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Differential radiosensitizing effect of valproic acid in differentiation versus self-renewal promoting culture conditions

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Abstract

Purpose—It has been shown that valproic acid (VA) enhances proliferation and self-renewal of normal hematopoietic stem cells and that breast cancer stem/progenitor cells can be resistant to radiation. Based on these data, we hypothesized that VA would fail to radiosensitize breast cancer stem/progenitor cells grown to 3D mammospheres.

Materials and Methods—We used the MCF7 breast cancer cell line grown under stem cell promoting culture conditions (3D, mammosphere) and standard non-stem cell monolayer culture conditions (2D) to examine the effect of pretreatment with VA on radiation sensitivity in clonogenic survival assays and on the expression of embryonic stem cell transcription factors.

Results—3D cultured MCF-7 cells express higher levels of Oct4, Nanog and Sox2. 3D passage enriched self-renewal and increased radioresistance in 3D mammosphere formation assays. VA radiosensitized adherent cells but radioprotected 3D cells in single fraction clonogenic assays. Moreover, fractionated radiation sensitized VA-treated adherent MCF7 cells, but did not have a significant effect in VA-treated single cells grown to mammospheres.

Conclusion—We conclude that VA may preferentially radiosensitize differentiated cells over those expressing stem cell surrogates, and that stem cell promoting culture is a useful tool for in vitro evaluation of novel cancer therapeutic agents and radiosensitizers.

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Conflicts of Interest: None

Keywords

Valproic Acid; Radiation; Breast Cancer Stem/Progenitors cells; Mammospheres; 3D culture

Introduction

Cancer stem cells are a small population of cells within a tumor that are capable of self renewal and differentiation into one or more cell types, two key features of normal stem cells. They are believed to be responsible for metastasis and recurrence of malignant tumors. Mounting evidence supports the hypothesis that cancer stem or progenitor cells are more resistant to radiation treatment than non-cancer stem cells. For example, stem or progenitor cells from breast cancer (1–3), glioma (4) and atypical teratoid/rhabdoid tumor (5) are found to be more resistant to radiation therapy than the non-stem cells. Thus, cancer stem cells could be an ideal model to test and develop cancer therapeutic agents and radiation sensitizers.

Valproic acid (VA), a short chain fatty acid, has been used to treat epilepsy since 1963 (6). In recent studies, VA was found to have potent anti-tumor effects in breast, colon and prostate cancer cells (7–9). The anti-tumor activity of VA was reported to be related to its ability to inhibit histone deacetylase 1 (HDAC1) (10–12). The effect of VA in the radiosensitization of cancer cells was investigated using adherent cultures of human glioma cell lines and in vivo tumor growth delay assays (13,14). In those studies, VA was shown to significantly enhance the in vitro and in vivo sensitivity of the cancer cells to radiation treatment. It can be argued that these in vitro assays use differentiation promoting adherent, serum containing culture conditions, and that tumor growth delay is not a measure of stem cell survival. Therefore, although VA has been described as a radiosensitizer in standard, but non-stem cell assays (13–15), its effect on cancer stem cell radiosensitization is unknown. Indeed, it has been shown that VA enhances proliferation and self-renewal of normal hematopoietic stem cells (HSCs) (16,17).

In the present study, we used the MCF7 breast cancer cell line to examine the effect of pretreatment with VA on radiation sensitivity of cells grown under stem cell promoting culture conditions (3D, mammosphere) and standard non-stem cell monolayer culture conditions (2D). We hypothesized based on data suggesting that breast cancer stem and progenitor cells can be resistant to radiation and the data suggesting VA supports self-renewal that VA would fail to radiosensitize breast cancer stem/progenitor cells grown to 3D mammospheres. Interestingly, we found that VA not only fails to radiosensitize MCF-7 cells in 3D culture but it protects them from radiation. This finding suggests that VA may work as radiosensitizer to reduce tumor volume in the short term but increase the chance of future cancer progression and relapse.

Materials and methods

Cell culture

MCF-7 breast cancer cells were cultured in log-growth phase in modified Eagle medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate, 5ug/ml insulin, 1ug/ml hydrocortisone and 1× antibiotic-antimycotic at 37 °C in a humidified atmosphere (5% CO₂). Cancer stem/progenitor cells can be enriched by propagating cells in serum free, growth factor enriched conditions - called mammosphere or tumorsphere (3D) culture (18–21). Briefly, cells were grown in 6-well ultralow attachment plates in serum free MEM supplemented with 20 ng/ml bFGF (Invitrogen), 20 ng/ml EGF (Invitrogen), and B27 (Invitrogen).

