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Advances in Brief

Accessibility to DNA in Carcinoma Chromatin Is Promoted by Nanomolar Okadaic Acid: Effect on AT-Rich DNA Binding Proteins[1]

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Abstract

Differential accessibility to DNA in tumor cell chromatin is important to growth, differentiation apoptosis, and the targeting of DNA modifying drugs. We now show that endonuclease accessibility to DNA in the nuclei of A431 human carcinoma cells is increased within 90 min by nontoxic nanomolar levels of okadaic acid, known to inhibit protein phosphatase 2A. This genomic hypersensitivity was partly enhanced by joint treatment with epidermal growth factor and okadaic acid but did not appear without the latter. Nuclei with greater DNA susceptibility showed a decrease in M_r 80,000 DNA binding protein doublet specific for dAT-rich sequences concurrent with the "apparent" hyperphosphorylation of a M_r 70,000 nuclear matrix protein.

We propose that some of the tumor-promoting effects of okadaic acid may be partly associated with its ability to promote genomic susceptibility.

Introduction

Differential gene expression in mammalian cells can involve changes in nuclear organization, in which transcriptionally competent genes are more exposed than repressed genes, as evidenced by differential endonuclease hypersensitivity (1, 2). Within a specialized cell type, changes in chromatin structure may be associated with the differential phosphorylation of nuclear proteins that directly bind to DNA (3) or have an indirect role in DNA organization by influencing nuclear matrix assembly (4).

Tumor promotion can also be correlated with protein hyperphosphorylation (5) resulting from the effect of protein phosphatase inhibitors like okadaic acid (6-8). This compound may have a role in nucleosomal structure since it favors the phosphorylation of histone H3 (9), which suggests its potential to influence chromatin structure (9).

We have now used A431 human carcinoma cells responsive to EGF,[3] to investigate whether okadaic acid induces changes in endonuclease hypersensitivity in chromatin and whether this is modified by EGF. Since differential endonuclease accessibility to DNA in nuclei is a measure of decreased DNA/protein interactions, we have also investigated whether okadaic acid induces differential DNA/protein associations. This has been studied by UV light induction of cross-linking between nucleic acids and nuclear proteins followed by nuclease digestion of unprotected nucleic acids and electrophoretic separation of cross-linked proteins (10, 11).

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[3] The abbreviations used are: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate.

Materials and Methods

Cell Culture and Preparation of Nuclear Fractions. A431 human carcinoma cells were maintained in Dulbecco's medium containing 10% fetal calf serum. Nuclei were prepared from subconfluent cells exposed to hypotonic conditions and then lysed with Nonidet P-40 for nuclease susceptibility assays, as shown elsewhere (10).

DNA-Protein Cross-Linking by UV Light. Nuclei were extracted with buffer C (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 × *g* for 10 min at 4°C (10). The supernatant was diluted 1:4 with H_2O, and equal protein concentrations were used in each case (70 μg) in a 50-μl final volume containing 1 mM $MgCl_2$, 40 μM spermidine, and 6 μg of competitor sonicated calf thymus DNA plus 0.5 μg of the ^{32}P-end-labeled 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·m^{5}dC)·poly(dG·m^{5}dC), poly(dI·dC)·poly(dI·dC), all from Pharmacia LKB Biotechnology. Aliquots of 0.5 μg of each one of these sequences were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase using the NEK-006 Kit from New England Nuclear (Boston, MA) for use in cross-linking in the presence of competitor DNA. Nuclear extracts plus end-labeled and competitor DNA were exposed to UV light (440 μW/cm^2) at 15 cm for 10 min on ice. Then 1 μg/μl of DNase I and 1.25 units/μl of micrococcal nuclease were added for 30 min at 37°C (11). Reactions were stopped by adding EDTA to 20 mM and 5× the following 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 (10).

