Sodium Phenylacetate Induces Growth Inhibition and Bcl-2 Down-Regulation and Apoptosis in MCF7ras Cells in Vitro and in Nude Mice

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Abstract

Using a highly tumorigenic human breast cancer model (Ha-ras-transfected MCF7 cell line) we analyzed the efficacy of the differentiation-inducing agent sodium phenylacetate (NaPA), both in vitro and in vivo. NaPA-treated MCF7ras cells showed dose-dependent growth inhibition from 2.5 to 15 mM without apparent toxicity. Western blot analysis showed a Bcl-2 down-regulation after 48 h treatment with 5 mM NaPA, together with apparition of apoptotic nuclei by DAPI staining. Mice bearing MCF7ras xenografts (n = 40) were treated for 2 weeks through s.c.-delivering osmotic pumps, followed by 6 weeks of daily i.p. NaPA administration. After 3 weeks, the treated tumors showed growth arrest without regression for the whole observation time, e.g., 12 weeks. Immunohistochemical analysis showed Bcl-2 down-regulation and differentiation patterns: decrease of Ki-67 and increase of steroid receptors (estrogen and progesterone receptors) compared to controls. Cells cultured from treated tumors (II.b) displayed pseudotrabecular disposition as MCF7ras cells treated in vitro. They also showed a higher NaPA sensitivity, together with 70% Bcl-2 down-regulation as compared to the derived cells of untreated tumors (II.a). When reinjected into nude mice, II.b cells induced only one poorly vascularized, noninvasive tumor (8%) with lower proliferation index, 100% progesterone receptor positive cells, and 35% terminal deoxynucleotidyltransferase-mediated dUTP-X nick end labeling (+) nuclei, as compared to 100% induction of highly vascularized and invasive tumors with 3% terminal deoxynucleotidyltransferase-mediated dUTP-X nick end labeling (+) nuclei induced by II.a cells.

Introduction

In the last few years, NaPA has been shown to possess antiproliferative and differentiating effects in vitro and in experimental trials on several hematological and solid tumors (1, 2). Experimental data indicate that NaPA can promote maturation of various human leukaemia cells, prostate carcinoma cells, and malignant glioma cells (3–5) at nontoxic concentrations readily achievable in humans (6).

The accumulating data that emphasize the differentiation potential of this compound led us to examine its activity in a breast cancer experimental model: MCF7ras cell line and MCF7ras tumors induced in nude mice. The estrogen dependency of MCF7 cell tumorigenicity (7) is bypassed in the MCF7 clone transformed with the v-ras oncoprotein (8). This derivative MCF7ras cell line no longer requires estrogen supplementation to induce a high incidence of tumors in nude mice, although it remains estrogen and progesterone receptor positive (9). It represents a model for aggressive breast cancer, since high levels of c-Hras expression have been detected in human breast carcinomas (10). It has been proposed that overexpression of p21 in tumor tissues or its presence in a mutated (activated) form could be acting as a cofactor with other oncogenes, yielding the tumorigenic phenotype (11, 12). Another feature of this model is that MCF7ras cells overexpress bcl-2 oncogene, which has been shown to inhibit apoptosis (13). As it was shown in other models, coexpression of c-myc with bcl-2 gives rise to a markedly enhanced incidence of tumors, significantly greater than that seen with either bcl-2 or c-myc alone (14, 15).

Using this experimental model, we investigated tumor growth suppression and phenotypical modifications induced by pharmacological concentrations of NaPA on MCF7ras cells cultured in vitro and xenografted in nude mice. Modifications concerning the NaPA resistance and the tumor induction of cells derived from treated xenografts were also studied. We show here that NaPA, used at nontoxic pharmacological doses: (a) displays an important growth inhibition potential in vitro and in vivo; (b) reverses the malignant phenotype of tumorigenic Ha-ras transfected MCF7 cells; (c) down-regulates Bcl-2 oncoprotein synthesis; and (d) induces a differentiation process together with apoptotic death.

Materials and Methods

Cell Lines and Reagents. A human malignant breast MCF7ras cell line derived from pleural effusion was kindly provided by Dr. F. Calvo (Hôpital St. Louis, Paris, France). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS (GIBCO Laboratories, Grand Island, NY), antibiotics, and 2 mm l-glutamine at 37°C in a 5% CO2-humidified atmosphere. Cells were routinely passaged once a week at a 1:10 split ratio. NaPA (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water, brought to pH 7.0, and stored in aliquots at 4°C.

Cell Culture and NaPA Treatment. Cells growing in logarithmic phase were seeded into 24-well plates at a concentration of 2 × 104 cells/well. The following day, various concentrations of NaPA were added. At indicated times, cells were harvested with 0.25% trypsin-0.2% EDTA and counted in a Coulter counter (Coultronics).

