Cancer Chemotherapy by Deoxynucleotide Depletion and E2F-1 Elevation

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Abstract

We propose that the lethality of commonly used anticancer drugs, e.g., methotrexate and cisplatinum, is due to, at least in part, to an increase of the E2F-1–mediated apoptotic cascade. The drugs directly or indirectly decrease deoxynucleoside triphosphates. The E2F family acts to provide control of S phase by transcribing genes required for deoxynucleoside triphosphate and DNA synthesis. Thus, a mechanism for control of E2F-1 is essential, a signal safeguarding against aberrant or uncontrolled cell proliferation. We have proposed a feedback control by NTPs that down-regulates E2F-1. Here, we provide evidence in support of this hypothesis. (Cancer Res 2005; 65(17): 7809-14)

Introduction

Functions of E2F-1. E2Fs are a family of transcription factors with pivotal roles in activating and coordinating events connected with DNA duplication, cell cycle arrest, and apoptosis (1). E2F-1 in particular is a master regulator of restriction (R) point and phase transition (2). E2F-1 activity is released in late G1 phase, triggered by dissociation from retinoblastoma protein (pRb). Injection of E2F-1 permits S phase entry (3). E2F-1 is a factor for its own transcription, and so its autocatalytic production increases rapidly. Then, bound to DP1 or DP2, it induces multiple enzymes needed for the synthesis of deoxynucleoside triphosphate (dNTP; thymidylate synthase and ribonucleotide reductase-2), DNA synthesis (DNA polymerase-α), damage repair, and regulation (cyclin A, cyclin E, and cdk2). G1 restriction point control of E2F-1 by pRb is lost in many cancer cells, as evidenced by observations of its elevation in many cancers (4). Although it is not mutated in human cancers, its expression is very frequently increased by other mutations, e.g., of pRb or cyclin A (5). E2Fs are induced by growth factors in normal melanocytes, but are five times higher in melanomas, probably because of release of control by pRb. The E2F family is up-regulated three to eight times in induced mouse tumors (6). Most human cancers are deregulated in transcriptions dependent on E2F-1 (7, 8). Their S phase enzymes and transcription factors are more active and dNTP concentrations are about 10 times higher (9).

A paradox is that excessive E2F-1 upon aberrant regulation of cell growth, such as that caused by drugs, stress, hypoxia (10), or in cancers is a signal for activation of apoptosis (11). Conversely, a deficiency of E2F-1 decreases S entry, activation of responsive genes and apoptosis (12). Only E2F-1 of the E2F family is involved in apoptosis (13, 14). Adenovirus-mediated overexpression of E2F-1 induces apoptosis in human carcinoma lines (15), which is inhibited by pRb (16). This lethal effect may function as a tumor surveillance mechanism, in which surplus or shortage of a molecule needed for proliferation signals apoptosis, thereby protecting the organism from potential cancers. This overexpression activates a fail-safe apoptotic pathway (17) for cancer cells and possibly for premalignant cells (18). E2F-1 is redirected from transcribing DNA synthetic genes to apoptotic ones (19). Transfection of E2F-1 initiates synthesis of S phase enzymes that is followed by apoptosis (20). Proof of principle is found in: (a) E2F-1 knock-out mice are highly tumor prone (21) partly because of lack of apoptosis (22); (b) E2F-1 inhibits activation of antiapoptotic nuclear factor-κB (17, 23); and that (c) β-lapachone blocks tumor growth in mice through an apoptotic mechanism that depends on activation of the E2F-1 mediated checkpoint (24).

Further research is required to determine the mechanisms involved in (a) the rapid E2F increase at G1-S, (b) removal of E2F at the end of S when DNA synthesis slows, (c) the basis for elevated E2F in tumor cells, (d) how elevated E2F is controlled to prevent apoptosis, and (e) how E2F can be manipulated for chemotherapy.

