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Effect of Nicotinamide on 12-O-tetradecanoyl-phorbol-13-acetate Exposed Mouse Skin Endonuclease Activity and DNA Synthesis [1]

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Nicotinamide (NA), a relatively nontoxic compound, has been shown to inhibit tumor development, induce differentiation, increase the sensitization of the anticancer drug resistant cancer cells and is being used in different skin ailments. But there are not many reports on its mechanism of action. Here we report that NA induced endonuclease activity. This endonuclease induction by NA appeared to be dose dependent and a function of time. As evident by the use of modifiers of DNase I, this endonuclease appeared to be like DNase type I. Increased [3H] thymidine incorporation in DNA in the presence of NA is possibly a consequence of increased 3-OH 'nicks due to increased DNA fragmentation by increased endonuclease activity. The present results would be of help in the better understanding of the mechanism of NA action and its improved use in cancer control.

INTRODUCTION

Nicotinamide (NA) is a well-established drug which is relatively nontoxic in both animals and humans (Horsman, Chaplin and Brown, 1987; Zackhein *et al.*, 1981) and has been used in the treatment of psoriasis (Zackhein, 1978), pellegra (Green, 1970) and schizophrenia (Greenbaum, 1970). It has also been shown that NA suppresses urethane initiated malformation in mice (Gotoh *et al.*, 1988), increases radiation sensitivity in several tumor models (Jonsson *et al.*, 1985), inhibits growth of transplanted tumors (Gotoh, Nomura, and Hasegawa, 1993) and inhibits chemically induced mouse skin tumor development (Ludwig *et al.*, 1990). However, the mechanism of action of NA is not well established. It may partially be ascribed to its inhibitory effect on the nuclear enzyme poly (ADP-ribose) synthetase which has been implicated in the cell differentiation process (Otonkoski *et al.*, 1993).

We and others have shown that NA suppresses skin tumor promotion following a two stage tumorigenesis protocol (Ludwig *et al.*, 1990; Gupta, 1999). We have also shown the effect of NA on the marker events of proliferation and differentiation (Gupta, 1999). 12-O-Tetradecanoylphorbol-13-acetate (TPA) is the most effective skin tumor promoter which alters the cascade of events (Diamond, 1984). While inducing the clonal expansion of cells, it inhibits the natural process of cell death, the programmed cell death or apoptosis (Wright, Zhong and Larrick, 1994). In order to make better use of NA as antitumor agent

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and to understand its mechanism of action, we carried out the present study. Here we report the effect of NA on Ca^{2+} Mg^{2+} dependent endonuclease and DNA synthesis in epidermis from TPA exposed mouse.

MATERIALS AND METHODS

Animals

We used swiss albino mice from the inbred colony at ITRC, Lucknow. Animals were 4-6 weeks old and weighed between 10g and 12g. They were fed with synthetic pellet diet and water *ad libitum*. Animals were shaved on the back in the interscapular region using surgical clippers two days before the start of experiment and were selected for the study only in their resting phase of hair growth.

Chemicals

TPA, NA and dexamethasone were purchased from Sigma Chemical Co., USA. [3H] Thymidine (sp. activity 18.5 Ci/m mol) was procured from Bhabha Atomic Research Centre, Mumbai, India. Plasmid PUC18 DNA was obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. Rest of the chemical and materials used were of molecular biology and analytical grade.

Animal Treatment

Animals were treated once topically in the interscapular region by acetone, TPA and or NA. Acetone was used as vehicle. The dose of TPA was 10 μg/animal in 0.1 ml acetone for all the experiments, whereas the duration of TPA treatment and the dose of NA varied from experiment to experiment, and are stated in the Figure legends. NA was also applied topically on the same region immediately after the application of TPA.

