Tumor Necrosis Factor α-dependent Drug Resistance to Purine and Pyrimidine Analogues in Human Colon Tumor Cells Mediated through IKK*

Ling-Chi Wang‡, Cindy Yen Okitsu‡, and Ebrahim Zandi‡§

From the Department of Molecular Microbiology and Immunology, USC/Norris Comprehensive Cancer Center, Keck School of Medicine at USC, Los Angeles, California 90033

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Development of drug resistance in cancer is one of the main challenges in chemotherapy, and many mechanisms are still unknown. In this study, we show that tumor necrosis factor α (TNFα) increases postdrug survival from 5-fluoro-2'-deoxyuridine (FdUrd) in two human colon tumor cell lines. This resulted in the development of drug-resistant cells in a TNFα-dependent manner. Interestingly, although the drug-resistant cells were selected using FdUrd, they are also resistant to a number of other antimetabolites in the DNA synthesis pathway in a TNFα-dependent manner. Only in the drug-resistant cells (p35-colo201) TNFα treatment resulted in G0-G1 arrest but not in the parental colo201 and other cell types. Blocking TNFα-induced cell cycle arrest sensitized drug-resistant cells to FdUrd. TNFα-induced cell cycle arrest required IKK. IKK inhibition by a small molecule inhibitor or by the knockdown of IKKα, IKKβ, or RelA/p65 using siRNA, but not the inhibition of JNK, MEK, p38, or caspase-8 pathways, blocked TNFα-induced G0-G1 arrest and restored sensitivity to FdUrd of drug-resistant cells. TNFα reduced the transscripts and protein levels of phosphorylated retinoblastoma protein (Rb), Rb, E2F1, and Cdk4 only in drug-resistant p35-colo201 cells. This effect of TNFα was reversed by IKK inhibitor, suggesting that TNFα-induced cell cycle arrest is probably due to the reduction of Rb, E2F1, and Cdk4. Taken together, this study shows that, in vitro, TNFα-induced cell cycle arrest through IKK can provide a mechanism for the development of drug resistance to anti-cancer drugs, purine and pyrimidine analogues.

Intrinsic or acquired resistance to anti-cancer drugs is the major cause of failure in cancer chemotherapy. Intrinsic resistance is present at the time of treatment and often can be diagnosed. Acquired resistance develops during and after chemotherapy (1, 2). Known intrinsic mechanisms of drug resistance in cancer include changes in drug influx and efflux, intracellular activation and catabolism, or drug target modifications (2–4). For example, increased expression of thymidylate synthase in some colon tumors is associated with intrinsic fluoropyrimidine resistance (5). However, there still are a large number of drug-resistant cancers with unknown mechanisms (6). Unraveling these mechanisms is essential for rational design of combination chemotherapy.

Antimetabolites of purine and pyrimidine nucleotide metabolism such as 5-fluorouracil, 5-fluoro-2'-deoxyuridine (FdUrd), methotrexate, 3-deazauridine, ribavirin, hydroxyurea, and cytosine arabinoside (Ara-C; an inhibitor of DNA polymerases α and β) inhibit enzymes at different steps of biosynthetic pathways of DNA and RNA (7) and are widely used in cancer chemotherapy. These agents inhibit the proliferation of dividing cells and very importantly exhibit relatively lower toxic side effects than other drugs such as DNA-damaging agents (8). However, a relatively high number of cancers have either intrinsic or acquired resistance to these agents (9). To overcome the drug resistance and increase efficacy, these antimetabolites are combined together or with cytotoxic agents (10). However, the high degree of systemic toxicity and harmful side effects of cytotoxic drugs limit their usage.

Purine and pyrimidine antimetabolites affect both DNA and RNA metabolism. The effects on RNA and proteins are transient, and thus the main pathway of cytotoxicity is through the arrest of DNA synthesis in the S-phase of the cell cycle (9). Fully differentiated or “quasy” quiescent cells, which are arrested in the G0-G1-phase of the cell cycle are resistant to the anti-proliferative activity of antimetabolites (7). Furthermore, slowly growing tumors show poor sensitivity to chemotherapy. Conditions or extracellular signals that cause a slow growth or a G0-G1 cell cycle arrest in cancer cells could provide conditions for survival against antimetabolites. Cytokines produced by tumors or by cells of the immune system and by cells in a tumor microenvironment could generate such signals.

Many cytokines regulate cell proliferation, differentiation, and death. Cytokines such as TNFα, TGFβ, and IL-1β, for instance, have cell-stimulatory or growth-inhibitory effects in different cell types. TNFα is a proinflammatory cytokine produced by macrophages and other immune cells in local responses to infection, tissue injury, and repair. TNFα has a wide range of functions including stimulation of the immune system, tissue differentiation, induction of programmed cell death, and tissue repair (11, 12). In some cells, TNFα induces cell cycle

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† These authors contributed equally to this work.