Because control of excessive E2F-1 is essential for the survival of normal cells (25), a mechanism that regulates E2F-1 is very important to prevent excess. We have proposed a negative feedback activity that could provide a regulation of E2F-1 in the unperturbed cell cycle, by which elevated dNTP pools decrease E2F-1 (26), dNTPs could directly bind to E2F-1 or to its accessory proteins, or act indirectly by modifying kinases that phosphorylate E2F-1.

“Thymineless” death, reported by Cohen and Barner 50 years ago for an Escherichia coli mutant (27), is effective in mammalian cells (28). Anticancer agents such as methotrexate, 5-fluorodeoxyuridine (5-FdUr), IMP dehydrogenase inhibitors (29), and hydroxyurea inhibit dNTP biosynthesis (30, 31). Their apoptotic action is prevented by supplying dNTPs via the salvage pathway (32). Some major anticancer agents induce DNA strand breaks, which by activating poly(ADP-ribose) polymerase (PARP) activity depletes nicotinamide adenine nucleotide (NAD), and thereby decreases dNTP synthesis catalyzed by ribonucleotide reductase (33). We propose that apoptosis is activated by imbalances of dNTPs which elevate E2F-1, and is one of several mechanisms by which antineoplastic drugs cause cell death.

Materials and Methods

Cell culture. Human colon cancer cell lines, SW 480 and DLD, and prostate cancer cell lines (LNCaP; DU145, and PC-3) were purchased from the American Type Culture Collection, Manassas, VA. LNCaP cells were grown in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS, 100 µg/mL streptomycin, 100 units/mL penicillin, and 10 nmol/L testosterone. DU145, PC3, and BPH-1 cells were grown in DMEM (Life Technologies) containing 10% FCS, 100 µg/mL streptomycin and 100 units/mL penicillin, in a humidified incubator with 5% CO2 and 95% air at 37°C (34).

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Cell proliferation/viability assay. LNCaP, DU145, PC-3, or BPH-1 cells were seeded at a density of 6,000 cells/well in 96-well plates in appropriate media and allowed to grow 24 hours. Cells were then treated with 5-FUdR (1 mmol/L), Methotrexate (10 μmol/L) or aphidicolin (1 μg/mL; Sigma Chemical Co., St. Louis, MO) for 48 hours. Concentration of each of the drugs used in this study was based on the doses required for 85% to 90% inhibition of DNA synthesis in LNCaP, PC-3, and DU145 cells as measured by 3H-thymidine incorporation into DNA, and are comparable to those used in studies with other cell types (1–3). Colorimetric assay to detect the live cells in each well was done by adding 20 μL/well of CellTiter96 AQ One Solution (Promega Corp., Madison, WI) and incubating for 1 hour in a humidified incubator with 5% CO2 and 95% air at 37°C. Absorbance at 450 nm was then recorded using microplate spectrophotometerμQuant equipped with KCJunior Data Analysis Software (Bio-Tek Instruments Inc., Winooski, VT). Each point represents the mean ± SD of four replicates.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. DLD1 cells were cultured in DMEM with 10% bovine serum. Ten thousand cells for each well in 96-well plates were applied. After proper treatment, 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich) was spotted into each well at final concentration of 0.5 mg/mL in the medium and incubated at 37°C for 2 hours. After incubation with solubilizing buffer (2 mN HCl, 1% SDS in isopropanol) at room temperature for 20 minutes, the plates were scanned at 570 nmol/L in microplate reader (Molecular Devices). Each piece of data represented the average of readings from six wells.

Western blot analysis. Cells grown in 150 mm cell culture dishes (Corning, Inc., NY) were treated with 5-FUdR, methotrexate, or aphidicolin for 24 hours and whole cell extracts were then prepared as described (34). An equal amount of protein in each fraction was subjected to denaturing 12% PAGE (SDS-PAGE) and then transferred to nitrocellulose membranes. Individual membranes were probed with monoclonal antibodies against pRB and p53, or polyclonal antibodies against E2F-1, p21Cip1, and cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using horseradish peroxidase conjugated secondary antibodies and SuperSignal West Pico chemoluminescent substrate (Pierce, Rockford, IL) and visualized using X-ray film.