Electrophoretic Analysis of Phosphorylated Products. Endogenous nuclear protein phosphorylation was now carried out with intact nuclei for 15 min at 20°C in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 μM [γ-^{32}P]ATP. This was stopped with 20 mM EDTA followed by the extraction of soluble proteins with NaCl to a final concentration of 0.4 M. Nuclear residues were extracted with 1 M NaCl and exposed to DNase I (50 units/ml) for 18 h for analysis of nuclear matrix phosphoproteins. In all cases protein phosphorylation was assayed by the addition of a 5× dissocation mixture to give a final concentration of 2% SDS, 0.1 M β-mercaptoethanol, 0.1 M Tris-HCl (pH 6.8), 2 mM phenylmethylsulfonyl fluoride for dissociation at 90° for 3 min and analysis on 7.5% SDS-polyacrylamide electrophoresis gels. Subsequently, the gels were fixed in 30% isopropanol:10% acetic acid and vacuum-dried, and the results were visualized by autoradiography on X-ray film.

Results

Okadaic Acid Promotes Endonuclease Susceptibility in Nuclei. Since A431 has an unusually high number of receptors for epidermal growth factor (12), we tested the effect of okadaic acid not only in control cells but also in cells exposed to EGF. To optimize the response to EGF, we used subconfluent cells in medium from which calcium was removed (13), to prevent the known effect of a calcium-activated protease that cleaves the native M_r 170,000 EGF receptor (14).

To assay the effect of okadaic acid on endonuclease accessibility to DNA in chromatin, nuclei were prepared from: (*a*) untreated cells; (*b*) cells treated with 8 nM of okadaic acid for

90 min; (*c*) those treated with okadaic acid as indicated previously, followed by a 15-min exposure to 50 ng/ml of EGF; (*d*) cells exposed only to EGF. The corresponding nuclei were exposed for 5 min to 0.2 μg/ml of DNase I in 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, and 0.005 M β-mercaptoethanol. Endonuclease treatment of nuclei was stopped with 20 mM EDTA, and DNA was deproteinased with 100 μg/ml proteinase K, 1% SDS, 20 mM EDTA for electrophoretic analysis in 1% agarose gels. This assay showed no significant endogenous DNA degradation in any nuclei (Fig. 1). However, preferential nuclease-mediated DNA cleavage was evident in nuclei from cells exposed for 90 min to 8 nM okadaic acid, in an effect further stimulated by joint EGF okadaic acid treatment (Fig. 1, *Lanes C* and *D*).

In contrast, cells treated only with EGF for 15 min yielded nuclei with a somehow decreased endonuclease susceptibility compared to that seen in nuclei from untreated cells (Fig. 1, *Lanes A* and *B*).

Susceptibility to Nucleases Induced by Okadaic Acid Correlates with a Decrease in a M_r 80,000 ATATAT Nuclear DNA-binding Protein. This was analyzed by studying UV light induction of cross-linking between end-labeled sequence-specific DNA and nuclear proteins, followed by nuclease digestion of unprotected nucleic acids and electrophoretic separation of cross-linked from uncross-linked proteins under high-resolution denaturing conditions (11). Since DNA organization may involve association with the nuclear matrix and the nuclear scaffold shows preferential binding to tracts of dA·dT-alternating sequences (15, 16), we investigated whether genomic susceptibility induced by okadaic acid involved a change in ATAT DNA-binding proteins. For these assays we also used end-labeled polymers enriched in either (dG·dC)(dG·dC) or (dA·dT)(dA·dT) alternating sequences. The corresponding analysis revealed essentially no binding of nuclear proteins with (dG·dC)(dG·dC) or (dI·dC)(dI·dC) sequences (not shown).