Primary human breast cancer cells were isolated from pleural effusion fluid obtained on a clinical protocol approved by the institutional review board from a patient with inflammatory breast cancer. Tumor cells were selected by serial transplant into the cleared mammary fatpads of immunocompromised mice and the resulting tumor tissue passaged in short-term 2D or 3D culture for the experiments described herein. 3D media is as above. 2D media consists of Ham's F-12 media supplemented with 10% fetal bovine serum, 1 µg/ml hydrocortisone, 5 µg/ml insulin and 1× antibiotic-antimycotic.

Valproic acid (sodium salt; Sigma, St. Louis, MO) was dissolved in PBS to a stock concentration of 1M and stored at −20°C. A final concentration of 1mM of VA was used to treat cells. Dose response studies of VA alone (0–10mM) revealed a significant decrease in sphere formation with increasing concentration: 0.5mM (0%), 1mM (35% reduction), and 2mM (55% reduction), $p = 0.04$ (data not shown). Since some aggregation of clonal spheres was observed at 2mM but not 1mM, we chose 1mM to test radiation sensitivity. The media contained 1mM VA throughout the duration of the clonogenic or 3D mammosphere formation assays.

RNA Isolation and RT-PCR

Total RNA was isolated from adherent (2D) or mammospheres (3D) using TRIzol reagent according to the manufacturer's protocol. After treatment with DNase I (Ambion), 2µg of the RNA samples were reverse-transcribed with random hexamers using Super Script III First-Strand Synthesis System (Invitrogen). Control reactions excluded reverse transcriptase. PCR was done with aliquots of the cDNA samples at annealing temperature of 60°C with the following primers: *Oct4* forward 5'- ccagtatcgagaaccgagtgag -3', reverse 5'- gcatagtgcgtgctgatcg -3'; *Nanog* forward 5'- gatgcctcacacggagactg -3', reverse 5'- gctgggtaggttaggtgctg -3'; *Sox2* forward 5'- tccacactcacgcaaaaacc -3', reverse 5'- ttgcaaaactctgcaaagc -3'; *Gapdh* forward 5'- caccagaagactgtggatgg -3', reverse 5'- ttctagacggcaggctcaggtc -3'. The resultant PCR products were resolved on 2% agarose gels stained with ethidium bromide.

Label Retention Assay

BrdU (10 µM) was added to MCF7 cells grown in 6-well ultralow attachment plates. At each passage (5 days), a single well was harvested for BrdU staining, while the other wells were enzymatically dissociated and subjected to serial mammosphere passages without further addition of BrdU. Intact spheres from all the passages were subjected to BrdU staining and viewed by confocal microscopy.

Western blot

Cells were incubated at 37°C with 1 mM VA at different time points. Cells were scraped into 1× RIPA lysis buffer (diluted from 10× RIPA from Cell Signaling) containing 1 µM PMSF and transferred to microcentrifuge tubes. Samples were rotated for 1 hr at 4°C and were centrifuged at 16,000g for 15 min. Aliquots of the supernatants containing 50 µg protein were electrophoresed on 4–20% gradient SDS-polyacrylamide gels (Invitrogen) and transferred to PVDF membranes (Biorad). Membranes were incubated in 5% nonfat milk for 1 hr at room temperature and were then incubated at 4°C for 16 hrs with acetyl-H3 (Cell Signal) or Beta-actin (Sigma) antibody. Membranes were washed three times and incubated with the corresponding secondary antibody conjugated with a horseradish peroxidase in 5% nonfat milk at room temperature. After incubation with secondary antibody, the membranes were washed three times and immunoreactivity was detected by enhanced chemiluminescence. Beta-actin was used as a loading control.