‡ A 2001 PEW Scholar; supported by National Institutes of Health Grant R01 MG065525. To whom correspondence should be addressed: USC/Norris Comprehensive Cancer Center, 1441 Eastlake Ave., Norris 6429, Los Angeles, CA 90033. Tel.: 323-865-0644; Fax: 323-865-0645; E-mail: Zandi@usc.edu.

1 The abbreviations used are: FdUrd, 5-fluoro-2'-deoxyuridine; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin; Rb, retinoblastoma protein; FACS, fluorescence-activated cell sorter; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; siRNA, small interfering RNA; CKI, cyclin-dependent kinase inhibitor; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase.
arrest at G1-G2 phase (13–16). TNFα was also shown to induce resistance to doxorubicin in a lung cancer cell line by shifting S phase to G0-G1 phase (17). TNFα is produced by many tumors (18, 19), and its level is elevated in the sera of cachectic patients with advanced tumors (20). TNFα at high levels (10–7 M) kills certain tumors, but it also causes systemic toxicity leading to septic shock (21). TNFα at levels comparable with its concentration in local inflammatory areas (10–3 M) causes G0-G1 arrest in the A375-C6 melanoma cell line (22). The mechanism of TNFα-induced cell cycle arrest includes the induction of hypophosphorylated retinoblastoma protein (Rb) in A375-C6 cells (23). Unphosphorylated or hypophosphorylated Rb is present at early to middle G1 phase, which associates with E2F1 and suppresses its activity. Phosphorylation of Rb by Cdk4 and Cdk6 at late G1 phase results in activation of E2F1 and S phase entry of cells (24).

TNFα exerts its biological functions by activating signaling pathways that regulate NF-κB, AP-1, p38, ERK, and death pathways. Activation of IKK/NF-κB by TNFα protects cells from apoptosis and is required for production of cell cycle and immune regulatory proteins (11). NF-κB-s are homo- and heterodimeric transcription factors that are kept in an inactive state by inhibitory IκB proteins. Activated NF-κB phosphorylates regulatory serines on IκB inhibitors, marking them for polyubiquitination and subsequent degradation. Free NF-κB-s binds to target gene promoters and activates the rate of transcription of a large number of genes including cyclin D and antiapoptotic proteins (25). Hence, IKK/NF-κB activation in most cell types results in increased proliferation and prevention of cell death. Activation of NF-κB by a number of anti-cancer drugs has also been shown to protect cells from death, thus reducing the cytotoxic effect of drugs (26). IKK complex is a multisubunit kinase. It is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory factor, IKKγ. IKKβ and IKKγ are required for activation of NF-κB by most of the proinflammatory cytokines such TNFα and IL-1 (27).

This paper reports that TNFα, in an IKK-dependent manner, increases postdrug recovery and survival of a human colon cancer cell line from FuUrd, resulting in the development of drug-resistant cells. Only in drug-resistant cells, through IKK, does TNFα prevent S phase entry by reducing phospho-Rb, Rb, E2F1, and Cdk4 levels.

**MATERIALS AND METHODS**

**Cell Culture and Small Molecule Inhibitors—**Human colon cancer cell lines, colo201 and colo320, were generously provided by Dr. Heinz Lenz. The cells are maintained in RPMI1640 (Cellgro) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the presence of 5% CO2. The supplement reagents were purchased from Invitrogen. Cells were trypsinized and split 1:10 every 3–4 days. On the day before each experiment, the cells were seeded at the indicated densities to maintain exponential growth throughout the duration of experiment. Small molecule inhibitors were purchased from Calbiochem. PS1145 was a generous gift from Millennium Pharmaceuticals, Inc.

**Cell Proliferation Assay—**Cells were seeded at 2500 cells/well in a 96-well plate in triplicate and treated with or without drugs the next day, and cell proliferation was followed each day using the Promega CellTiter 96AQuous One Solution Cell Proliferation Assay kit according to the manufacturer’s recommendations. The concentration of TNFα was 10–20 ng/ml, and the concentration of FuUrd (Sigma) was 10 μM unless otherwise stated. The percentage of cell survival was calculated by CellTiter 96AQuous One Solution Cell Proliferation Assay kit according to the manufacturer’s recommendations. The concentration of TNFα was 10–20 ng/ml, and the concentration of FuUrd (Sigma) was 10 μM unless otherwise stated. The percentage of cell survival was calculated by CellTiter 96AQuous One Solution Cell Proliferation Assay kit according to the manufacturer’s recommendations.

**Cell Death Assay—**colo201 cells were seeded at 500,000 cells/well in 6-well plates in triplicate and grown for 24 h. Cells were left untreated or treated with FuUrd (10 μM) or FuUrd + TNFα (20 ng/ml) for 24, 48, 72, or 98 h. At the indicated times, cells were harvested by scraping and counted, and apoptosis was determined using the ApoAlert Annexin V staining kit (BD Biosciences) using the manufacturer’s specifications. The results are shown as mean ± S.D. of three independent experiments.