To test for E2F-1 induction in SW480 cells, methotrexate (5 μmol/L) was added and Western blot analyses was done. Culture conditions were like those described (24, 35). For E2F-1 knockdown, DLD1 cells were transfected with Smartpool E2F-1 small interfering RNA (siRNA; Dharmacon, Lafayette, CO) for 4 days. After transfection for 24 hours, cells were treated with 10 μmol/L cisplatin for 48 hours. Cisplatin was not added to the control samples. Then Western blot analyses were done.

Results

Methotrexate increases E2F-1. Methotrexate blocks dihydrofolate reductase and thereby thymidylate synthesis. To test whether this nucleotide depletion increases E2F-1, we treated the SW480 human colon cancer cell line with 5 μmol/L methotrexate and at intervals determined E2F-1 protein. It was found to increase greatly by 6 hours (Fig. 1). This E2F-1 is functional because cyclin A, which is transcribed by E2F-1, later increased. An extensive (8-fold) increase was observed by 48 hours. Similar results were obtained
with human colon cancer cell line DLD1 exposed to methotrexate or to 10 to 20 μmol/L cisplatin (data not shown).

Role of E2F-1 in cisplatin lethality. There are a few reports of increased E2F-1 following DNA damage by an antineoplastic drug (10) or radiation (36). Knockout experiments were applied to test an E2F-1 link between DNA damage and cell death. Seventy-two hours after E2F-1 siRNA was introduced into DLD1 tumor cells, they contained very little E2F-1 (90% reduction) relative to controls. The treated cells were more resistant to up to 30 μmol/L cisplatin, supporting the role of E2F-1 in damage-induced death (Fig. 2A). DLD1 cells were stably transfected with an E2F-1 expression construct inducible by tetracycline, and then treated with 10 μmol/L cisplatin for 48 hours. Half the cells with high E2F-1 (tetracycline + cisplatin) were killed (Fig. 2B). There was <15% killing by cisplatin in low E2F-1 conditions. These results are consistent with a major role of E2F-1 in lethality of cisplatin.

Effect of inhibitors of DNA synthesis on viability and E2F-1 levels of prostate cancer cells. Prostate cancers are relatively resistant to chemotherapy (37). Treatment with 5-FUdR, methotrexate, and aphidicolin at concentrations that caused 85% to 90% inhibition of DNA synthesis in LNCaP, PC-3, and DU145 cells had varied effects on viability (Fig. 3) and E2F-1 levels (Fig. 4). At the concentration of each of the drugs studied, 5-FUdR was most effective in decreasing the viability of LNCaP and PC-3 cells, as compared with methotrexate or aphidicolin (Fig. 3). Under exponentially growing (control) conditions, E2F-1 levels were 5- and 10-fold higher in LNCaP cells (pRB wt, p53 wt, p16Ink4a null) than in PC-3 (pRB wt, p53 null, p16Ink4a null) or DU145 (pRB mutant, p53 mutant, p16Ink4a mutant) cells, respectively (Fig. 4).

Drug-induced changes in E2F-1 levels varied depending on pRB and p53 status of each of these cell lines. For instance, LNCaP cells with wild-type pRB and p53 showed very little change in the presence of methotrexate or aphidicolin, but decreased 2-fold in response to 5-FUdR. PC-3 cells with wild-type pRB but p53 null showed a 2-fold increase in response to 5-FUdR and a 3-fold increase in response to methotrexate and aphidicolin. DU145 cells with mutated pRB and mutated p53 showed a moderate increase but overall levels were 2- to 4-fold lower than those in PC-3 cells. Some of the drugs also increased cyclin A, pRB, and p21Cip1. Changes in these proteins were more evident in LNCaP cells that have both pRB and p53 wild-type. Cyclin A levels were highest in DU145 cells, but lower in LNCaP and PC-3 cells. pRB levels were highest in LNCaP cells, and p53 was highest in PC-3 cells. p21Cip1 levels were highest in DU145 cells.