Preparation of Nuclei for Endonuclease Assay

Nuclei were purified from mouse epidermis by a modification of a method described by Rebeiro and Carson (1993). Tissue was homogenized in 10 mol/L Tris HCl pH 7.5 containing 1 mmol/L $MgCl_2$ and 0.5 mmol/L DTT (Buffer A) and supplemented with 0.32 mol/L sucrose. Homogenate (15%) was centrifuged at 10,000 × *g* for 30 min and pellets were resuspended in buffer A containing 1.7 mol/L sucrose, homogenized and centrifuged as before. This step was repeated once more, and the new pellet was resuspended in buffer A containing 0.32 mol/L sucrose and 0.2% Triton and centrifuged as before. The nuclear pellet was resuspended directly in 10 mmol/L Tris pH 7.5 containing 1 mmol/L EDTA, 0.5 mmol/L DTT and 100 mmol/L NaCl. This suspension (2-4 mg prot./ml) was used as a source of endonuclease enzyme. Protein was estimated following Lowry's method (Lowry *et al.*, 1951).

Endonuclease Assay

Endonuclease activity was determined using the plasmid degradation assay. Activation of endonuclease can be detected by plasmid degradation (Rauch *et al.*, 1997). 30 μl of re-

action mixture contained 1 μg supercoiled plasmid PUC 18 DNA as substrate, 20 mmol/L Tris acetate pH 7.4, 2 mmol/L $CaCl_2$ and 2 mmol/L $MgCl_2$. Reaction was started by adding 5 μg of nuclear protein as a source of endonuclease. After 90 min of incubation at 37℃, the reaction was stopped by adding 100 mmol/L EDTA. The incubation mixture was loaded on a 1% agarose gel and electrophoresed in Tris (89 mmol/L)-borate (89 mmol/L)-EDTA (2.5 mmol/L) buffer of pH 8.0. Gel was stained with ethidium bromide, visualized under UV transilluminator and photographed.

DNA Synthesis

DNA synthesis was followed by *in vivo* [3H] thymidine incorporation into DNA (Gupta and Mehrotra, 1992). Animals were treated with TPA and/or NA. One hour before sacrificing the animals, 30 μCi of [3H] thymidine was administered intraperitoneally. Animals were sacrificed and epidermal DNA was measured in acid insoluble fraction as described by Burton (1956) following diphenylamine method. Radioactivity was also counted in the same fraction. [3H]- Thymidine incorporation was expressed as cpm/μg DNA.

RESULTS

Effect of NA on Endonuclease Activity

Animals were treated topically once with 10 μg of TPA followed by topical application of acetone or 6 mg NA. Animals were sacrified at 1, 2, 4, 8, 16 or 24 h. After the treatment and epidermal nuclei were prepared as mentioned. We looked for the status of endonuclease, a Ca^{2+}, Mg^{2+} dependent enzyme responsible for the DNA fragmentation during programmed cell death. Effect of NA and or TPA on endonuclease activity are shown in Fig. 1. NA induced the TPA inhibited enzyme activity at all the time points. Fig. 1 clearly shows that NA induced the endonuclease activity maximum at 1 h after the treatment. This indicated that the effect of NA on endonuclease is an early event and is a function of time.

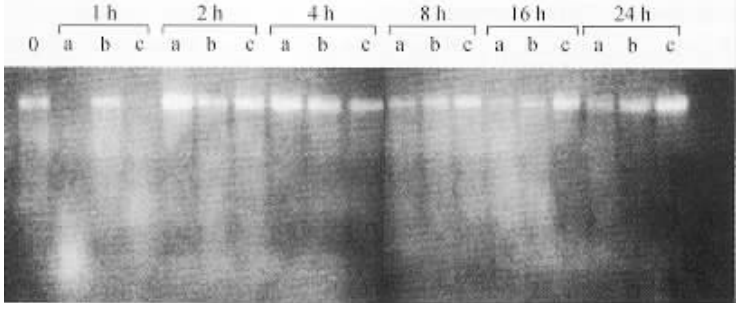

FIG. 1. Time dependent effect of NA and/or TPA on mouse skin endonuclease activity. Animals were treated topically once with 6 mg NA in presence or absence of 10 μg TPA. Animals were sacrificed at different times as indicated. 0 = Vehicle control, a = NA 6mg, b = TPA 10μg, C = NA 6mg plus TPA 10μg.