**Survival Plate Assay—**Cells were seeded at 250,000 cells/well in a 6-well plate in triplicates. FuUrd at 10 μM or other drugs as indicated in the figures were added individually or in combination with TNFα at 20 ng/ml for 5 days. Cells were then washed free of the drugs, fresh media were replenished every 3–4 days thereafter, and each plate was followed for 9–21 days postrelease. The plates were stained and documented using a scanner, and colonies were counted manually. Drug combination usually consisted of 1–6 h of TNFα pretreatment, followed by FuUrd or other drugs unless otherwise stated.

**Cell Cycle Analysis by a Fluorescence-activated Cell Sorter (FACS)—**For each assay ~5 × 106 cells were used. At the time of harvest, cells were washed with phosphate-buffered saline and removed from dishes by trypsin. Cells were fixed by 70% ethanol and stained in the dark with 1 μg/ml propidium iodide in phosphate-buffered saline containing 1% Triton X-100 and 1 mg/ml RNase A. Cell cycle profiles were analyzed by FACS.

**μRNA (H)Thymidine Incorporation Assay—**Cells were seeded in a 96-well plate at 1000 cells/well. After 24 h, drugs and/or TNFα were added as indicated in the figures. One μCi of [3H]thymidine (0.5 Ci/mmol; Amersham Biosciences) was added 24 h later, and cells were harvested on a glass fiber filter using a Skatron cell harvester (Skatron Instruments, Lier, Norway) and analyzed in the Tri-Carb Liquid Scintillation Analyzer (model 2100TR, Packard Instrument Co.). Results are mean cpm of triplicate wells.

**Cell Lysate Preparation—**Whole cell lysates were prepared as described (28). Briefly, cells were lysed in an all purpose buffer (consisting of 20 mM Tris, 20 mM β-glycerophosphate, 10 mM sodium orthovanadate, 2.5 mM metabisulphite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, pH 7.6) supplemented with 300 mM NaCl, 1% Triton X-100 and freshly added protease/phosphatase inhibitors (2.5 μg/ml aprotinin, 20 μg/ml aprotinin, 8.5 μg/ml bestatin, 2 μg/ml pepstatin A, 2 mM dithiorthiol, 1 mM phenylmethylsulfonyl fluoride, 1 mM p-nitrophenol phosphate). Lysed cells were centrifuged in Biofuge pico at maximum speed for 30 min at 4 °C, and whole cell lysates were separated and stored at −80 °C after protein concentrations were determined (Bio-Rad).

**In Vitro Kinase Assay—**Whole cell lysates (100 μg) were immunoprecipitated with 1 μg of rabbit anti-IKKα antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight in the presence of Protein G-Sepharose (Amersham Biosciences). Kinase activities were determined as described (29). Briefly, kinase activity was assessed after 30 min at 30 °C in 30 μl of kinase buffer (20 mM Tris, 10 mM MgCl2, pH 7.5) mixture containing 20 μM ATP, 2 mM dithiothreitol, 5–10 μg of [γ-32P]ATP, and 1 μg of glutathione S-transferase-IκBα–1–54 protein as substrate, followed by 10% SDS-PAGE. The unbound [γ-32P]ATP from the dye front was removed before the remaining SDS-polyacrylamide gel was subjected to protein transfer onto a polyvinylidene difluoride membrane (Bio-Rad) at 300 mA for 2 h. The membrane was exposed to a PhosphorImager (Amersham Biosciences) overnight and analyzed via ImageQuant (Amersham Biosciences) software. Each membrane was blocked in 5% milk and then probed with mouse anti-IKKα and mouse anti-IKKβ antibodies (Pharmingen) to judge immunoprecipitation efficiency. After stripping (50 mM glycine, 200 mM NaCl, pH 3.0, 2 h at 50 °C), each membrane was blocked and probed with mouse anti-IKKβ antibodies (Imgenex, CA) again to evaluate IKKβ levels immunoprecipitated.

**Immunoblot Assay and Antibodies—**For immunoblot, 20–40 μg of whole cell lysates was used. The following antibodies were purchased from Cell Signaling: anti-phospho-Rb (catalog no. 9308; phosphoserines 807 and 811), anti-phospho-c-Jun (catalog no. 9261; phosphoserine 63), anti-phospho-ATF2 (catalog no. 9221; phosphothreonine 71), anti-Rb (catalog no. 9309), anti-phospho-IκBα (catalog no. 9241), anti-IκBα (catalog no. 9242), anti-Cdk4 (catalog no. 2906), and anti-Cdk6 (catalog no. 3136). The following antibodies were purchased from Santa Cruz Biotechnology: anti-E2F1 (catalog no. 190) and anti-phospho-ERK (catalog no. 7383). Anti-p53/RelA (catalog no. 20681415) was purchased from Zymed Laboratories Inc.

