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Inhibition of plasma membrane NADH oxidase activity and growth of HeLa cells by natural and synthetic retinoids

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Abstract

Several retinoids, both natural and synthetic, were evaluated for their ability to modulate NADH oxidase activity of plasma membranes of cultured HeLa cells and the growth of HeLa cells in culture. Both NADH oxidase activity and the growth of cells were inhibited by the naturally-occurring retinoids all *trans*-retinoic acid (tretinoin) and retinol as well as by the synthetic retinoids, *trans*-acitretin, 13-*cis*-acitretin, etretinate and arotonoid ethylester (Ro 13-6298). For all retinoids tested, inhibition of NADH oxidase activity and inhibition of growth were correlated closely. With tretinoin, etretinate and arotonoid ethylester, NADH oxidase activity and cell growth were inhibited in parallel in proportion to the logarithm of retinoid concentration over the range of concentrations 10^{-8} to 10^{-5} M. Approximately 70% inhibition of both NADH oxidase activity and growth was reached at 10 μM. With retinol, *trans*-acitretin and 13-*cis*-acitretin, inhibition of NADH oxidase activity and growth also were correlated but maximum inhibition of both was about 40% at 10 μM. The possibility is suggested that inhibition of the plasma membrane NADH oxidase activity by retinoids may be related to their mechanism of inhibition of growth of HeLa cells in culture. (Mol Cell Biochem **166**: 101–109, 1997)

Key words: NADH oxidase, plasma membranes, growth, retinol, retinoids, HeLa cells

Introduction

Synthetic retinoids have exhibited a wide range of therapeutic potential and some have been introduced into clinical practice. Two synthetic retinoids, for example, are used widely in dermatology. Isotretinoin [13-*cis*-retinoic acid (Accutane®)] is used for the treatment of severe cystic acne [1] and etretinate (Tegison®) is used either alone or in combination with other agents for the treatment of psoriasis, particularly the erythrodermic and pustular varieties [2]. These retinoids are effective in the treatment of cutaneous disorders of keratinization as well. A naturally occurring metabolite of retinol, all-*trans*-retinoic acid (RA; tretinoin), has found utility in the topical treatment of dermatoses [3, 4]. It is the currently accepted standard for topical therapy for acne vulgaris. It has exhibited activity as well as a differentiation-inducing agent for acute promyelocytic leukemia [5, 6].

Retinoids are considered widely as prime candidate agents for cancer chemoprevention and, to a lesser extent, as cancer therapeutics [7]. Cancer is associated with uncontrolled growth and with the loss of differentiation. Retinoids regulate cell proliferation and differentiation and thus may produce a beneficial response in cancer chemoprevention. A deficiency in dietary vitamin A leads to a clinical syndrome characterized by growth retardation, degeneration of reproductive organs, and metaplasia and hyperkeratinization of epi-

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thelial tissues [8]. Animals deficient in vitamin A exhibit increased susceptibility to chemical carcinogens than do nondeficient animals [9, 10]. Exogenously administered retinoids may, under certain conditions, inhibit or reverse carcinogen-induced epithelial changes in appropriate target tissues [8, 11]. In addition, inverse relationships between vitamin A intake and the risk of developing cancer [12, 13] has been noted from epidemiological studies. That retinoids can reverse premalignant changes has been studied in both *in vivo* and *in vitro* models [7]. Examples include epithelia of mouse prostate glands in organ culture [14, 15], phorbol ester-induced tumor promotion [16, 17] in mouse skin and the malignant transformation of cells in culture where transformation was induced by ionizing radiation [18] chemical carcinogens [19], or transforming polypeptides [20]. These observations have culminated in the establishment of clinical trials to evaluate benefits of retinoids to patients with a high risk for cancer [21].

Much effort has been devoted to an understanding of the mechanism whereby retinoids modulate growth and differentiation of cancer cells. One of the difficulties was to understand how such a diversity of retinoids ranging from retinol to the newest arotonoids and including retinoid acid derivatives can all elicit dermatological and antineoplastic responses despite having apparently diverse abilities to affect gene regulation [22].

