Radiation and the Apo2L/TRAIL Apoptotic Pathway Preferentially Inhibit the Colonization of Premalignant Human Breast Cells Overexpressing Cyclin D1

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Abstract

The role of cyclin D1 overexpression in human breast premalignancy was investigated using immortal, nontumorigenic MCF-10A cells. Previous work documented that cyclin D1 overexpression promoted in vitro anchorage-independent colonization. We now report that the colonization of MCF-10A cyclin D1 transfectants was preferentially inhibited by γ-radiation and specific classes of apoptosis inducers [Apo-2 ligand (Apo-2L), but not tumor necrosis factor α]. Antibody inhibition studies and semiquantitative PCR indicated that radiation inhibition of colonization was partially mediated via the Apo2L/TRAIL pathway. The apoptotic removal of cyclin D1-overexpressing, colonization-competent premalignant breast cells by Apo2L/TRAIL or other biologics may represent a novel approach to the prevention of breast cancer.

Introduction

The elucidation of signaling pathways underlying human breast cancer development can lead to the design of rational prevention strategies. Indeed, it has been speculated that the interruption of signaling pathways in premalignant cells in a prevention setting may be more effective than that in carcinoma cells in a therapeutic setting, where overall levels of instability have increased. We and others have reported that cyclin D1 overexpression is a frequent marker of high risk for the development of invasive breast cancer. In cohort studies of human biopsy lesions, cyclin D1 was frequently overexpressed in ductal lobular units in the margin of the specimen or premalignant lesions, which confer lower risks (1–4). The consequences of cyclin D1 overexpression on breast cancer development may be more complex than G1-S-phase cell cycle progression, based on studies in other cell types that indicate roles in transcriptional regulation, gene amplification, regulation of DNA repair, and the apoptotic process (5–11). The phenotypic consequences of cyclin D1 overexpression were therefore studied in a relevant model system of human breast premalignancy using the MCF-10A cell line, which was derived from a mastectomy specimen containing low-risk premalignant lesions (see the references in Ref. 12). Cyclin D1 overexpression augmented anchorage-independent colonization without conferring full tumorigenicity (12), suggesting that it plays a participatory role in promoting colonization competence in breast neoplastic development. We now report a second function for cyclin D1 overexpression in this premalignancy model system: colonization by cyclin D1-overexpressing MCF-10A cells was preferentially inhibited by γ-radiation, which is used in the treatment of DCIS, and by a specific class of apoptosis inducer, Apo-2L. The latter data suggest that the removal of colonization-competent, cyclin D1-overexpressing premalignant breast cells by specific apoptosis-inducing agents can be hypothesized as a prevention strategy. Moreover, we present evidence that the inhibitory effect of radiation on a breast cell may be mediated by the Apo-2L apoptosis pathway.

Materials and Methods

Cell Culture and Transfection. MCF-10A cells were transfected with culture supernatants from the amphotropic packaging cell line PA317 (American Type Culture Collection) transfected with pBabe retrovirus vector with or without a cyclin D1 cDNA insert under the control of a Moloney murine leukemia virus long terminal repeat. Three cyclin D1-overexpressing clones (D1, D2, and D3) and three randomly selected control transfectants (C1, C2, and C3) were characterized (12); where indicated, pools were made by combining equal numbers of cells from the three clonal transfectants. Anchorage-independent growth was determined in cultures containing soft agar or methylcellulose as described previously (12). Each point represents the mean of three cultures, and each experiment shown is representative of at least three experiments conducted.

Apo-2L Reagents. Soluble Apo-2L (amino acids 114–881) was produced as a His-tagged protein in Escherichia coli, purified by nickel chelate chromatography (13), and diluted in 20 mM sodium acetate, 8% trehalose, and 0.01% Tween (pH 5.5) and stored at −70 °C. For experiments using antibodies, 10 µg/ml antibody to Apo-2L (mouse IgG2a; anti-Apo2L 5C2.4.9 blocking monoclonal antibody) or a control isotype-matched antibody to ragweed (IgG2a; Genentech) was added to the anchorage-independent cultures before solidification.

Flow Cytometry. Cells (1 × 10⁶) from exponential cultures were grown for 24 h in 15-ml conical tubes. Flow cytometry was performed as reported previously (12). For apoptosis analysis, 5 × 10⁵ MCF-10A cells in 15-ml methylcellulose-containing conical tubes were treated for 2–6 h and analyzed for early apoptosis using the Immunotech annexin V-FITC kit according to the manufacturer’s instructions.