Clonogenic/3D mammosphere formation assay

In order to evaluate radiosensitivity, monolayer cultures of MCF7 cells were trypsinized into single cells and were seeded into individual wells of a 6-well tissue culture plate (for 2D) or ultralow attachment plates (for 3D) with or without 1mM VA. Both 2D and 3D 6-well plates containing seeded single cells were exposed to increasing doses of gamma-irradiation (2, 4 or 6 Gy) 4 hrs after plating with a Cs-137 source Nasatron (U.S. Nuclear, Burbank, CA) for Figure 2, all else with Shepherd Irradiator (J.L. Sheperd and Associates, San Fernando, CA). 2D plates were incubated for 14 days and colonies were stained with crystal violet. For mammosphere formation (we designate this assay as 3D mammosphere formation assay), cells were incubated in mammosphere media for 7 days and spheres were stained with MTT to improve visualization. Spheres with a minimum size of 50 μ M were counted using a Gelcount colony counter (Oxford Optronix, Oxford, UK). Average sphere size was 120 μ m, ranging from 101–147 μ m in MCF-7 cells and 111 μ m, ranging from 107–115 μ m in short term pleural effusion cells. Experiments represented by figure 2 were performed prior to the purchase of the Optronix gel counter and as such spheres were counted manually. Survival curves were generated using Sigmaplot 8.0. Where appropriate, the two-tailed Student's t-test was used to compare group means with $p < 0.05$ considered to be significant.

Results

The study of cancer stem cells has been made easier through an in vitro enrichment and propagation technique called mammosphere culture (18). Using this method as a surrogate to enrich the cancer stem cell population, we first examined whether MCF7 mammospheres undergo self renewal. Adult stem cells have been identified by label retention assays wherein the slow dividing property of stem cells causes them to retain labels such as BrdU while the fast proliferating progenitors dilute them out with time (22). In order to explore whether MCF7 mammospheres contain such label retaining cells, we pulsed mammospheres with BrdU for 5 days and thereafter chased the label for over 4 weeks with serial passaging of the mammospheres without additional label (Fig.1A). While cells in passage 0 (P0) mammospheres were 100% labeled (not shown), some P1 mammospheres retained the BrdU-positive cells. By the second (P2) and subsequent passages, mammospheres containing a single long term label-retaining cell were observed suggesting asymmetric self-renewal and existence of a hierarchy within mammospheres. Similar observations were made recently in primary mammospheres derived from human breast organoids (23). Likewise, 3D mammospheres selectively expressed embryonic stem cell transcription factors Oct4, Nanog and Sox2 (Fig. 1B). These genes are essential for the self renewal and pluripotency of ES cells (24,25).

In order to investigate the radiosensitivity of MCF7 grown under adherent and stem-promoting culture conditions, we performed survival assays using increasing doses of radiation. As shown in Fig. 2, cells grown in mammosphere-culture (3D) were found to be more radioresistant than cells in adherent culture (2D). Similar results were reported from a previous study by Phillips et al. that compared the radiosensitivity of adherent and floating cells of MCF7 cells (2). We also found that the second-generation spheres (P1; a further enrichment for self-renewing sphere forming cells) are more resistant to radiation than the first-generation spheres (P0) of MCF7 cells. This is consistent with the hypothesis that self-renewing cells are more radioresistant than differentiated cells, although it cannot be excluded that radioresistance may have increased as a function of increasing stemness of the cells (Fig. 2). To explore the effect of VA on radiosensitivity of cancer cells grown under 2D or 3D culture conditions, we initially determined the time course of H3 hyperacetylation in cells exposed to 1mM VA and grown under 2D or 3D culture conditions by western blot analysis. Increases in the levels of acetylated histone H3 was detected by 6 hr after the addition of VA in 2D monolayer cells reaching a maximum by 48 hr but no histone H3 acetylation was detected in 3D mammospheres treated

with VA (Fig. 3A). We then examined the effect of VA on sensitivity to radiation of MCF-7 cells grown to 2D colonies or 3D mammospheres. VA can sensitize the adherent cells to radiation (Fig. 3B) while it radioprotects the cancer stem cell population (Fig. 3C). We further expanded the relevance of these interesting findings using short term human primary culture of pleural effusion cells from an inflammatory breast cancer patient. A similar radioprotective effect was observed in VA-treated single cells grown to 3D mammospheres compared to non treated cells (Fig. 3D). Furthermore, we investigated this phenomenon in MCF cells using VA and fractionated radiation. Cells were irradiated with a single dose of 10 Gy or with five daily doses of 2 Gy or two daily doses of 5 Gy. As shown in Fig. 4, fractionated doses of radiation sensitized VA-treated adherent cells but does not have a significant effect in mammospheres (Fig. 4B; $P < 0.001$). We also examined the clonogenic potential of VA-treated cells isolated from mammospheres in the 2D clonogenic assay system. The standard, 2D clonogenic fraction is enriched after VA and radiation treatment of cells in mammosphere culture conditions (Fig. 5).