Dose Dependent Effect of NA of Endonuclease Activity

In order to see whether the effect of NA on endonuclease is dose dependent, we used 0.1, 1, 3, 6 or 12 mg of NA for topical application in presence of absence of TPA and assayed the epidermal endonuclease activity as mentioned. The results are shown in Fig. 2. NA induced the TPA inhibited endonuclease activity. Degradation of plasmid DNA was increased at all the concentrations of NA but the maximum effect was observed at the highest dose of NA used i.e. 12 mg/mouse.

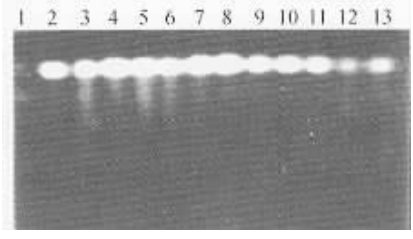

FIG. 2. Dose dependent effect of NA on TPA inhibited mouse skin endonuclease activity. Animals were treated topically once with 10 μg TPA in presence or absence of different doses of NA. Same doses of NA were also applied alone to the other animals. All the animals were sacrificed at 1 h after the treatment. 1 = Reaction mixture without enzyme; 2, 3 = TPA 10μg; 4 = NA 0.1 mg, 5 = TPA + NA 0.1mg; 6 = NA 1mg; 7 = TPA + NA 1mg; 8 = NA 3mg; 9 = TPA + 3mg NA; 10 = NA 6mg; 11 = TPA + NA 6mg; 12 = NA 12mg; 13 = TPA + NA 12mg.

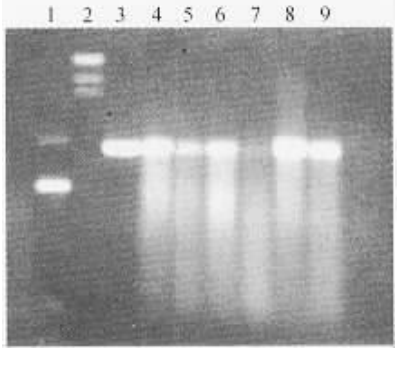

FIG. 3. Comparative effect of NA and dexamethasone on TPA altered mouse skin endonuclease activity. Animals were treated with 6 mg NA or 10μg dexamethasone along or in combination with acetone or 10 μg TPA. 1 = Reaction mixture without enzyme, 2 = phase Hind III digest, 3 = PUC 18, 4 = TPA 10 μg, 5 = Vehicle treated control, 6 = NA 6 mg, 7 = TPA 10 μg + NA 6 mg. 8 = Dexamethasone 10 μg, 9 = TPA + Dexamethasone.

We compared the effects of NA and dexamethasone, a known inducer of apoptosis on endonuclease induction. NA (6mg), compared to dexamethasone (10μg), had greater effect on TPA inhibited endonuclease activity, as shown in Fig. 3.

In vitro Effect of NA on Endonuclease Activity

NA induced endonuclease activity was found to be dependent on the duration of treatment and dose of NA used. To determine at what stage this induction of enzyme occurred and what were the associated changes, we prepared the enzyme from control (acetone treated) and TPA treated skin. The enzyme assay was done in presence of different concentrations of NA. The NA concentrations used were 1, 5, 10, 20 or 40 mmol/L. The results are shown in Fig. 4. We noticed that NA induced the enzyme activity in both control and TPA treated samples but the effect was more profound in TPA treated samples. These results indicate that NA increased the activity of the existing enzyme may be by post-translational modification. These results also indicate preferential effect of NA on endonuclease modification for TPA exposed skin.

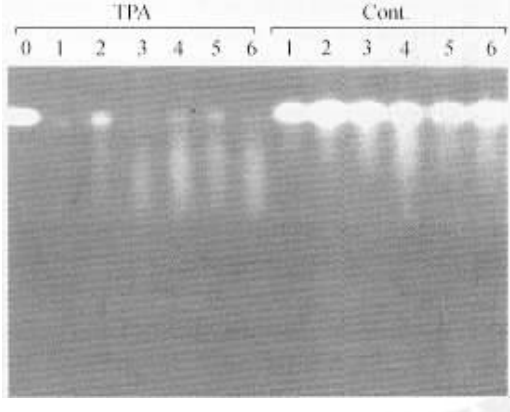

FIG. 4. *In vitro* effect of NA on TPA exposed and unexposed mouse skin endonuclease activity. Animals were treated topically once with 10μg TPA or acetone and were sacrificed after 1 h of treatment. 0 = 0mmol/L; in TPA-treated group 1, 2 = 1mmol/L NA; 3 = 5mmol/L NA; 4 = 10 mmol/L NA; 5 = 20 mmol/L NA; 6 = 40 mmol/L NA. In control group 1 = 0mmol/L NA; 2 = 1 mmol/L NA; 3 = 5mmol/L NA; 4 = 10mmol/L NA; 5 = 20mmol/L NA; 6 = 40 mmol/L NA.