Xenografts in Nude Mice. Virgin female athymic mice CD-1 (nu/nu), 5 weeks old, were obtained from Charles River, France. Animals were kept in a temperature-controlled room on 12/12 light/dark schedule with food and water ad libitum. All surgery was performed under ether anesthesia, and animals were sacrificed by spinal elongation. MCF7ras cells were cultivated in 10% DMEM and 1% FCS, and stored in aliquots at −80°C.

Animals (40 mice) were placed in a temperature-controlled room on 12/12 light/dark schedule with food and water ad libitum. All surgery was performed under ether anesthesia, and animals were sacrificed by spinal elongation. MCF7ras cells were cultivated in 10% DMEM and 1% FCS, and stored in aliquots at −80°C.

Two experimental protocols were developed. The first investigated the NaPA treatment of established tumors. MCF7ras tumors were induced as described above. Tumor measurements were begun at week 3 and followed for 2 weeks to evaluate the rate of tumor growth prior to treatment. NaPA treatment of established tumors. MCF7ras tumors were induced as described above. Tumor measurements were begun at week 3 and followed for 2 weeks to evaluate the rate of tumor growth prior to treatment. NaPA treated tumors showed growth arrest without apparent toxicity. Western blot analysis showed a Bcl-2 down-regulation after 48 h treatment with 5 mM NaPA, together with apparition of apoptotic nuclei by DAPI staining.
EFFECCRS OF PHENYLACETATE IN A HUMAN BREAST TUMOR MODEL

and protein loading was verified by Comassie Blue staining. Each point represents five different assays.

Tissue Storage and Immunohistochemical Analysis. Tumor specimens were frozen in liquid nitrogen and/or formalin fixed immediately after surgical resection, together with liver, lung, and spleen for further microscopic examination. The fixed samples were processed to paraffin in the usual way, and 5-μm sections were examined in hematoxylin and eosin preparations. Usual Mason’s trichroma staining, as well as immunohistochemical staining using Universal Kit of LSAB2 (K675-DAB; DAKO S.A. France) were also performed. Monoclonal mouse antibodies against human Bcl-2 (M-887; DAKO S.A. France), Ki-67 (MB-1; Immunotech), and steroid receptors using ER/POR Kit (TEBU) were detected, following the manufacturer’s specifications. The number of positive cells for each monoclonal antibody or of TUNEL (+) apoptotic nuclei was estimated in six high-power fields containing 60–80 cells/field (×400). Results were described as follows: ++, >90%; +++, 50–80%; ++, 30–50%; +, 5–20% of cells stained; and −, no or weak staining in a few cells (<0.01%). For stromal fibroblasts: ++, dense; +, moderate; +, sparse; and −, none. Number of vascular formation: +, few (3–4); ++, intense (7–10); +++ (+>10) at ×100; and ±, very rare (1–2/tumor).

Apoptotic Death Assay. We used DNA fragmentation as the criteria for apoptotic cell death (17). Formalin-fixed tissue sections were processed for individual apoptotic death cells identified by using a TUNEL reaction-based in situ cell death detection kit, POD, according to the manufacturer’s protocol (Boehringer Mannheim). Each sample was performed in triplicate. For in vitro apoptotic death assay, cells cultured on Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) were treated with several concentrations of NaPA. At different times from NaPA addition are shown in the histogram analysis. Results represented were obtained from at least three independent experiments, and a minimum of 150 cells were scored for each time point; bars, SD.

The second protocol investigated tumor induction of MCF7ras from NaPA-treated and nontreated biopsies obtained in protocol I. Three groups of mice were injected with “parental” MCF7ras cells (12 mice; Lo cells), cells from NaPA treated tumors (10 mice; II.b cells), and cells from untreated tumors (11 mice; II.a cells). Cells derived from biopsies (II.a and II.b cells) were cultured in 10% FCS for two or three passages, and then 3 × 10⁶ cells were reinoculated to mice as described in the first protocol. Tumor cells derived from explants grow rapidly, and a dilution factor of 10 at each passage is almost always sufficient to select the rapidly growing MCF7ras cells. Confirmation of the human origin of cells was done by assaying samples for vimentin expression, an intermediate filament normally absent in MCF7ras cells (16) but which stains mesenchymal cells from the host animal monoclonal antibody (VIM-13.2; Sigma).

Western Blot Analysis. Cells were plated in T-150 plastic dishes (Falcon) in 10% FCS supplemented DMEM. When cells reached 80% confluence, the media were replaced with 10% FCS containing DMEM with or without NaPA at indicated concentrations. Cells were harvested after 48 h, and protein extracts were processed according to the protocol provided by the manufacturer (Amersham). Hyperfilm MP (ECL detection system; Amersham Co., Les Ulis, France.) were then quantified by absorbance-based image treatment with a Biocom Prolinea 4/33 Compaq Computer and expressed in relative percentages to control. Protein extracts were quantified using the Bradford method, and protein loading was verified by Comassie Blue staining. Each point represents five different assays.