Discussion

We report that 3D mammosphere culture of MCF-7 cells selects for radioresistant self-renewing clonogens that express increased levels of embryonic transcription factors. VA, a commonly used anti-seizure medication with anti-tumor activity in preclinical studies not only fails to radiosensitize but also radioprotects putative cancer stem cells cultured as 3D mammospheres. VA and another class I HDAC inhibitor trichostatin-A have been shown to protect HSCs (26), and VA has been shown to stimulate the self-renewal of HSCs (16,17). Moreover, the recent finding that VA can be used to reprogram human fibroblasts into pluripotent stem cells along with other genes (27) suggests that in spite of the purported function of HDAC inhibitors to promote differentiation of tumor cells, dedifferentiation of cancer cells may occur with VA treatment that in turn increases self renewing cancer stem cells (reviewed in (28)).

The neuropsychiatric effects of VA have been attributed to numerous mechanisms. VA has been reported among other things to function indirectly as a gamma-aminobutyric acid agonist to increase stem cell survival signaling effectors Wnt/beta-catenin through inhibition of GSK3-beta, and to stimulate the ERK pathway (reviewed in (29)). Interestingly, it can also alter the epigenome through inhibition of HDAC1 a mechanism believed to be responsible for its potent anti-tumor activity in vitro and in vivo (10–12). While most of the data regarding the use of HDAC inhibitors as cancer therapy stems from the hematologic malignancy experience, HDAC inhibitors, hydroxamates and benzamide analogs have been shown to induce differentiation in breast cancer cells (30,31), and quinidine was reported to induce breast tumor cell differentiation through proteasome-dependent HDAC1 degradation (32).

Based on similar pre-clinical studies suggesting that HDAC inhibitors may target cancer cells by inducing differentiation, VA has been clinically tested in patients with myelodysplastic syndrome (MDS) and acute myelogenous leukemia with disappointing results. Using VA alone only a small subset of patients improved, and complete or partial remissions were rare (33). Similarly disappointing results were seen with combined regimens incorporating retinoic acid (16,33,34), and with more potent HDAC inhibitors (Depsipeptide, MS-275, LBH589, MGCD0103 and Vorinostat (reviewed in (35)).

Viewed in the context of data demonstrating that VA may also promote stem cell self-renewal, the clinical results using non-specific HDAC inhibitors are not necessarily unexpected. De Felice and co-workers investigated the effect of VA on CD34+ cells isolated from cord blood, mobilized peripheral blood and bone marrow and found that VA strongly enhanced the effect of Flt3L, thrombopoietin, stem cell factor and IL3 on the maintenance and expansion of

primitive HSC population (17). The expansion was due to an increased self-renewal ability of HSCs. HDAC inhibition therefore has important biological effects on normal HSCs. It might be expected that tumor stem cells may be similarly impacted and indeed, it has been shown that VA treatment enhanced the maintenance and clonogenic capacity of CD34+ AML progenitor cells (36). Since VA induces differentiation and/or apoptosis on the bulk of AML blasts, it clearly has a different effect on the small population of leukemic stem cells. Interestingly, treatment of AML cells with more potent HDAC inhibitors resulted in a loss of clonogenic survival and the enhancement of differentiation of leukemia cells, indicating specific HDAC targets may play an important role in tumor stem cell expansion (37).

While the data are more limited in solid tumors, Camphausen et al demonstrated that VA can not only radiosensitize adherent human glioma cell lines *in vitro* but it also causes tumor radiosensitivity *in vivo* (13). These *in vitro* studies were done using gold standard monolayer clonogenic assays, and we propose that this culture environment may not fully reflect the impact of therapy on the most primitive stem or progenitor cells that may be selected against in this culture (reviewed in (38)). Indeed, while we report the same effect using monolayer 2D culture with MCF7 cells, we observed opposite results in culture promoting self-renewal. Similarly, while tumor growth delay as was assessed in these *in vivo* studies may be a good indicator of death of the differentiated cell types of a tumor, cancer stem cells may not be fully evaluated with this assay. This hypothesis is supported by a recently published paper (21) that clearly demonstrated differences on tumor seeding, growth and metastasis *in vivo* of salinomycin (a drug that effectively reduced sphere formation) and the common chemotherapeutic drug Taxol (which did not). While both drugs showed comparable tumor growth delays, tumors from the Taxol-treated cohort had an increase in tumorsphere-forming cells. This highlights the value of sphere assays in predicting *in vivo* response and indicates that cancer stem cells may not be fully evaluated with tumor growth delay experiments. Tumor radiocurability, which is quantified by tumor control dose 50% (TCD 50), may more accurately address the cancer stem cell *in vivo* (39,40).