Effect of Modifiers on Endonuclease Activity

To determine the type of NA induced endonuclease, we looked for the effect of known modifiers of DNase I on endonuclease activity.

We used the enzyme preparation obtained from NA and TPA treated mouse. The modifiers used for the study were spermine, spermidine, putrescine, $ZnCl_2$, EDTA and EGTA at the concentrations mentioned in the legend of Fig. 5. Spermine, $ZnCl_2$ and EDTA inhibited the enzyme activity when incubated with the assay mixture whereas EGTA and putrescine induced the enzyme activity.

Spermidine showed little effect on enzyme activity. The similarity of effects of these compounds has been reported by other researchers using DNase I preparation. Hence we

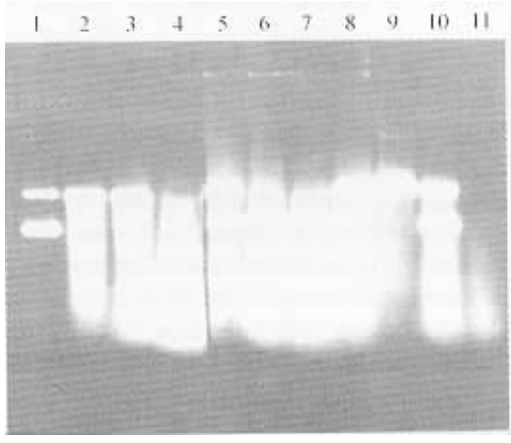

FIG. 5. Effect of modifiers of DNase I on TPA/NA exposed mouse skin endonuclease activity. Enzyme was prepared from mouse skin exposed for 1h to 10μg TPA or TPA + 6mg NA. 1 = Blank, 2 = Control, 3 = 10μg TPA, 4 = TPA + 6mg NA, 5 = spermine 1mmol/L, 6 = Spermidine 1mmol/L, 7 = Putrescine 1 mmol/L, 8 = $ZnCl_2$ 15μmol/L, 9 = $ZnCl_2$ 40μmol/L, 10 = EDTA 20mmol/L, 11 = EGTA, 20mmol/L. The same enzyme preparation was used in lane No. 4-11.

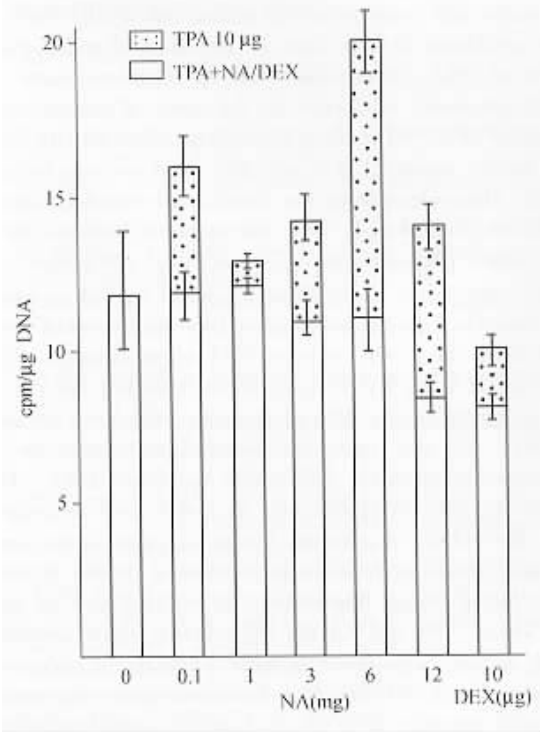

FIG. 6. Effect of NA on TPA altered [3H] thymidine incorporation in mouse skin DNA. Values are $\bar{x} \pm s$ obtained from 3 experiments.

conclude that the NA induced the endonuclease which is more or less similar to DNase I, type.