Our laboratory has recently described a cell surface NADH oxidase activity inhibited by the naturally-occurring quinone analog 8-methyl-N-vanillyl-6-nonenamide (capsaicin) with plasma membrane vesicles isolated from transformed human cell lines but which was unaffected by capsaicin in nontransformed cell lines [23]. This activity was hormone- and growth factor-stimulated with rat liver plasma membranes [24, 25] but unregulated with plasma membranes from rat hepatoma [26] or hyperplastic liver nodules [27]. In an earlier paper we reported that retinoic acid and calcitriol inhibited the NADH oxidase activity of normal and immortalized human keratinocytes and that this inhibition correlated with inhibition of cell proliferation [28]. In this report, we have examined the effect of retinoic acid on the NADH oxidase and growth of HeLa cells (human cervical carcinoma origin) and compared the response given by retinoic acid to that of retinol and several synthetic retinoids. The experimental systems used were cultured HeLa cells and plasma membranes isolated from cultured HeLa cells.

Materials and methods

Source of retinoids

Retinoids were dissolved in DMSO or ethanol Controls received an equivalent of solvent (0.01%). All retinoids were supplied by Hoffmann LaRoche Ltd, Basel. Retinol was from Sigma (St. Louis, MO) and was dissolved in ethanol.

Growth of HeLa cells

HeLa S cells for isolation of plasma membranes were grown as described [2] on Minimal Essential Medium (S-MEM) (Jolik modified) with glutamine (244 mg/l) and phosphate (1.3 g/l Na_2HPO_4) plus 5% donor horse serum but without $CaCl_2$. Gentamicin sulfate (50 mg/l) and sodium bicarbonate (2 g/l) were added.

Attached HeLa cells (ATCC CCL2), were grown in 150 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7 .0, at 37°C with 10% bovine calf serum (heat inactivated), plus 50 mg/l gentamicin sulfate (Sigma). Growth was determined after 72 or 96 h by counting the number of cells over defined areas consisting of a grid of 1 mm squares.

Cell fractionation

Cells were collected by centrifugation for 6 min at 3,000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10–30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30–40 sec at 10,500 rpm using an ST-10 probe and 7–8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1,000 rpm (175 g) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 g min (e.g. 1 h at 23,500 g) to prepare a plasma membrane enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto an aqueous two-phase partitioning system constituted on a weight basis [29, 30].

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate, pH 7.2. The plasma membrane-enriched microsome fraction [29] was added to the two-phase system and the weight was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm (150 × g) in a Sorvall HB 4 rotor for 5 min. The upper phases, enriched in plasma membranes, were carefully withdrawn with a Pasteur pipette, diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation (10,000 × g in a HB rotor for 30 min). The purity of the plasma membrane was determined to be 90% by electron microscopy and analysis of marker enzymes [29, 31]. The yield was 20 mg plasma membrane protein from 10^{10} cells [31].

Spectrophotometric assay

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any potential mitochondrial oxidase activity, and 150 μM NADH at 37°C with constant stirring. Activity was determined from the decrease in absorbance of NADH monitored at 340 nm with reference at 430 nm using an SLM-2000 (Aminco) dual-beam wavelength spectrophotometer in the dual wavelength mode of operation. Recording was continuous over two 5 min intervals. A millimolar extinction coefficient of 6.22 was used to determine NADH disappearance.

Results

NADH oxidase activity of isolated plasma membrane vesicles and the growth of attached HeLa cells were inhibited markedly by the natural retinoid tretinoin (all *trans*-retinoic acid). Both NADH oxidase activity and growth were inhibited with an EC_{50} of about 1 μM (Figs 1A and 1B). Inhibition of NADH oxidase activity and growth were directly proportional to the logarithm of tretinoin concentration over the range of 10^{-8} to 10^{-5} M for NADH oxidase activity and between 10^{-8} M and 10^{-4} M for growth.