A potential limitation in assessing radiation resistance in 2D monolayer culture compared to 3D sphere culture is the inherent differences in culture conditions and cell adhesion (41–43) which can also contribute to radiation resistance. Phillips et al have examined the impact of mammosphere media on radioresistance of 2D MCF-7 cells and report that growth factors in the media do not account for the full differences in radioresistance between spheres and monolayer, and indeed promote the formation of spheres in the monolayer culture thus confounding the results (2). In addition, we find that there is increased radioresistance between P0 and P1 spheres (Fig. 2) which cannot be due solely to differences in cell adhesion (41,42), although some contribution may be likely given that cell adhesion molecules (i.e beta1 integrin) are included among the cell surface markers used to identify cancer stem cells. Further studies to elucidate the relationship/correlation between cell adhesion, stemness, and radioresistance are clearly warranted.

In summary, we found that although VA sensitized the adherent or differentiated primary and immortalized breast cancer cells to ionizing radiation in standard clonogenic assays, it did not radiosensitize stem/progenitor cells as assessed by 3D mammosphere formation assay. Indeed, it protected the cancer stem/progenitor cells during radiation treatment. Based on these *in vitro* studies, we would expect that VA may work as radiosensitizer to shrink the tumor in short term but increase or fail to reduce the chance of cancer relapse in the future. Moreover, it suggests that the widely used standard clonogenic assays and tumor growth delay experiments may not optimally select anti-cancer stem cell agents for clinical trials in breast cancer (reviewed in (38)). Further studies are necessary to prove the clinical relevance of utilizing the 3D mammosphere formation assay as a model to test and develop cancer therapies.

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Figure 1. Mammospheres (3D) self renew and express stem cell markers

A. MCF-7 cells were pulse labeled with BrdU for 5 days and grown as mammospheres. Cells in passage 0 (P0) mammospheres were 100% labeled (not shown). Serial passage for more than 4 weeks without additional label reveals serial dilution to a single long term label-retaining cell per mammosphere. Green-anti-BrdU; Red-Topro-3. **B.** RT-PCR demonstrated higher expression of embryonic stem cell specific genes including Oct4, Nanog and Sox2 in 3D mammospheres but not in 2D MCF7 cells.

Figure 2. Mammospheres (3D) are resistant to radiation compared to monolayer (2D) cells
Survival assay of cells derived from MCF-7 monolayer cultures (2D) or first (3D P0) or second (3D P1)-generation mammospheres. MCF7 cells in 3D are more resistant to radiation than cells grown in 2D. Second-generation mammospheres (3D P1) are also more resistant than first-generation spheres (3D P0). Single cells were seeded in 2D or 3D culture conditions and irradiated with single, increasing doses (0, 2, 4, 6 Gy) of radiation.

Figure 3. Effect of VA treatment on radiation response of MCF7 cells

A. MCF-7 cells were cultured in adherent (2D) or stem cell enriching culture (3D) conditions. Cells were treated with 1mM VA for indicated time points followed by western blotting using Acetyl-H3 and actin antibodies. **B.** VA-treated monolayer (2D) MCF7 cells showed radiosensitization relative to the untreated cells. **C.** VA-treated 3D MCF7 cells showed radioprotection effect compared to untreated cells. **D.** Short-term culture of pleural effusion cells from an inflammatory breast cancer patient showed radioprotective effect in 3D cells treated with VA. Single cells were seeded in 2D or 3D culture conditions were irradiated with single, increasing doses (0, 2, 4, 6 Gy) of radiation. In Figures 3C and 3D, mammosphere radiosensitivity assay was done with the first generation (P0).

Figure 4. Effect of fractionated irradiation on VA-treated MCF7 cells

A. Fractionated doses of radiation radiosensitizes MCF7 2D cells treated with VA. **B.** Fractionated irradiation does not alter the radiosensitivity of MCF7 3D cells treated with VA. Cells were treated with a single dose of 10 Gy or with five daily doses of 2 Gy or two daily doses of 5Gy. In Figures 4B, mammosphere radiosensitivity assay was done with the first generation (P0).

Figure 5. 2D clonogenic potential of mammospheres obtained from VA-treated MCF7 cells
Mammospheres (3D P0) were dissociated into single cells and plated in 2D clonogenic assays to assess the fraction of clonogenic cells after radiation. VA enriched the clonogenic fraction in mammospheres after radiation ($P<0.001$).