PCR Methods. Cells (1 × 10⁶) from exponential cultures were grown in 8 ml of 1% methylcellulose in 15-ml conical tubes. The cells were γ-irradiated at a dose of 0–8 Gy and harvested after 1, 3, 7, and 10 days. Total RNA from these cells was purified using Trizol reagent (Life Technologies, Inc.), and 1 µg of each RNA was treated with 1 unit of RNase-free DNase (Life Technologies, Inc.). The enzymes were heat-inactivated in EDTA, and the total RNAs were reverse-transcribed with superscript II (Life Technologies, Inc.). After titration of the amount of cDNA templates with various primers to linear range, 50 ng of cDNA templates were used in semiquantitative PCR for DcR1 (primers, 5′-GTGGTTGTGGAAAGACTTCACTTGC-3′ and 5′-GCAG-GGCTTCTGTCTGTGGGAAAC-3′; 140-bp fragment expected) and Apo2LL (primers, 5′-TGGCTATGATGAGGTCCAG-3′ and 5′-GGTGGCACCATT-
GACTTGCCAG-3'; 249-bp fragment expected), and 5 ng of cDNA templates were used in semiquantitative PCR for DR4 (primers, 5'-CGATGTGGTCAGAGCTGGTACAGC-3' and 5'-GGACACGGCAGAGCCTGTGCCATC-3'; 217-bp fragment expected), DR5 (primers, 5'-GGGAGCCGCTCATGAGGGAAGTTGG-3' and 5'-GGCAAGTCTCTTCCACGGTCTC-3'; 182-bp fragment expected), DcR2 (primers, 5'-CTTCAGGAAACCAGCTTCCTC-3' and 5'-TTCTCCGTTTTGCTTATCACGC-3'; 200-bp fragment expected), and \( \beta \)-actin (primers, 5'-AAAGACCTGTACGCCAACACAGTGGCTGTCTGG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATCGCT-3'; 220-bp fragment expected). PCR was performed at 93°C for 2 min (first denaturation) and then performed at 93°C for 1 min (denaturation), 52°C for 1 min (annealing), and 72°C for 1 min (extension) for 35 cycles, with a 10-min final extension at 72°C. DNA fragments were electrophoresed on agarose gels, visualized by ethidium bromide staining, and quantitated by densitometry.

**Results and Discussion**

**Preferential Inhibition of Cyclin D1-overexpressing MCF-10A Cell Colonization by \( \gamma \)-Radiation and Apo-2L.**

Three independent MCF-10A transfectants overexpressed cyclin D1 at levels comparable to those observed in DCIS and without concomitant alterations in other cyclin, cyclin-dependent kinase, and inhibitor genes; when compared with randomly selected control transfectants, the major phenotypic change in the cyclin D1 transfectants was increased anchorage-independent colonization in vitro without in vivo tumorigenicity (12). Colonization is thought to be an important facet of the tumorigenesis process, permitting the growth of cells in foreign environments; the demonstration of cyclin D1 promotion of colonization suggests its use as an intermediate end point (biomarker) in breast cancer prevention studies. Clinical trials have indicated that for patients with DCIS, lumpectomy and radiation resulted in local recurrence rates that favored lumpectomy alone (14). Given the frequent overexpression of cyclin D1 in DCIS, the effect of \( \gamma \)-radiation in the anchorage-independent colonization assay was determined (Fig. 1A). Three control transfectants produced 2–10-fold fewer colonies than three cyclin D1 transfectants, confirming our previously published observation (12). Irradiation of cultures with 0.1 Gy did not change this trend. This represents a higher dose than single mammographic exposures (15). The colonization of the control transfectants was unaffected by 1.0 Gy of radiation, but the irradiated cyclin D1 transfectants produced 30% fewer colonies than unirradiated cyclin D1 transfectants. Doses of 1–2 Gy are frequently used for breast cancer radiation therapy (15). Preferential inhibition of colonization was noted in the cyclin D1 transfectants at higher radiation doses of 4 and 8 Gy as well. At 4 Gy of radiation, the control transfectants produced 67% unirradiated colonies/culture, whereas the cyclin D1 transfectants were reduced to approximately 5% of unirradiated colonization. At an 8-Gy dose, colonization was low in all transfectants, but the cyclin D1 transfectants were inhibited by 99% versus 90% for the control transfectants. Similar trends were observed when the three

![Fig. 1. Preferential inhibition of cyclin D1-overexpressing MCF-10A human breast cell colonization by \( \gamma \)-radiation. A, three independent control transfectants (C1, C2, and C3) and three cyclin D1 transfectants (D1, D2, and D3) were irradiated with 0–8 Gy of \( \gamma \)-radiation immediately after plating in anchorage-independent cultures, and colony formation was determined 2 weeks later (mean ± SE). The number in parentheses above each bar indicates the percentage of unirradiated colonization produced by that transfectant. B, flow cytometry of pooled control and cyclin D1 transfectants on day 1 of culture. \( \square \), \( G_0/G_1 \); \( \blacksquare \), \( S \) phase; \( \lozenge \), \( G_2/M \) phase. C, annexin V staining of apoptotic cells from pooled control and cyclin D1 transfectants on day 1 of culture. The percentage of apoptotic cells is indicated.
independent clonal transfectants were pooled and cultured in either soft agar or 1% methylcellulose, which permits harvest of the cells for characterization (data not shown); these trends are consistent with the results of Martin et al. (9) in a breast carcinoma cell line.