With all retinoids tested it was not possible to assay concentrations greater than 10^{-5} M for inhibition of NADH oxidase activity in the spectrophotometric assay due to interference of the high concentrations of retinoids in the assay. DMSO or ethanol blank rates were determined in all experiments and were found to be insignificant.

Although not as effective as tretinoin, retinol also inhibited NADH oxidase activity and growth (Figs 1C and 1D). NADH oxidase activity of isolated HeLa plasma membrane vesicles was inhibited by about 26% at a retinol concentration of 10^{-5} M compared to 33% inhibition of growth at that same concentration of retinol. Both the inhibition of the NADH oxidase and the inhibition of growth by retinol appeared to be biphasic although the effect was most apparent with growth where a concentration of 10^{-4} M was tested. With growth, a first phase of inhibition by retinol was observed at 10^{-7} M followed by a plateau and then a second phase of inhibition beginning at 10^{-5} M. A similar response was seen with NADH oxidase activity of isolated plasma membrane vesicles. The first phase was observed at 10^{-7} M followed by a plateau. The 10^{-4} M concentration could not be tested and even the response to 10^{-5} M retinol became very variable.

Among the synthetic retinoids tested, etretinate was the most inhibitory of both growth and NADH oxidase (Figs 2A and 2B). NADH oxidase activity of isolated HeLa plasma membrane vesicles was 50% inhibited at a concentration of about 3 μM (Fig. 2A). Growth also was inhibited although somewhat higher concentrations of etretinate were required with an EC_{50} of about 10 μM. Results with arotonoid ethylester yielded log linear dose response curves similar to those of etretinate (Figs 2C and 2D).

The dose-response curves for the synthetic retinoids *trans*-acitretin and 13-*cis*-acitretin were similar to those observed with retinol (Fig. 3, compare with Figs 1C and 1D). The NADH oxidase activity of HeLa plasma membrane vesicles was inhibited by 33% at a concentration of 10^{-5} M *trans*-acitretin (Fig. 3A) whereas at that same concentration growth was inhibited by about 25% (Fig. 3B). With 13-*cis*-acitretin, NADH oxidase activity was inhibited by about 40% at 10^{-5} M and growth was inhibited by 35% at 10^{-5} M by 96 h of treatment (Fig. 3D).

With 13-*cis*-acitretin, the inhibition of NADH oxidase activity was markedly time-dependent. At the highest concentration tested, approximately 20 min of incubation was required for maximum inhibition to be observed (Fig. 4A). In contrast to 13-*cis*-acitretin, *trans*-acitretin did not exhibit a marked time-dependent inhibition of NADH oxidase activity (Fig. 4B).

At sublethal concentrations of all retinoids tested, the inhibition of both growth and of NADH oxidase appeared to be reversible (not shown). Oxidation of NADH by HeLa plasma membranes was inhibited by 50% with 10^{-7} M retinol but the inhibition was lost when the contents of the cuvette were oxygenated and irradiated for 15 min to destroy the retinol. The response of growth of HeLa cells to retinol at high concentrations also was reversible. Cells grown for 72 h with 10^{-5} M retinol grew only at about 25% of the control rate. Following removal of the retinol-containing medium and replenishment with fresh medium, the cells now grew during a subsequent 96 h observation period at 75% of the control rate. If the retinol was added every day for the first 3 days at 10^{-5} M, the cells again grew at about 25% of the control rate. With removal of retinol and addition of fresh medium, the retinol effect was at least partially reversible and the retinol-treated cells grew at 50% of control rate over the next 96 h.

Overall, the percentage inhibition of growth and percentage inhibition of oxidation of NADH by the several retinoids were highly correlated ($r = 0.91$) (Fig. 5). For individual retinoids, r values varied from 0.89 for etretinate to 0.99 for acitretin. With etretinate, which was the most potent inhibitor of NADH oxidase, apoptotic cell death was induced at 10^{-4} M as evidenced by nuclear fluorescence using DAPI, chromatin clumping and patterns of DNA fragmentation [32].