**Cell Transfection and siRNAs—** Cell transfection for siRNAs was done using Oligofectamine reagent (Invitrogen) as described by vendor protocol. In preliminary experiments, various concentrations (1–100 nM) of duplex oligonucleotides were used to determine the optimal inhibitor concentration of siRNAs as described in the figures. The sequences of oligonucleotides used were as follows: IKKα siRNA, sense strand (5′-AGGAAGACCCAGUGGUCAGU-3′); antisense (5′-AAGGCUGAAAGCCUCACUCUTT); IKKβ siRNA, sense strand (5′-UGGUAGUGUAGUGAGGACTT); and antisense (5′-UCUCGGUCUGUGAAGUGATT) and antisense (5′-UCUCGGUCUGUGAAGUGATT); pS7/RelA siRNA, sense (5′-AGAGCATCAUUGAGG-
UGUAUTT) and antisense (5'-AUACCCUCAUUGUCUCCUTT).

Ribonuclease Protection and RT-PCR Assay—RNA isolation and the ribonuclease protection assay were performed as described by the BD RiboQuant (BD Biosciences) instruction manual. The multiprobe template set hCC2 (catalog no. 556160) containing cell cycle regulator templates p130, Rb, p107, p53, p27, p21, p19, p18, p14/15, L32, and GAPDH was used. For RT-PCR, a total of 10 μg of RNA was first reverse transcribed using ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) in 16-μl reactions. For PCR, 1–3 μl of first strand cDNA was used. The following primer sets were used to amplify GAPDH, E2F1, and Cdk4: Cdk4, sense (5'-GAGAGTCCCAATG) and antisense (5'-GTGGGGGTGCCTTG); E2F1, sense (5'-

FIG. 1. Short and long term effects of TNFα on cytotoxicity of FdUrd in two human colon tumor cell lines. A, effect of TNFα on proliferation of colo201 cells. Cells were seeded at 2500 cells/well in a 96-well plate in triplicate. After 24 h, cells were treated with 10 μM FdUrd with or without different concentrations of TNFα. Cell proliferation was measured at 24, 48, 72, and 96 h as described under “Materials and Methods.” The percentage of cell survival was plotted against time. B, effect of TNFα on FdUrd-induced cell death. colo201 cells were seeded at 500,000 cells/well in 6-well plates in triplicate and grown for 24 h. Cells were left untreated or treated with FdUrd (10 μM) or FdUrd + TNFα (20 ng/ml) for 24, 48, 72, or 98 h. At the indicated times, cell death indices in each plate were determined as described under “Materials and Methods.” Mean average of triplicates ± S.D. percentage of cell death were plotted against the time of cell incubation with or without TNF. C, TNFα increases postdrug survival in FdUrd-treated colo201 and colo320 cells. Cells were seeded at 250,000 cells/well in a 6-well plate in triplicate and grown for 24 h, at which time cells were or were not treated with 20 ng/ml TNFα for 1 h. FdUrd (10 μM) was then added. 5 days later, floating cells were washed off of the plates, and drug-free medium was added and replenished every 3–4 days. After 3 weeks, cells were fixed and stained, and plates were documented by a scanner. Duplicates representative of plates are shown. D, pretreatment with TNFα is important for its colony survival effect in FdUrd-treated colo201 cells. Colony survival assay was performed as in C. TNFα (20 ng/ml) was either omitted or added 1 h before or 6, 12, 24, or 72 h after FdUrd. Mean colony counts of triplicate plates are shown.
TNF-Dependent Drug Resistance to Antimetabolites

RESULTS

TNFα Increases Postdrug Survival of a Small Number of FdUrd-treated colo201 and colo320 Cells—To investigate whether proinflammatory cytokine TNFα modifies cancer cell response to antimitabolites of purine and pyrimidine nucleotides, we examined its effect on the short term cytotoxicity and postdrug survival of FdUrd in two human colon tumor cell lines, colo201 and colo320. We first examined the effect of TNFα by itself and in combination with 10 μM FdUrd on the cell proliferation of colo201. The 10 μM FdUrd concentration is 100-fold above the 50% growth-inhibitory (GI50) concentration of FdUrd in colo201 cells in culture for up to 4 days of treatment. We determined the GI50 of FdUrd for these cells to be about 0.1 μM (data not shown). TNFα at 5, 10, or 20 ng/ml reduced cell proliferation only 10–15% (Fig. 1A). Treatment of cells with 10 μM FdUrd alone reduced cell proliferation up to 95% after 4 days of treatment (Fig. 1A). Pretreatment of the cells with TNFα did not significantly reduce or increase the cytotoxicity of FdUrd (Fig. 1A). Since cell proliferation assay is not an indicator of cell death, we also examined the combined effect of TNFα and FdUrd on the cell death by annexin V assay (see “Materials and Methods”). TNFα by itself did not increase cell death in colo201 cells above the basal level in culture (Fig. 1B). TNFα also did not change cell death induced by FdUrd (Fig. 1B). Thus, TNFα does not seem to alter the cytotoxicity of FdUrd in an overwhelming majority of colo201 cells. However, with or without TNFα, consistently a small fraction (about 5%) of cells surviving 4–5-day FdUrd treatment (Fig. 1A).

