were associated with genomic susceptibility, comparable aliquots of

nuclei from cells grown as described in the previous section were exposed to the 0.4 M NaCl solution C described under Methods, to solubilize nuclear components, for ultraviolet light-mediated cross-linking to identify proteins that contact DNA (10). Preliminary experiments with various sequence-specific polymers, showed no significant reactivity of nuclear proteins with end-labelled poly (dI.dC) (dI.dC), poly (dG.dC).(dG.dC), or poly (dG.m⁵dC).(dG.m⁵dC), in contrast with a greater interaction with poly (dA.dT) (dA.dT), when assayed under comparable conditions in the presence of competitor calf thymus DNA (not shown). Hence, we chose to investigate whether exposure to okadaic acid or cholera toxin which influenced genomic susceptibility in melanoma cells, (Fig 2) also had an effect on DNA-protein interactions. When comparable amounts of nuclear proteins were used in cross-linking, we detected a clearly greater cross linking in control melanoma nuclei which showed minimal genomic susceptibility. This was paralleled by a decreased DNA-protein cross-linking in nuclei from cells exposed to cholera toxin or okadaic acid. Although the reactions with melanoma nuclei differed quantitatively in an inverse manner with genome susceptibility, reactive areas were essentially seen within the 70 to 43 kd region as well as in faster migrating components (Fig 3, a-d). A parallel assay with melanocytes showed negligible cross-linking in nuclear proteins prepared from extracts with maximal genomic

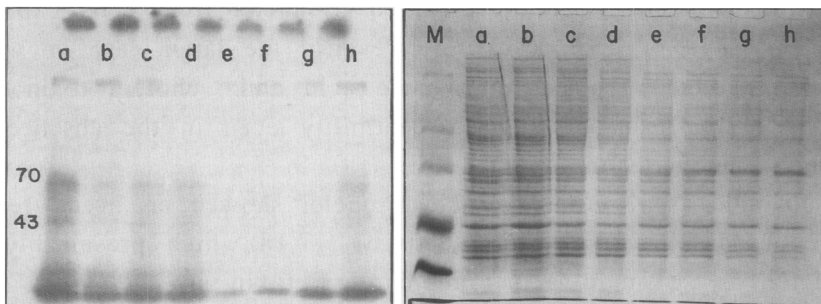


Fig. 3. Differential DNA/Protein Cross Linking in Melanocytes and Melanoma Cells.

Proteins extracted from nuclei like those used in Fig. 2 were used for ultraviolet light cross linking with end-labelled poly (dA.dT) (dA.dT) for nuclease treatment and electrophoretic analysis described in Methods (10). Left, autoradiography; right, Coomassie blue protein stain. a and b, proteins from control melanoma and melanocytes, respectively. b and f, proteins from melanoma and melanocytes exposed to okadaic acid, respectively. c and g, proteins from melanoma and melanocytes exposed to okadaic acid and cholera toxin, respectively. d and h, proteins from melanoma and melanocytes exposed only to cholera toxin, respectively.

susceptibility, in contrast with detectable cross-linking mainly in the 70 kd region in extracts from cholera toxin-treated cells in which genomic susceptibility was partly reversed compared to that seen in the other melanocyte nuclei. (Fig 2,e-f;3,e-f).

DISCUSSION

In agreement with the postulate that cancer is a disease in DNA organization(2,3), we have now shown that malignant melanoma reveal a clearly decreased genomic susceptibility compared to that of normal melanocytes. In addition, we have shown a novel effect of okadaic acid, an inhibitor of phosphoserine/phosphothreonine protein phosphatases(14) as a regulator of genome susceptibility and DNA/protein interactions. This report is the first to demonstrate that okadaic acid modifies DNA/protein cross linking and genome susceptibility in malignant melanoma. In the presence of cholera toxin by itself or with okadaic acid, genomic susceptibility was apparent even without DNase I addition, suggesting that melanoma cells exposed to these agents have increased levels of endogenous endonucleases(16,17), effect which is also assumed in normal melanocytes in which significant basal DNA degradation was observed. This effect was weakly reversed by exposure to cholera toxin, which also promoted poly (dA.dT) (dA.dT) interaction mostly with a 70 kd nuclear protein. The differential genomic susceptibility now reported correlates well with the cell-type dependent DNA/protein interactions evidenced by cross-linking. The reactivity now demonstrated with the alternating copolymer dA.dT.dA.dT which presents ATATAT sequences, suggests that the proteins cross-linked to this sequence may relate to the family of transcription factors TF that show affinity for TATA-box sequences which play a central role in eukaryotic promoter activation (18). The participation of proteins that bind to cyclic AMP-responsive elements (19) in genomic susceptibility, is also possible, since the toxin is known to elevate cyclic AMP levels in both melanoma and melanocytes (15) and now showed preferential effects on DNA/protein interactions and genomic susceptibility of melanocytes. In summary, we have now shown that malignant transformation of melanocytes is associated with changes in genome susceptibility paralleled by altered expression and regulation of sequence specific DNA binding proteins.

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