Discussion

Effects of retinoids on cell growth and differentiation have been evaluated in numerous cell and/or organ cultures. Some

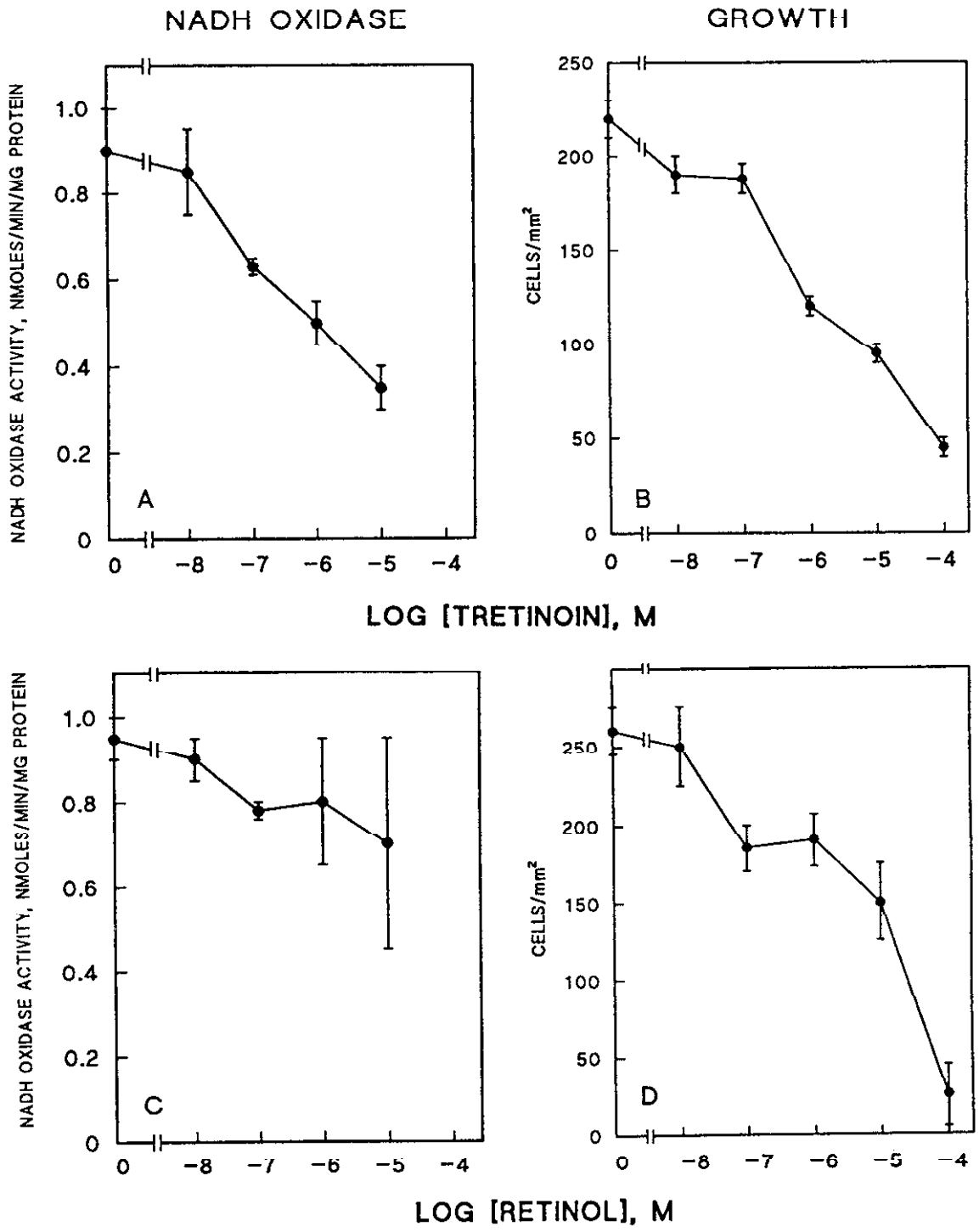

Fig. 1. NADH oxidase of plasma membranes from HeLa cells (A, C) and growth of HeLa cells in culture (B, D) as a function of the logarithm of concentration of tretinoin (all *trans* retinoic acid) (A, B) and retinol (C, D). Results are from 3 experiments for retinol and 6 experiments for tretinoin ± standard deviations. Growth was determined over 72 h.