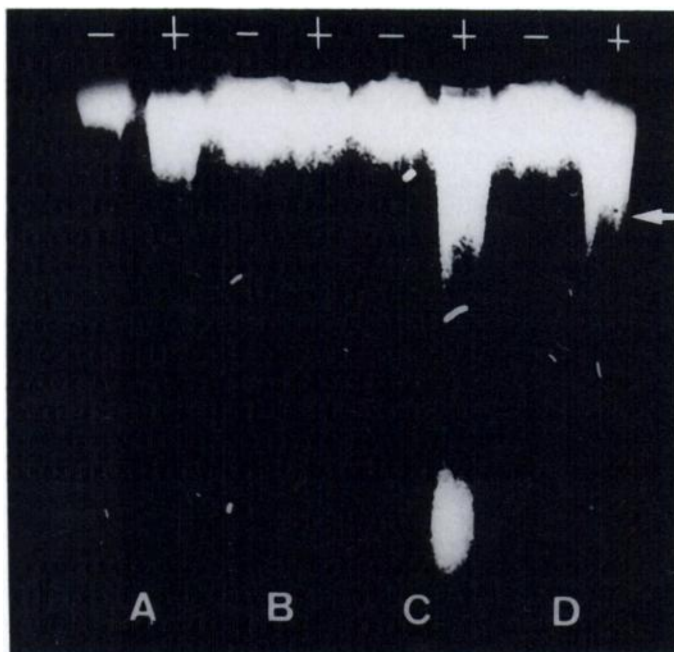

Fig. 1. Okadaic acid promotes susceptibility to DNase I. Nuclei from control cells (*A*), cells treated with EGF (*B*), cells treated with EGF plus prior exposure to okadaic acid (*C*), or cells exposed only to okadaic acid (*D*) were used for DNA preparation and agarose electrophoretic analysis, either as such (–) or after brief exposure to DNase I (+), for UV detection following ethidium bromide staining.

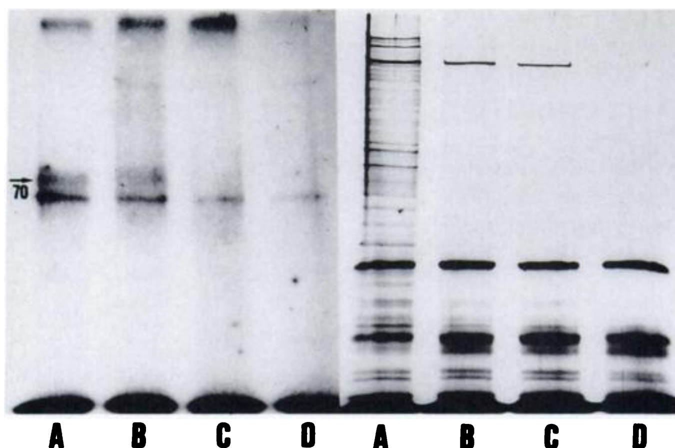

Fig. 2. Effect of okadaic acid on DNA/protein cross-linking. Comparable protein concentrations from nuclei were used for UV cross-linking with end-labeled (dA·dT)(dA·dT) for nuclease treatment and analysis as described under "Materials and Methods." The autoradiography with the *arrow* shows (*from left*) a doublet around the M_r 80,000 region in control (*A*) and EGF-treated (*B*) cells. In contrast, these are decreased in nuclear proteins from cells treated with EGF plus okadaic acid (*C*) or in the corresponding samples from cells treated with okadaic acid (*D*). The profile *to the right* shows that the corresponding protein concentrations used for the UV cross-linking were similar in all samples, as depicted by the Coomassie blue stain.

However, a significant but differential binding was detected following the cross-linking of ^{32}P-end-labeled (dA·dT)(dA·dT) polymer, in the presence of competitor calf thymus DNA. This showed that a 90-min exposure to okadaic acid was associated with a decrease in a M_r ~80,000 nuclear protein doublet with affinity for ATATAT-rich DNA sequences (Fig. 2, *right lanes*) in comparison to that seen with comparable protein concentrations of controls without okadaic acid (Fig. 2, *left lanes*).

Okadaic Acid Promotes the Phosphorylation of a M_r 70,000 Protein Associated with the Nuclear Matrix. Since we showed above that okadaic acid promoted a decrease in DNA-binding proteins with affinity for ATAT sequences, which are believed to mediate binding to the nuclear scaffold (15, 16), we investigated the possibility that this was paralleled by changes in proteins associated with the nuclear matrix.

For this, we chose nuclear matrix proteins from cells exposed jointly to okadaic acid and EGF, for comparison with those from cells treated with only EGF or those from untreated cells, since these gave the maximal difference in endonuclease susceptibility assays shown in Fig. 1.

The protein electrophoretic profile of nuclear matrix proteins from control cells differed considerably from that of EGF-treated cells. However, although the corresponding polypeptides from EGF-treated cells were quite similar to those of EGF-treated cells pretreated with okadaic acid, only the latter revealed the greater phosphorylation of a M_r 70,000 component (Fig. 3). This also correlates with the altered genomic organization induced by okadaic acid in A431 cells, as implied from its promotion of endonuclease susceptibility in nuclei (Fig. 1).