Flow cytometry of the pooled transfectants is shown in Fig. 1B. Whereas the increased colonization of the unirradiated cyclin D1 transfectants was accompanied by the expected G1-S-phase progression, the most evident change in the irradiated transfectants was a G2-M-phase buildup characteristic of radiation-treated cells, which did not vary between the control and cyclin D1 transfectants. The data indicate that the traditional G1-S-phase progression role of cyclin D1 was not evident on radiation treatment, suggesting that other mechanisms may be involved. Annexin V staining, which is shown in Fig. 1C, indicated a selective increase in apoptotic cells in the irradiated cyclin D1 transfectants, suggesting increased apoptosis as a mechanism of increased sensitivity.

Preferential Inhibition of Cyclin D1 Transfectant Colonization by Apo-2L. If the induction of apoptosis mediated preferential radiation inhibition of cyclin D1 MCF-10A transfectant colonization, then it could be hypothesized that specific apoptosis inducers might mimic this effect. Fig. 2A shows the effect of the Apo-2L (TRAIL) apoptosis agent in this model system. At 50 ng/ml, the control transfectants produced 59–110% of the colonies that occurred in the absence of Apo-2L; the cyclin D1 transfectants produced 18–27% of the untreated cultures. Similarly, at 100 ng/ml Apo-2L, the control transfectants were inhibited to 30–60% of untreated cultures, whereas the cyclin D1 transfectants were further reduced to 6–8% of untreated cultures. In contrast, Fig. 2B shows the effect of TNF-α, another class of apoptosis inducer that signals through a distinct pathway, on the colonization of pooled transfectants. Equivalent inhibition of the control and cyclin D1 transfectants was observed. Similar trends were observed when individual clonal lines were tested (data not shown).

The data indicate that colonization of cyclin D1-overexpressing MCF-10A cells was preferentially inhibitable by radiation or the Apo-2L class of apoptosis inducer. Flow cytometry confirmed that Apo-2L selectively increased apoptosis in the cyclin D1 transfectants (Fig. 2C).

Does the Apo-2L Pathway Mediate γ-Radiation Inhibition of Cyclin D1-overexpressing MCF-10A Cells? Given the similarity of the inhibitory effects of radiation and Apo-2L in this model system, experiments were performed to determine whether Apo-2L mediated the radiation effect. The mechanism of action of radiation is not completely understood but is thought to reflect DNA damage, membrane-signal events, mitotic-linked cell death, and/or apoptosis in various model systems. Pooled cyclin D1 transfectants were irradiated with 0–4 Gy and plated in soft agar anchorage-independent cultures, and anti-Apo-2L monoclonal antibody 5C2.4.9 or a class-matched control antibody was added to the cultures before semisolidification (Table 1). As a control, irradiation of the cyclin D1 transfectants inhibited colonization in a dose-dependent manner. Addition of anti-Apo-2L had no significant effect (in more than three experiments) on the colonization of unirradiated cyclin D1 transfectants or the colonization of pooled control transfectants under irradiated or unirradiated conditions (Table 1; data not shown). Anti-Apo-2L partially blocked the inhibition of cyclin D1 transfectant colonization by γ-irradiation, whereas the control antibody was without significant effect. At 1 Gy, anti-Apo-2L restored colonization to untreated levels. At higher radiation doses, anti-Apo-2L significantly augmented colonization but did not completely restore wild-type colonization. A precedent for the use of neutralizing antibodies under semisolid culture conditions has been reported previously (16, 17), although it is conceivable that the antibody could have more efficacy where diffusion was not hampered. These data are consistent with the hypotheses that Apo-2L mediated the preferential aspect of radiation inhibition of
cycalin D1 transfectants or that it constituted one of multiple mechanisms involved.