Using a colony survival assay, we next examined whether TNFα alters the postdrug recovery and survival of the small percentage of cells that survived. We pretreated or not colo201 and colo320 cells with 20 ng/ml TNFα for 1 h prior to treatment with 10 μM FdUrd. Cells were treated for 5 days, after which the floating cells were washed off of the plate, and FdUrd- and TNF-free culture medium was added. The cells that remained adhered to the plate were grown for 3 weeks, after which the cells were fixed and the colonies were counted. In both cell lines, significantly more colonies grew on TNFα-pretreated plates than the FdUrd-only treated plates (Fig. 1C). Thus, TNFα increased the recovery of a small fraction of colo201 and colo320 cells treated with 10 μM FdUrd. Pretreatment of cells with TNFα prior to FdUrd was essential for the TNFα-induced postdrug recovery, since TNFα added to cells 6 h or longer after FdUrd did not have the effect (Fig. 1D).

Increased Postdrug Recovery Induced by TNFα Results in Selection of Drug-resistant Cells—The data above indicate that TNFα increases postdrug survival of colo201 and colo320 cells. To test whether surviving colonies are resistant to FdUrd in a TNF-dependent manner, several single colo201 colonies from the TNFα + FdUrd plate were separately grown for 2 weeks and subjected to colony survival assays as described above. TNF-dependent colony survival was on average only 2–3-fold higher for cells that originated from the colonies of the first round of the survival assay relative to those of parental colo201 cells (data not shown). This indicated that only a small number of cells originating from these colonies used the TNFα signal for postdrug survival. Thus, the TNF-dependent postdrug recovery does not seem to be based on a permanent genetic change. Nevertheless, since there was a 2–3-fold increase in TNF-dependent colony survival, we examined whether this trend would continue and whether a population of TNF-dependent drug-resistant cells would emerge. For this, colonies from TNFα + FdUrd-treated plates were pooled, grown for 1–2 weeks, and subjected to another round of colony survival assay. This procedure was repeated for 35 rounds. Indeed, after each round of TNFα + FdUrd treatment and postdrug recovery selection, the number of TNF-dependent colonies increased slowly at first, but it grew exponentially after round 15 (Fig. 2A). To allow a more quantitative colony count, after rounds 10 and 17, the colonies were evaluated after 12 and 9 days postrelease, respectively, as opposed to 3 weeks. After 30 rounds of treatment/recovery, the entire plate was covered with confluent colonies. Representative colony survival plates comparing the TNF-dependent colony survival of the parental (P1) colo201...
cells with the cells after 35 rounds (called hereafter p35-colo201) of FdUrd + TNFα treatment and recovery illustrate a large difference (Fig. 2B). It is important to note that even after 35 rounds of treatment/recovery, not the entire population, but more than 30% of p35-colo201 are resistant to FdUrd in a TNF-dependent manner. The data clearly demonstrate that TNFα treatment has selected a conditional drug-resistant cell population. Furthermore, TNF-independent drug-resistant colonies began to emerge as well but with a much slower rate than TNF-dependent colonies (Fig. 2A).

Incorporation of FdUrd Is Not Affected by TNFα Treatment—
One of the common causes of drug resistance is reduced drug uptake and/or increased drug efflux. To determine whether these mechanisms are at play in TNF-induced resistance to FdUrd, we determined the total incorporation of FdUrd into the p35-colo201 cells in the presence or absence of TNFα at various times as a means for drug uptake and efflux. Cells were incubated with 10 μM [14C]FdUrd in the presence and absence of TNFα. Cells were harvested at various times from 5 min to 24 h (Fig. 3). TNFα treatment did not significantly change the amount of [14C]FdUrd incorporation into cells, indicating that drug uptake and efflux are not altered by TNFα.