![Fig. 1. A, growth inhibition of MCF7ras breast cells in vitro. Cells were seeded for 24 h in DMEM supplemented with 10% FCS and treated with various concentrations of NaPA for 10 days. Each point represents the mean of two experiments in which the measurements were made in triplicate; bars, SD. B, percentage of cells presenting condensed nuclei (apoptotic-like phenotype) at different times from NaPA addition are shown in the histogram analysis. Results represented were obtained from at least three independent experiments, and a minimum of 150 cells were scored for each time point; bars, SD.](image1)

![Fig. 2. Effect of NaPA on morphology of MCF7ras cells; phase-contrast microscopy. Untreated cells cultivated in 10% FCS containing DMEM (a); treated for 48 h with 5 mM NaPA (b); cells derived from untreated xenografts (c); and cells derived from NaPA treated xenografts and cultured for 3 passages in DMEM supplemented 10% FCS (d) are shown. a and b, ×250; c and d, ×100.](image2)
times after NaPA treatment, adherent cells were stained with the DNA-specific fluorochrome diamino-2-phenylindole (Boehringer Mannheim Biochemica) in a 1-μg/ml methanol solution. Cell counts were performed within 20 min of staining on a Zeiss Axiophot epifluorescence microscope. Experiments were performed in triplicate with at least 150 cells scored at each point. Fragmented or condensed nuclei were scored as apoptotic. Intact or mitotic nuclei were scored as normal.

**Statistics.** In the figures, the data are presented as the mean values for a 95% confidence interval. Multiple statistical comparisons were performed using ANOVA in a multivariate linear model. Other statistical comparisons

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Fig. 3. Effects of NaPA on xenografts and on cells derived from xenografts. Tumors were measured at weekly intervals beginning 3 weeks after 6 × 10⁶ cells/animal inoculation. Treatment was started 2 weeks after apparition of single palpable fat pad tumors. Tumors in the NaPA-treated group were significantly smaller than those in the control group, *P* < 0.001 (A); 3 × 10⁶ cells derived from NaPA treated MCF7ras (II.b) xenografts and from untreated xenografts (II.a) were inoculated s.c. in the right fat pad and compared with in vitro cultured MCF7ras (I.o) for their tumor induction potential. Note the long delay and poor tumor incidence in II.b cells (B), percentage of change in tumor volume/week for the same cells (C), and dose-dependent inhibition of NaPA pretreated MCF7ras xenografts. Twenty-four h after seeding the cells, various concentrations of the drug were added. Note the drastic growth inhibition of the cells beginning from 2.5 mM of NaPA (D). The induction of apoptosis, under various concentrations of NaPA at 3, 5, and 7 days after treatment, was scored morphologically as described in “Materials and Methods,” and viability was determined by trypan blue exclusion in II.b cells cultured in DMEM supplemented with 10% FCS (E). The percentage of floating cells is also represented. The controls are indicated by light stippling and the treated cells by heavy stippling. Bars, 95% confidence interval.
NaPA Effects on MCF7ras Cells Cultured in Vitro. NaPA inhibited the growth of tumorigenic MCF7ras cells in a dose-dependent manner (Fig. 1A). Cells grown on plastic substratum in 10% FCS containing DMEM display typical epithelial patterns: a cluster of cells growing in a polygonal shape with low contact inhibition (Fig. 2a). Within 48 h of treatment with 5 mM of NaPA, MCF7ras cells exhibited a marked change in morphology, showing pseudotrabecular disposition and round-up of the cells (Fig. 2b). Exposure of MCF7ras cells to NaPA triggered apoptosis, which was recognized by characteristically altered cell morphology and the appearance of condensation of chromatin and nuclear fragmentation. The cell response by apoptosis was relatively rapid and dose dependent (Fig. 1B).

Effect of NaPA on MCF7ras Cells Xenografted in Nude Mice. When 6 × 10⁶ MCF7ras cells were inoculated in the right fat pad of nude mice, palpable tumors appeared in 70% of mice 3 weeks after cell injections. Treatment started 2 weeks after the appearance of tumors, when average tumor volume had reached 350 mm³. After 2 weeks of treatment with continuous perfusion of NaPA as described in “Materials and Methods,” the tumor growth rates already presented a 20% growth inhibition as compared to control (Fig. 3A). After an additional 6 weeks of daily i.p. NaPA injections, tumor growth was completely abolished without regression (P < 0.001).