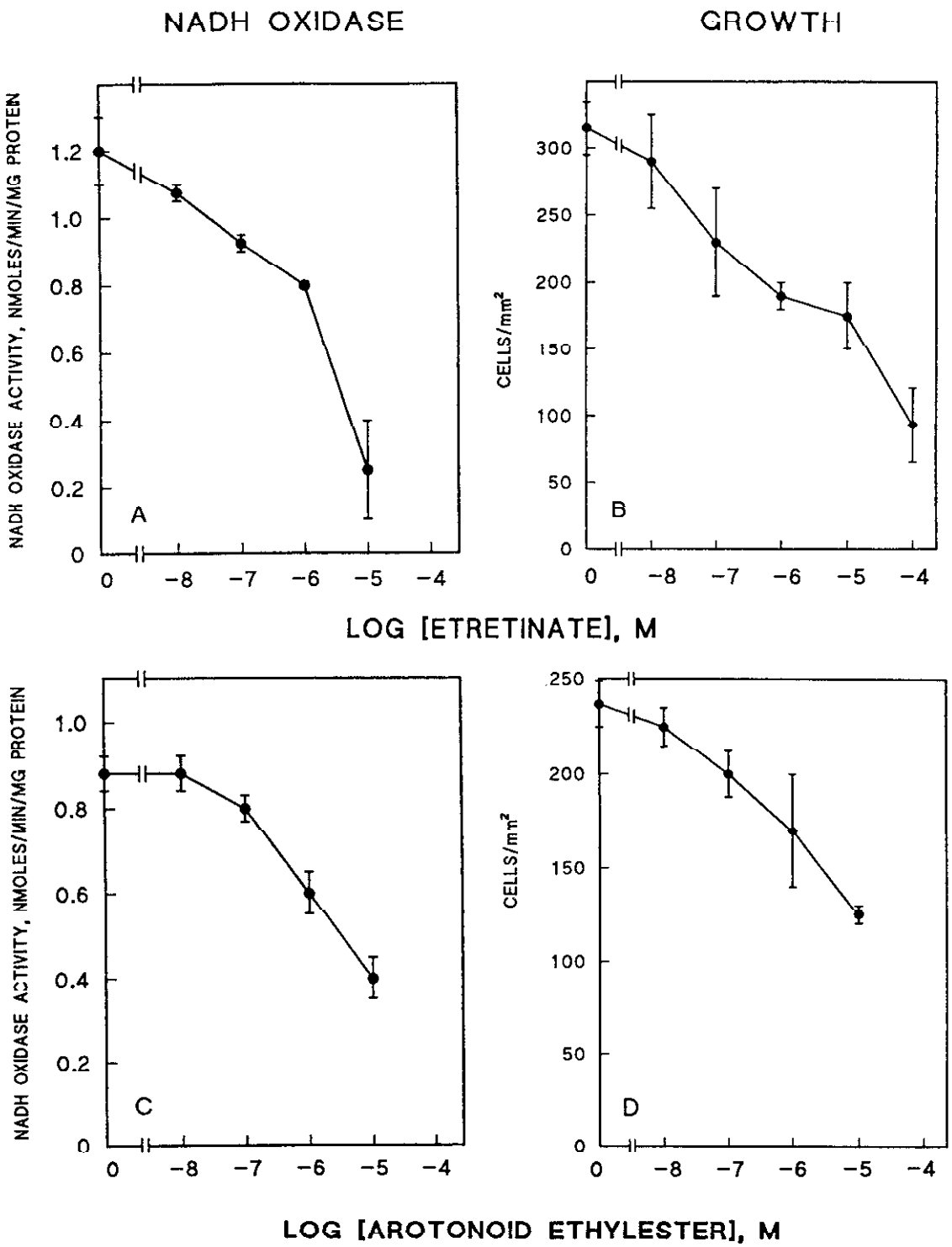

Fig. 2. NADH oxidase of plasma membranes from HeLa cells (A, C) and growth of HeLa cells in culture (B, D) as a function of the logarithm of concentration of etretinate (A, B) and arotonoid ethylester (Ro 13-6-6298) (C, D). Results are from 3 experiments ± standard deviations. Growth was determined over 72 h.