TNFα Prevents S-phase Entry Specifically in p35-colo201 Cells—The cytotoxicity of purine and pyrimidine nucleotide antimetabolites is associated with the arrest of proliferating cells at S-phase (8). TNFα has been reported to be cytostatic in...
certain cell lines (13–16). We examined by FACS and \(^{3}H\) thymidine incorporation whether TNF\(\alpha\) alters cell cycle (e.g. prevents S-phase entry) in p35-colo201 cells. TNF\(\alpha\) did not cause a detectable change of cell cycle profile in colo201 parental cells after 1, 2, 3, or 4 days of treatment (Fig. 4A). On the other hand, in p35-colo201 cells after 2, 3, and 4 days of treatment, TNF\(\alpha\) increased cell cycle arrest at G\(_{0}\)-G\(_{1}\) by about 20, 25, and 22\%, respectively (Fig. 4A). Concomitantly with increased cell populations in G\(_{0}\)-G\(_{1}\), the fraction of cells in S-phase decreased in TNF-treated cells (Fig. 4A, p35-colo201 panel). To examine whether the G\(_{0}\)-G\(_{1}\) arrest in p35-colo201 is due to cell confluence in culture after 4 days, we cultured the cells with or without TNF\(\alpha\) for 4 days, after which TNF\(\alpha\) was removed and fresh medium was added. Cell cycle profiles were analyzed at day 4 of TNF\(\alpha\) treatment and 1 or 2 days after TNF\(\alpha\) was removed (Fig. 4B). The cell cycle profiles show that 4 days of TNF\(\alpha\) treatment increased the G\(_{0}\)-G\(_{1}\) arrest as before (Fig. 4B). 1 or 2 days after TNF was removed, the G\(_{0}\)-G\(_{1}\) fraction of cells decreased concomitant with an increase of S phase cells (Fig. 4B). The untreated cells did not show any cell cycle arrest after 4 days or after 1 or 2 days upon replenishing the culture medium (Fig. 4B). Thus, under the culture conditions we used here, the cell cycle arrest observed in p35-colo201 is mediated by TNF\(\alpha\). To test whether TNF\(\alpha\) indeed arrests p35-colo201 before the S-phase, we measured \(^{3}H\) thymidine incorporation in the presence or absence of TNF\(\alpha\). Untreated cells incorporated increasing amounts of \(^{3}H\) thymidine into their DNA after 1, 2, 3, and 4 days (Fig. 4C). TNF\(\alpha\) treatment strongly reduced \(^{3}H\) thymidine incorporation to almost basal levels (Fig. 4C). Thus, TNF\(\alpha\) prevents a significant number of p35-colo201 cells from entering S-phase.

To further examine whether TNF\(\alpha\) induces G\(_{0}\)-G\(_{1}\) arrest in other commonly used cell lines, we compared the effect of TNF\(\alpha\) on p35-colo201 with HeLa, COS-7, and HEK293 cells. Again, TNF\(\alpha\) caused G\(_{0}\)-G\(_{1}\) arrest of p35-colo201 cells, whereas it did not alter cell cycle profile of the other cells (Fig. 4D). TNF\(\alpha\)-dependent sensitivity of HeLa, COS-7, and HEK293 cells to FdUrd in a survival assay is comparable with parental colo201 cells (data not shown).

**colo201 Cells Selected for TNF-dependent Resistance to FdUrd Are Also Resistant to Other Purine and Pyrimidine Analogues**—If the cytostatic function of TNF\(\alpha\) is a mechanism for p35-colo201 cell resistance to FdUrd, we reasoned these cells to also be resistant to other purine and pyrimidine analogues after TNF treatment. We tested the effect of TNF\(\alpha\) on the colony survival in p35-colo201 cells treated with 5-fluorouracil, methotrexate, AraC, hydroxyurea, 3-deazauridine, and ribavirin. A DNA-damaging agent, cisplatin, was used as a control. Each of these antimetabolites inhibits different steps of the biosynthesis of DNA (7). 5-Fluorouracil and methotrexate inhibit a similar pathway as FdUrd. Hydroxyurea inhibits ribonucleotide reductase. 3-Deazauridine inhibits CTP synthase. AraC inhibits DNA polymerases \(\alpha\) and \(\beta\). TNF\(\alpha\) indeed reduced the effectiveness of all of these antimetabolites to a similar degree as that of FdUrd (Fig. 5). p35-colo201 cells were resistant to ribavirin independent of TNF\(\alpha\). TNF\(\alpha\) reduced cytotoxicity of cisplatin only marginally (Fig. 5). Thus, since the above tested purine and pyrimidine analogues inhibit different steps in DNA synthesis and taking into consideration that AraC inhibits DNA polymerases directly, the mechanism of TNF\(\alpha\) action must either render the cell cycle arrest induced by antimetabolites at S-phase ineffective or prevent the cells from reaching S-phase.