Discussion

We have now shown that the organization of DNA within the nucleus is altered in human carcinoma cells by short-term exposures to nanomolar levels of okadaic acid, known to preferentially inhibit protein phosphatase 2A (5, 7). These changes were reflected by the greater endonuclease susceptibility in the chromatin of cells treated with okadaic acid, which correlated with a decrease in a M_r 80,000 AT-rich DNA binding protein and with an apparent hyperphosphorylation of a M_r 70,000

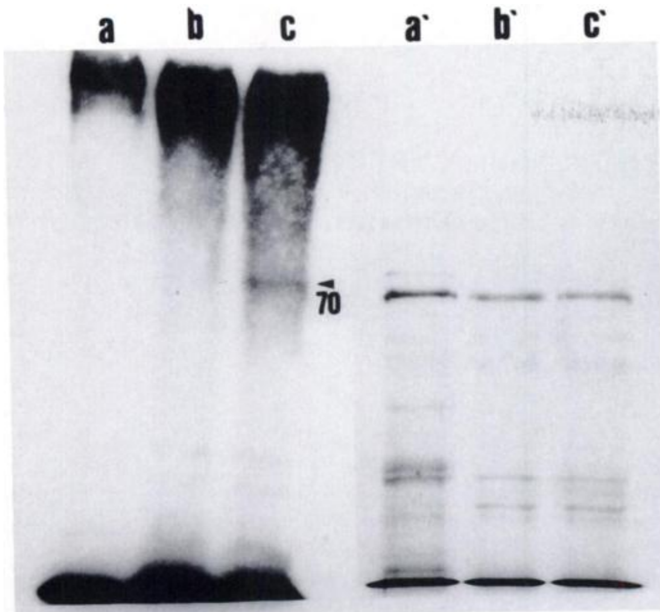

Fig. 3. Okadaic acid increases the phosphorylation of a M_r 70,000 protein associated with the nuclear matrix. Nuclear matrices from control cells (*a, a'*) and cells treated with EGF (*b, b'*) or with EGF plus okadaic acid (*c, c'*), as shown in Fig. 1, were examined for protein profile (*right*) or for endogenous protein phosphorylation (*left*). *Arrow*, position of the M_r 70,000 protein whose labeling is increased by okadaic acid.

nuclear matrix protein. The latter phenomenon is compatible with the expected effect of okadaic acid as an inhibitor of protein phosphatases (5–7) but also suggests that prolonged phosphorylation of specific nuclear matrix proteins may lead to changes in proteins recognizing AT-rich sequences that bind DNA to nuclear matrix attachment regions (15, 16).

Since the 3-dimensional organization of DNA within the nucleus is dictated in part by DNA interactions with the nuclear matrix, the okadaic acid induction of a differential organization of DNA in the nucleus may partly explain its tumor-promoting effects, since recent studies have also suggested that modifications in nuclear structure may represent preneoplastic stages in multistep carcinogenesis (17).

Moreover, the observation that joint exposure to okadaic acid and epidermal growth factor leads to greater changes in endonuclease susceptibility implies that tumor-promoting effects may be more pronounced in conjunction with growth factors. Our results showing greater susceptibility to exogenous endonuclease treatment, after short-term exposure to nanomolar levels of okadaic acid, could explain reports indicating that treatment of a number of cell types with okadaic acid in the 100–1000 nM range induces morphological changes typical of apoptosis (18). The latter could result as well from a change in the interaction of DNA with the nuclear matrix, leading to greater access of endogenous endonucleases to DNA.

We postulate that low doses of okadaic acid, which preferentially inhibit protein phosphatase 2A could lead to greater nuclear matrix ser/thr phosphorylation (5–7) and to a change of DNA organization in chromatin. Prolonged treatment or higher doses of okadaic acid may allow a greater risk of DNA fragmentation, leading to cell death or to a greater risk of DNA damage in the surviving cells, which would partly explain why okadaic acid acts as a tumor promoter.

Nevertheless, since okadaic acid has the potential to change accessibility to DNA in chromatin, it may be useful to study its effect and that of functionally similar nontumorigenic analogues, in conjunction with other DNA-modifying analogues in experimental chemotherapy with other human carcinoma cells.

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