Figure 3. Effect of inhibitors of DNA synthesis on the viability of prostate cancer cells. Exponentially growing LNCaP, PC-3, and DU145 cells were treated with 1 mmol/L 5-FUdR, 10 μmol/L methotrexate (MTX), or 1 μg/mL aphidicolin (Aph) for 48 hours, and the growth and viability was determined as described in Materials and Methods.

Figure 4. Effect of inhibitors of DNA synthesis on E2F-1 levels in prostate cancer cells. Exponentially growing LNCaP, PC-3, and DU145 cells were treated with 1 mmol/L 5-FUdR, 10 μmol/L methotrexate (MTX), or 1 μg/mL aphidicolin (Aph) for 24 hours, cell extracts were prepared and the level of E2F-1 and other cell cycle regulatory proteins was determined as described in Materials and Methods. Density of E2F-1 bands was determined using Stratagene Eagle Eye II Still Video System and EagleSight software (version 3.2; Stratagene, La Jolla, CA). All values are normalized to the density of E2F-1 band in control LNCaP cells.
p53 wild-type than in PC-3 or DU145 cells that have pRB and/or p53 mutated (Fig. 4). Lethality by E2F-1 is therefore variable with the cells and applied drugs. Results with nontumorigenic BPH-1 cells were similar to those observed with DU154 (data not shown).

**Discussion**

We present here evidence for a novel mechanism for the lethality of frequently applied antineoplastic agents, based on the demonstration that drugs which alter dNTPs increase the transcription factor E2F-1, which is apoptotic at elevated concentrations (17). Methotrexate applied at low toxicity raised E2F-1 more in PC-3 than in LNCaP or DU-145 prostate cells. The proposed mechanism of the activity thus depends on critical genetic differences manifest in the cell lines used. E2F-induced apoptosis is suppressed by pRB, LNCaP cells that are pRB+ are less susceptible to agents that increase E2F-1 than are pRB- cells. Others have shown that antineoplastic drugs including methotrexate caused apoptosis of Rb−/− mouse embryo fibroblasts. In contrast, Rb+/− and Rb+/+ cells were only growth-arrested. p53 accumulated in all of these cells (12). Because pRB inactivates E2F-1, these results suggest that this apoptosis involves E2F-1.

We have proposed a feedback control that regulates E2F-1 (Fig. 5). E2F-1 (with DP) is a transcription factor *(top, dashed lines)* for its own production, for enzymes of dNTP and DNA synthesis, and for cyclin A. Degradation of E2F-1, which has a 70-minute half-life (38), via cyclin A/cdk2-skp2 ubiquitin ligase-proteosome activities is shown (center). Inactivation of cdk2/cyclin A is linked to elevated E2F-1, and this process is apoptotic (8, 39, 40). Degradation of E2F-1 is proposed to be activated by excessive dNTPs *(diagonal dashed line)*. E2F-1 therefore would increase when dNTPs are depleted, as by methotrexate or by DNA damage *(right)*. This elevated E2F-1 then activates apoptosis through a process that involves p53 or p73, cytochrome c, and caspases *(left)*.

We report here that methotrexate raised E2F-1 in cancer cell lines. dNTPs supplied by the salvage pathway can prevent the apoptosis caused by inhibitors including methotrexate (32), consistent with the depletion of these compounds causing apoptosis. And providing the four dNTPs counteracts hydroxyurea lethality, although it did not prevent inhibition of DNA synthesis (41). Fluorodeoxyuridine (42, 43) decreased E2F-1 in LNCaP cells (Fig. 4), possibly by mimicking dTTP.