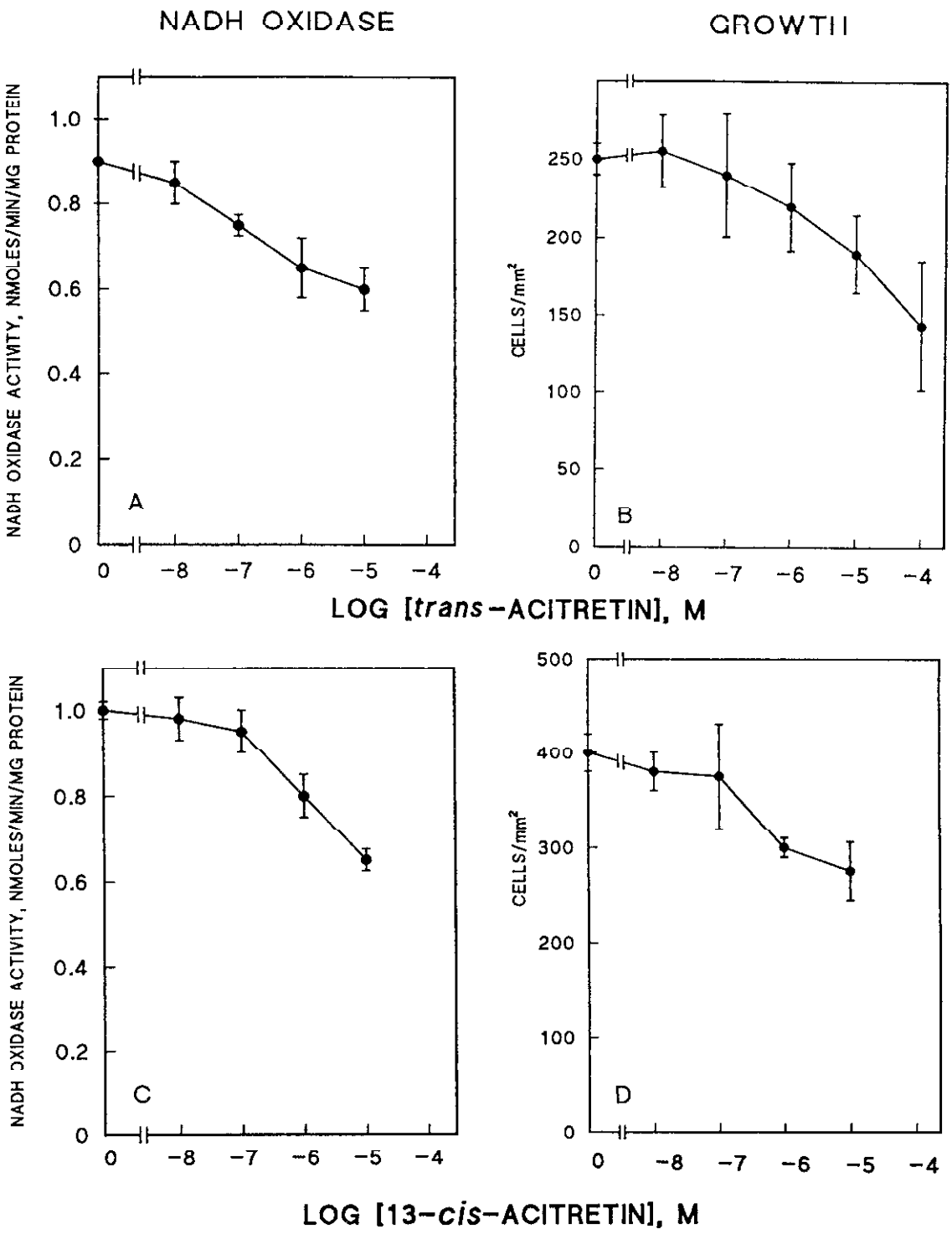

Fig. 3. NADH oxidase of plasma membranes from HeLa cells (A, C) and growth of HeLa cells in culture (B, D) as a function of the logarithm of concentration of *trans*-acitretin (A, B) and 13-*cis*-acitretin (C, D). Results are from 3 experiments ± standard deviations. Growth was determined over 72 h for *trans* acitretin and over 96 h for 13 *cis* acitretin.