**Inhibition of IKK but Not JNK, p38, ERK, or Caspase-8 Blocks both the TNF-induced Cell Cycle Arrest and Colony Survival in p35-colo201**—To examine the specificity of antimetabolite activity of TNF\(\alpha\), we tested whether IL-1\(\beta\), LPS, and TGF\(\beta\) would have such effect. IL-1\(\beta\) and LPS did not reduce the killing of FdUrd in parental colo201 cells (data not shown). IL-1\(\beta\) and TGF\(\beta\) are known to induce cell cycle arrest (13, 30) and activate signaling pathways that are overlapping (IL-1\(\beta\) and LPS) and different (TGF\(\beta\)) from those of TNF\(\alpha\). This would also shed light on the specificity of signaling pathways responsible for TNF-induced drug resistance. We treated cells with TGF\(\beta\), IL-1\(\beta\), and LPS or a combination of TGF\(\beta\) plus IL-1\(\beta\) followed by FdUrd, and survival assays were carried out. None of these cytokines or LPS had a survival effect as TNF\(\alpha\) did (Fig. 6A).

IL-1\(\beta\) and TGF\(\beta\) have been shown to arrest the cell cycle at G\(_{0}\)-G\(_{1}\) in a cell type-specific manner (13, 30). We examined whether these agents cause a cell cycle arrest in p35-colo201 cells. After 48 h of treatment, TNF\(\alpha\) induced G\(_{0}\)-G\(_{1}\) arrest, but IL-1\(\beta\), TGF\(\beta\), and LPS did not alter the cell cycle in p35-colo201 cells (Fig. 6B).

TNF\(\alpha\), IL-1\(\beta\), and LPS activate IKK/NF-\(\kappa\)B in many cell types (31). We examined whether activation of IKK by these agents is comparable in p35-colo201 and whether it plays a role in TNF-induced protection from purine and pyrimidine analogues. TNF\(\alpha\) strongly activated IKK in p35-colo201 cells, whereas IL-1 and LPS, surprisingly, did not (Fig. 6C). TGF\(\beta\), as expected, did not activate IKK (Fig. 6C). Similarly NF-\(\kappa\)B was activated only by TNF\(\alpha\) and not by IL-1\(\beta\), LPS, or TGF\(\beta\) in these cells (data not shown).

We next investigated whether IKK and other pathways downstream of TNF\(\alpha\) play a role in TNF-induced protection from FdUrd in p35-colo201 cells. TNF\(\alpha\) activates multiple
TNF-induced degradation of IκB, JNK, p38, MEK, and caspase-8 did not have any effect on the FdUrd activity. To detect the cell cycle protective activity against FdUrd, we knocked down p35-colo201 cells from FdUrd, we treated p35-colo201 cells with the indicated cytokines for 10 and 30 min. IKK activation was determined as described under "Materials and Methods." Kinase activity (KA) of IKK complex and immunoblot (IB) of IKKβ are shown.

FIG. 6. Specificity of TNF-induced drug resistance, cell cycle arrest, and IKK activation in p35-colo201 cells. A. IL-1β, TGFβ, or LPS does not mimic TNF-induced colony survival. p35-colo201 cells were pretreated with TNFα, TGFβ, IL-1β, TGFβ + IL-1β, or LPS prior to treatment with FdUrd. The survival assay was performed as in Fig. 1. Representative plates are shown. B. cytokines used in A do not cause cell cycle arrest in p35-colo201 cells. p35-colo201 cells were not treated or were treated with cytokines used in A for 48 h. Cell cycle phases were determined by FACS. The histograms show the percentages of cells in different phases. C, TNFα but not IL-1β, LPS, or TGFβ activates IKK in p35-colo201 cells. p35-colo201 cells were treated with the indicated cytokines for 10 and 30 min. IKK kinase activity was determined as described under "Materials and Methods." Kinase activity (KA) of IKK complex and immunoblot (IB) of IKKβ are shown.

Pathways including IKK/NF-κB, JNK/AP1, p38/ATF, ERK/mitogen-activated protein kinase kinase, and caspases (32). We used small molecule inhibitors utilized widely as blockers of these pathways. These inhibitors have a reasonable degree of specificity, but they may also have yet unknown targets and activities. Nevertheless, they do inhibit their respective targets. We used inhibitors of IKK, PS1145, JNK, SP10125, p38 kinase, SB203580, MEK, PD98059, and caspase-8, benzyloxy-carbonyl-IETD-fluoromethyl ketone in colony survival assays. In a dose-dependent manner, IKK inhibitor, PS1145, was the only agent that blocked TNF-induced colony survival in FdUrd-treated p35-colo201 cells (Fig. 7A). None of the other inhibitors altered the effectiveness of FdUrd in the absence or presence of TNFα (Fig. 7A). The effective concentration of PS1145 to inhibit the TNFα effect was between 1 and 10 μM. To ensure that inhibitors of the other pathways functioned properly in blocking their respective known targets, we determined their inhibitory effect on their specific pathways. The IKK, JNK, MEK, and p38 inhibitors did inhibit IκBα, c-Jun, ERK, and ATF2 phosphorylation, respectively, at the concentration range used for the colony survival assay (Fig. 7B). The inhibitors of JNK, p38, MEK, and caspase-8 did not have any effect on the TNF-induced degradation of IκBα (Fig. 7B). Together, the data show that IKK activation, but not the other known downstream pathways of TNFα, is required for the TNF-induced anti-FdUrd activity.