Few experiments connect changes of E2F-1 with levels of the four dNTPs. Which dNTP is active? All have been implicated. The literature is not specific, and imbalanced dNTPs are often cited as causing apoptosis (44). A linkage of antineoplastic drugs to apoptosis would be strengthened by data on concentrations of functional dNTPs. Numerous data on drugs, dNTP pool changes and apoptosis have been summarized (32, 45). Concentrations of total cellular dNTPs are not meaningful because they are out of proportion with those in sequestered pools and microcompartments involved in regulation of DNA synthesis (46, 47), and in the protein complex that synthesizes DNA (48).

Many anticancer agents directly damage DNA and these can raise E2F-1 and can cause apoptosis (25). These include ionizing radiation, alkylators, cisplatin, Adriamycin, and etoposides. Knockout of E2F-1 with siRNA decreased lethality of the apoptotic anticancer agent cisplatin, and elevated E2F-1 increased its lethality. The mechanism involves enzymes that assemble at these sites of DNA damage and regulate arrest, repair, and apoptosis (49, 50). PARP, a component of the 40-protein DNA replication complex, is activated when it binds to DNA strand breaks, and it is a positive regulator of the resulting responses (51, 52). Initially, PARP promotes repair and prevents genomic instability, and then is involved in DNA damage–induced apoptosis (53). PARP uses NAD to synthesize poly-ADP-ribose chains of up to 200 units/site, and also monomers, attached to DNA binding proteins, and has numerous regulatory functions (54). PARP inhibitors preserve NAD and membrane potential, prevent release of apoptotic factors, and reduce death (55, 56). A rapid PARP-dependent increase of E2F-1 is caused by Adriamycin or etoposide (57). PARP depletes the entire cytosolic NAD pool in only a few minutes (33, 45, 51). Thereby, DNA-damaging agents block dNTP synthesis, similarly to the antimetabolites listed above (58). Added NADH prevents increased p53 and apoptosis.
caused by X-rays, which was proposed to block free radicals that damage DNA (59). These results support a major role of NAD depletion in damage-induced apoptosis.

That conventional cancer chemotherapeutic drugs can decrease dNTPs, increase E2F-1, and thereby cause apoptosis (60), provides opportunities for the development of novel anticancer agents (8). E2F-1 could be increased because imbalanced dNTPs either activate its synthesis or inhibit its degradation. Engineered mutations of E2F-1 that block binding of cyclin A/cdk2 neutralize its effect to increase the amount. This result led to synthesis of short peptides that compete with E2F-1 for interaction with cyclin A/cdk2. These selectively cause apoptosis if the peptide is able to prevent changes in cell cycle phase of transformed but not of untransformed cells (61, 62), and are active in vivo (63).

Therapeutically possible include kinase inhibitors that block E2F-1 degradation (40). Numerous inhibitors of cdk2, such as roscovitinin (64), flavopiridol (65), and other kinase inhibitors (66) prevent proteolysis and raise E2F-1, causing apoptosis (67). Also, the transcription inhibitor actinomycin D (68) is being developed as an anticancer agent (69). We hypothesize that drugs which deplete NTPs could also selectively apoptotic against cancer cells by further raising their already elevated E2F-1. Some compounds can however arrest growth without causing DNA damage (70). β-Lapachone, an ortho-naphthoquinone, can cause apoptosis or necrosis without DNA damage. It increases E2F-1 (24) and decreases dNTPs and ATP (71).

Combined therapies, with drugs that increase E2F-1 plus others are possible. High E2F-1 sensitizes apoptosis of colon cancer cells by camptothecin, in culture and in mice (72). PARP inhibitors and ATP depletion are a useful combination therapy (49, 73). Drugs that prevent Skp2-mediated degradation of p27 and arrest proliferation (74) should also block ubiquitination-prolyosylation of E2F-1 and cause apoptosis. There are as yet few reports of such effects and are open for investigation.

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