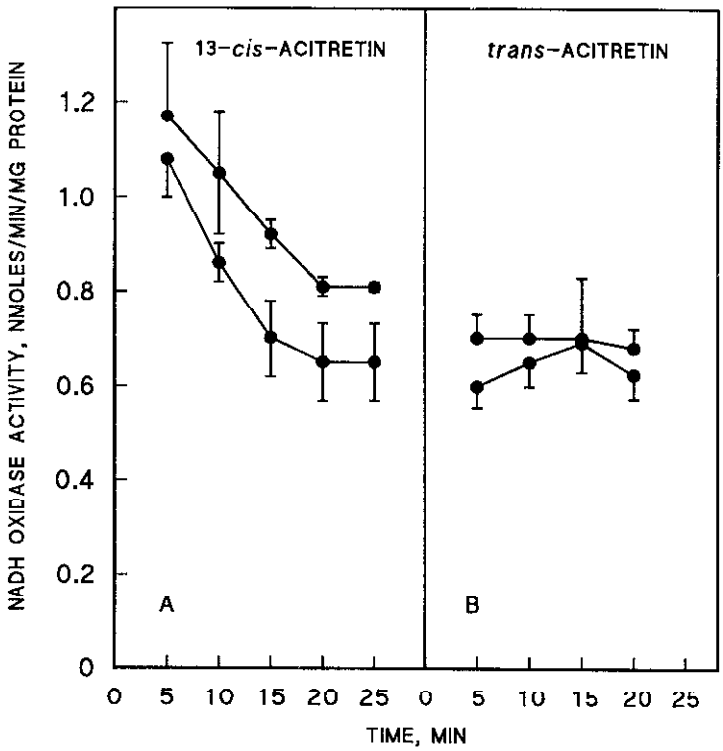

Fig. 4. Time dependency of inhibition of NADH oxidase activity comparing 13-*cis*-acitretin (A) and *trans*-acitretin (B). Rates given were averaged over 5 min of incubation at two concentrations of retinoid. Values are averages of two (A) or three (B) determinations ± mean average or standard deviations.

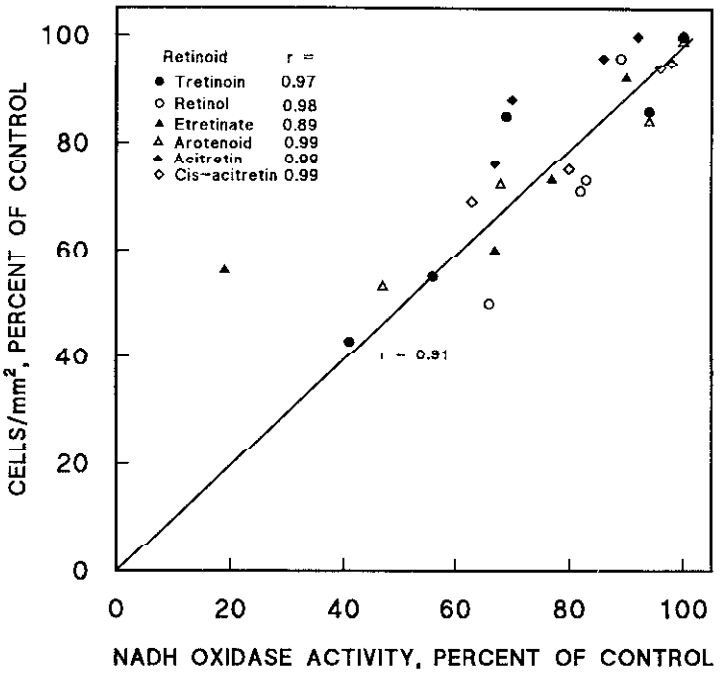

Fig. 5. Correlation between NADH oxidase activity and cells/mm² as percent of control for all treatments. Correlation coefficients and symbols for individual retinoids are given in the inset.