By immunohistochemical analysis, we measured several tumoral markers in paraffin-embedded biopsies (Table 1). We have observed in NaPA-treated tumors a lower proliferation index, measured with Ki-67 marker (18) and a lower Bcl-2 content (P < 0.05) (Fig. 3B). Treated tumors were also less vascularized, with stromal development and multifocal necrotic areas. In contrast, NaPA increased the percentage of estrogen receptor-positive cells very significantly from 20 to 60% of volume increase per week (Fig. 3C). They did not only grow significantly faster than I.o-derived tumors but also displayed growth rates more than 10 times faster than that of the II.b-derived tumor cells. The NaPA-treated, biopsy-derived cells (II.b) developed only 1 slowly growing tumor among 11 inoculated mice with a latency period of 5 weeks. Histological analysis of this tumor revealed a poor supporting vascularization, no sign of invasion in surrounding tissues, stromal development, and 100% intensive, progesterone receptor-positive cells (Table 1). A more differentiated phenotype induced by NaPA was obtained without toxicity on any of the internal organs studied as well as no modification of the body weight of the treated animals. Furthermore, cells derived from treated tumors did not develop any resistance to the compound when they were reexposed in vitro to the same concentrations of NaPA.

Phenotypical Modifications of in Vivo NaPA-Treated MCF7ras Cells. To investigate possible phenotypical modifications induced by pharmacological nontoxic concentrations of NaPA, we have cultured MCF7ras cells from 4-week, NaPA-treated (II.b) and untreated (II.a) tumors. When II.b cells were cultured in the presence of increasing concentrations of NaPA for several days, we observed a strong growth inhibitory effect at 2.5 mM as soon as 3 days of treatment and a complete cytostatic effect with a dose and a time effect on apoptosis induction (Fig. 3D). Apoptosis and cell viability were monitored after 3 days of treatment with various NaPA concentrations. As shown in Fig. 3E, the control cells had a baseline level of 12%, which increased to 40% after 5 days of 2.5 mM NaPA treatment (compared to over 20% for parental MCF7ras cells, as shown in Fig. 1B). The viability

![Fig. 4. Western Blot analysis of Bcl-2 protein. One μg of total protein (quantified by Bradford and confirmed by Blue Comassie staining of gels) from MCF7ras cells cultivated in DMEM supplemented with 10% FCS (Io-), or 5 mM NaPA treated cells for 48 h (Io+), as well as of untreated xenografts (II.a) and NaPA-treated xenografts (II.b) were immunoblotted and probed for Bcl-2. Values shown below are from wide-beam densitometric scanning of individual lanes expressed in relative percentages to controls (parental MCF7ras cells Io-). Five experiments were done on two different extracts and scored for each point. The 20% difference in the amounts of Bcl-2 in Io- and Io+ cells is significantly different (P < 0.01).](image-url)
of II.b cells was measured by trypan blue exclusion, which confirmed that the loss of viability paralleled the induction of apoptosis (Fig. 3E). It should be noted that since loss of membrane integrity is preceded by morphological changes of apoptosis, the numbers of nonviable cells did not correspond to the absolute percentages of apoptotic cells. Apparition of 99% nonviable cells detached from the tissue-culture support was monitored separately. They represent 10% of cells treated with 5 mm of NaPA and 30% of cells treated with 7.5 mm NaPA.

Cell morphological modifications appeared exclusively in II.b cells as compared to II.a cells (Fig. 2, c and d). Pseudotrabecular disposition of II.b cells appeared without addition of NaPA. Any mesenchymal cell contamination from the host was eliminated due to specific MCF7ras growth patterns as well as the negativity of all samples for vimentin (see "Materials and Methods").

Western blot analysis of II.b cells showed a decrease of the Bcl-2 oncoprotein content of 70% as compared to II.a cell extracts (Fig. 4, Lanes 3 and 4, respectively). A slight although significant (P < 0.001) down-regulation of 25% was also observed after 48 h of I.o cell treatment with 5 mm NaPA (Fig. 4, Lanes 1 and 2, respectively). Furthermore, TUNEL(+) nuclei (apoptotic-like phenotype) were numerous (generally over 35%) in the NaPA pretreated biopsy (II.b). In contrast, II.a and I.o samples displayed only 1% and 4% TUNEL(+) nuclei, respectively (Table 1). This assay uses terminal deoxynucleotransferase to label free 3′OH ends in genomic DNA with fluoresceindUTP and allows the detection of a very early apoptotic event (19).

The amount of DNA strand breaks in apoptotic cells is so important exerted on the stromal cells, since preliminary experiments in our laboratory show also that NaPA may block the induction of extracellular matrix elements responsible for increased invasiveness and neovascularization in tumors.4

It remains now to explore the effects of NaPA on other genes such as mdr, heat shock proteins, and genes involved in the cell cycle, as well as a possible synergistic effect of NaPA and cytostatics and/or hormone inhibitors on tumor repression.

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References

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