Status of DNA Synthesis in Presence or Absence of NA

In order to see the effect of NA on TPA induced DNA synthesis, we followed [3H] thymidine incorporation into mouse epidermal DNA exposed to TPA in the presence or absence of different concentrations of NA. We did not notice any inhibition of thymidine incorporation by NA, but as shown in Fig. 6, nicotinamide increased the thymidine incorporation in epidermal DNA from TPA exposed mouse. The induction was more or less dependent on the dose of NA used. This observation is different from that of other inhibitors of tumor development where they inhibited the thymidine incorporation into epidermal DNA from TPA exposed mouse. The increase in thymidine incorporation may possibly be due to the increase in 3′-OH nicks in DNA which occurs when DNA is acted upon by endonuclease and is capable of thymidine incorporation. Our results showed that dexamethasone, a known inducerof apoptosis, also increased the thymidine incorporation into epidermal DNA from TPA exposed mouse.

DISCUSSION

We wanted to investigate how the suppression of skin tumor promotion by NA takes place. Does NA modulate any event related to natural cell death ?Since the internucleosomol cleavage of DNA is considered to arise from the activation of endonuclease (s) and forms a characteristic pattern of DNA cleavage into 180 bp oligonucleosome integer fragments, a probable indicator of apoptosis, we looked for the status of endonuclease activity after NA treatment in presence of TPA. NA induced the endonuclease activity inhibited by TPA. This increase in enzyme activity appeared to be an early event and was found to be dependent on the dose of NA used. The endonuclease was found to be smimlar to that of DNase I, as far as the effect of modifiers is concerned. Zinc and spermine inhibited the induced enzyme activity whereas spermidine, putrescine or EGTA increased the enzyme activity. It appeared that the Ca^{2+}, Mg^{2+} dependent endonuclease induced by NA is more or less similar to DNase I and not DNase II. This is also supported by the increased thymidine incorporation into DNA. DNase I forms the 3′-OH nicks in DNA where radioactive thymidine gets incorporated where as DNase II forms 5′-OH nicks where thymidine can not be incorporated. Increased thymidine incorporation into DNA of apoptotic cells have already been reported (Otonkoski *et al.*, 1993). We also report that dexamethasone, a known inducer of apoptosis, increased the thymidine incorporation into mouse epidermal DNA. This explains the increased incorporation of [3H] thymidine and its involvement in programming the cell for their natural death. We did not observe any ladder formation in our study under any condition (data not shown). In fact, it is doubtful whether double stranded internucleosomol cleavage of DNA to 180 bp integer fragments is an essential step in the apoptosis (Tomei, Shapior, and Cope 1993). We did not see DNA ladder either because there is no ladder formation or if there is any, it is not detectable. Activity of endonuclease is believed to function after the phagocytosis of cells which have undergone programmed cell death. This supports the idea that degradation of DNA to oligonucleosome fragments is a late event and that in mammalian epithelial and mesenchymal cells it is either similarly late or is absent (Oberhammer *et al.*, 1993). Oberhammer *et al.* (1993) have already reported that the appearance of a DNA ladder is not mandatory in an apoptotic cell and that this appears to be

a late event, not coincident with chromatin condensation. DNA fragmentation, however, does not represent a constant feature of apoptosis and thus is not an absolute requirement for cell death (Lockshin and Zakeri, 1991).

We infer that probably NA programs the system for their natural death by inducing the DNase I type endonuclease. In other laboratories, ongoing attempts to identify and purify the endonuclease (s) responsible for the DNA cleavage are driven by the idea that the signals for cell death may be transduced directly to the endonuclease.

Now the mechanism of action of NA as inhibitor of tumor promotion may at least partially be ascribed to its inducing effect on endonuclease which has been implicated in cell death. Since the upcoming strategy in cancer treatment/prevention is to induce death of preneoplastic or malignant cells through apoptosis, NA, as shown here, may be a better candidate for the detailed study in cancer prevention alone or in combination with other tumor inhibitory agents.

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