The Apo-2L signaling pathway is complex and incompletely understood. Apo-2L induces apoptosis though two functional receptors, DR4 and DR5, in a Fas-associated death domain-independent manner. Decoy receptors TRID/DcR1 and DcR2 can bind Apo-2L but lack a functional death signaling domain and can therefore competitively inhibit the effect of DR4 and DR5 (13, 18, 19). Semiquantitative reverse transcription-PCR was performed on pooled transfectants from methylcellulose cultures to determine whether changes in ligand or receptor expression could contribute to radiation or Apo-2L pathways involved. Decoy receptors TRID/DcR1 and DcR2 can bind Apo-2L but lack a functional death signaling domain and can therefore competitively inhibit the effect of DR4 and DR5 (13, 18, 19). Semiquantitative reverse transcription-PCR was performed on pooled transfectants from methylcellulose cultures to determine whether changes in ligand or receptor expression could contribute to the effects observed (Fig. 3). Pooled control (Fig. 3, Lanes C) or cyclin D1 (Fig. 3, Lanes D) transfectants expressed comparable amounts of Apo-2L mRNA under all culture conditions tested on day 1 of culture, indicating constitutive Apo-2L production. Similar results were observed in mRNA preparations harvested from days 3, 7, and 10 of culture (data not shown).

The relative mRNA levels of the Apo-2L receptors in methylcellulose cultures are shown in Fig. 3. DR4 receptors were present in both the control and cyclin D1 transfectant pools and were not altered by radiation. In contrast, DR5 receptor mRNA was more abundant in unirradiated or low-level (1 Gy) irradiated control transfectants than cyclin D1 transfectants. At 2-4 Gy doses of radiation, both the control and cyclin D1 transfectant pools produced relatively high levels of DR5. The magnitude of the increase from unirradiated conditions was higher for the cyclin D1 transfectants than for the control transfectants. Similar trends were observed on days 3, 7, and 10 of culture (data not shown). DcR2 decoy receptor was present in all cultures but exhibited a slight (30% by densitometry) decrease in expression in the 2-4 Gy-irradiated cyclin D1 transfectants.

The responsiveness of breast epithelial cells in vivo to Apo-2L may reflect the receptor expression level changes noted above, as well as other influences. These include the total amount of available Apo-2L, i.e., the epithelial contribution quantified in Fig. 3, possible stromal or other microenvironmental production, and bloodstream levels. Other factors could include the threshold amount of ligand:receptor interaction needed for cellular death as well as the existence and activity of signaling pathways that could interrupt the Apo-2L signaling pathway. With regard to the latter, the p53 gene in MCF-10A cells is wild type, and p53 expression levels under the conditions examined in this report did not change significantly, suggesting that mutations have not occurred that could potentially contribute to radiation or Apo-2L pathway effects (data not shown). Within these limits of our knowledge, two hypotheses can be advanced: (a) the increased colonization potential of cyclin D1 transfectants may reflect increased G1-S-phase progression as well as decreased apoptosis in response to Apo-2L due to poor DR5 expression. The mechanism for the increased sensitivity of cyclin D1 transfectants to Apo-2L inhibition of colonization (Fig. 2) is not obvious, but it may reflect our preliminary data that treatment of transfectants with Apo-2L alone caused additional changes in receptor expression (data not shown); and (b) the preferential sensitivity of cyclin D transfectants to γ-irradiation is accompanied by a striking increase in DR5 receptor and a minor decrease in DcR2 decoy receptor mRNA levels, which may lead to increased Apo-2L-induced apoptosis.

Cyclin D1 overexpression is a frequent event in DCIS, which confers a high risk for the patient’s development of invasive breast cancer. Many studies have investigated the functional impact of cyclin D1 overexpression in breast cancer and other systems (reviewed in Ref. 20). Our data in breast premalignancy are confined to intermediate end points and indicate that cyclin D1 overexpression functionally contributes to increased anchorage-independent colonization (12). We now demonstrate that colonization in cyclin D1-overexpressing cells is preferentially inhibitable by γ-radiation and by Apo-2L. Two lines of evidence suggest that the Apo-2L pathway mediates, at least in part, the γ-radiation effect: (a) anti-Apo-2L partially blocked radiation inhibition of cyclin D1 transfectant colonization; and (b) a specific molecular change in the Apo-2L signaling pathway, increased DR5 expression, was associated with irradiation of cyclin D1-overexpressing cells. It will be of great interest to investigate whether specific molecular changes in the Apo-2L pathway occur in human breast lesion cohorts to determine the generality of the findings reported herein. Cohorts of interest include premalignant and DCIS breast lesions that confer stratified risk estimates for the patient’s development of invasive breast cancer and DCIS specimens that did or did not recur after radiation therapy. Finally, the data permit the development of hypotheses concerning breast cancer prevention in women at high risk: if the cyclin D1 functional connection to apoptosis via the Apo-2L or a similar signaling pathway is confirmed in human tumor cohort or other studies, the data suggest the hypothesis that preferential elimination of cyclin D1-overexpressing, colonization-competent cells from the ductal system by an apoptosis-related biological could represent a chemoprevention approach.
References


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