types of transformed cells can be induced to undergo terminal differentiation in response to treatment with retinoids [7]. Associated with terminal differentiation is a loss of their neoplastic character. Examples of this phenomenon include the induction of terminal differentiation in F9 teratocarcinoma cells [33, 34, 35] and promyelocytic leukemia cells [36, 37]. Despite the observations that the dramatic effect of retinoids has been limited to only a few cell types, other cell lines that have been routinely used to evaluate the effects of retinoids or to understand the mechanism of retinoid action include melanoma [38, 39], 10T1/2 cells [40], and tracheal epithelial cells [41].

The findings with cultured HeLa cells and plasma membrane isolated from cultured HeLa cells using several synthetic retinoids confirm earlier observations with retinoic acid and calcitriol with human keratinocytes [28] that growth inhibitory concentrations resulted in a parallel inhibition of the NADH oxidase activity of isolated plasma membrane vesicles.

A growth factor-sensitive NADH oxidase has been identified in plasma membrane preparations of both plant [42] and animal [24, 25] cells. In liver plasma membranes, the activity is stimulated by transferrin and insulin [25]. However, in hepatoma plasma membranes, while the NADH oxidase activity is present, it is no longer hormone responsive [26]. Similar findings were observed with hyperplastic liver nodules induced by the hepatocarcinogen 2-acetylaminofluorene [27]. The NADH oxidase of liver plasma membranes was stimulated by treatment with transferrin. However, that of the plasma membranes of liver nodules was not, despite the observation that the number of plasma membrane-associated transferrin receptors was increased in the nodules. On this basis, some relation of the plasma membrane oxidase to growth or growth control was postulated [25].

In a subsequent study, a correlation between inhibition of NADH oxidase and inhibition of clonal growth for the immortalized HKc/HPV16 cells was observed for both retinoic acid and calcitriol [28]. However, such a correlation was less clear for normal HKc. With normal HKc, response to retinoic acid of the oxidase as well as growth was complicated by a tendency for both to be stimulated at concentrations of retinoic acid around 10^{-8} M. The result was an optimum curve rather than the simple log-linear dose-dependent inhibition response exhibited by HKc/HPV16. A similar situation occurred in the response of the NADH oxidase of normal HKc to calcitriol, where a tendency for the oxidase to be stimulated at calcitriol concentrations of about 10^{-8} M. Rather, only log-linear growth inhibition was found. While correlations do not establish causality, the findings suggested that immortalized transformed cells may exhibit a greater susceptibility of the NADH oxidase and growth to retinol. Subsequently, the NADH oxidase was examined in plasma membrane vesicles from HeLa cells. Here, as in vesicles of hepatoma plasma membranes [26], the activity appeared to be constitutively activated and inherently more responsive to growth-inhibitory agents [23].

One of the recurring difficulties in evaluating the clinical potential of the retinoids is a general lack of correlation between the effects of retinoids observed in cell culture with those observed in *in vivo* experimental models [7]. While cell cultures have contributed much useful information, the basis for growth inhibition and why *in vivo* and *in vitro* results are not correlated are not understood. Further studies of the function of the plasma membrane NADH oxidase activity and its role in the control of cell growth may help resolve these anomalies by providing an alternative basis for understanding responses of growth and differentiation to retinoids.

Studies with hormones, growth factors and anticancer drugs that stimulate or inhibit growth suggest that growth and plasma membrane NADH oxidase are related. This, however, may be unrelated to the previously described trans plasma membrane redox system, ubiquitous among eukaryotic cells that transfer electrons from reducing agents to external impermeable oxidants [43] or to molecular oxygen [44].

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