To support the involvement of IKK in the TNF-induced protection of p35-colo201 cells from FdUrd, we knocked down IKKα and IKKβ individually or together and the RelA (p65) member of NF-κB using specific siRNAs. The siRNAs used against each protein were effective and reduced the respective protein levels by about 70–80% 2 days after transfection (Fig. 8). Transfection of cells with IKKα and IKKβ, individually or together, or RelA reduced TNF-induced colony survival of FdUrd-treated cells (Fig. 8). The inhibition of TNF-dependent colony survival using siRNAs of IKKα, IKKβ, and RelA, although clear and reproducible, is not as impressive as the inhibition seen with IKK inhibitor, PS1145. The reason may be due to the fact that siRNA does not completely eliminate IKK and RelA proteins, as shown also in Western blots (Fig. 8, top). Nevertheless, together the data from inhibitor and RNAi experiments support the notion that TNFα requires IKK for its cell protective activity against FdUrd.

IKK Inhibitor Blocks TNF-induced Cell Cycle Arrest—Prevention of S-phase entry in p35-colo201 cells by TNFα could be a plausible mechanism for its anti-antimetabolite activity. Since IKK inhibitor efficiently blocked TNF-induced colony survival in FdUrd-treated cells (Fig. 7A), we asked whether IKK inhibitor prevents TNF-induced G2-M cell cycle arrest in p35-colo201 cells. Treatment of p35-colo201 cells with PS1145 alone for 1, 2, 3, or 4 days did not change the cell cycle profile when compared with the untreated cells (Fig. 9, A and B). Treatment with TNFα resulted in G2-M arrest, as also shown in Fig. 4 (Fig. 9C). PS1145 indeed blocked TNF-induced G2-M arrest, since cells co-treated with PS1145 and TNFα did not show the G2-M cell cycle arrest (Fig. 9D). Thus, PS1145 pre-
vented TNF-induced cell cycle arrest, indicating that TNFα requires IKK for its cytostatic function in p35-colo201 cells.

**TNFα Reduces Phospho-Rb, E2F1, and Cdk4 Protein Levels in an IKK-dependent Manner**—Cell cycle progression from G0-G1 to S-phase is controlled by regulating the expression levels and activities of a complicated network of cyclin-dependent kinases, their upstream kinase or phosphatase activators/inhibitors, cyclins A, D, and E, and their direct inhibitors (CKIs; p27, p21, p19, p18, p16, and p14/p15). For S-phase transition, Cdk4 and/or Cdk6 phosphorylate Rb proteins, resulting in activation of bound E2F1 transcription factor. E2F1 increases the transcription of genes required for G1 to S-phase transition and DNA synthesis. Elevated expression of CKIs is often associated with cell cycle arrest (reviewed in Ref. 33). By RNase protection assays, we first examined whether TNFα increases the mRNA levels of a panel of CKIs (p27, p21, p19, p18, p16, and p14/p15). TNFα did not alter CKIs, p53, and p57 mRNAs in p35-colo201 cells (data not shown). Similar results were observed when we examined the protein expression of CKIs (data not shown). It is important to note that p53 is mutated in colo201 cells (34). Thus, TNF-induced cell cycle arrest was not due to increased CKI levels.

Next, we examined the effect of TNFα on the protein levels of Rb, phospho-Rb, E2F1, and Cdk4 in parental and p35-colo201 cells. It is well documented that the levels of expression and the activities of these proteins increase at the G1-S transition (35, 36). We compared the protein levels of phospho-Rb, residues Ser807/811, reported to be phosphorylated by Cdk4 (24), Rb, E2F1, Cdk4, and Cdk6 of p35-colo201 cells before and after 48-h TNFα treatment. TNFα did not alter the levels of these proteins in parental colo201 cells (Fig. 10A, lanes 1 and 2).
However, TNFα significantly reduced the levels of Rb, E2F1, and Cdk4 but not Cdk6 in p35-colo201 cells (Fig. 10A, lanes 3 and 4). TNFα also reduced the phosphorylation of Rb, because a second faster migrating band appeared in the Rb immuno-blot, and the phospho-Rb band was significantly reduced (Fig. 10A, lanes 3 and 4). Reduced Cdk4 protein correlates well with the reduced phosphorylation of Rb and the appearance of the faster migrating band in Rb immunoblot. This faster migrating band was not detected by phospho-Rb antibody. Thus, TNFα reduces the levels of three major proteins required for G to S transition in drug-resistant p35-colo201 but not in parental colo201 cells.

In the same experimental regimen, we also examined whether IKK plays a role in TNF-induced reduction of these cell cycle regulators. p35-colo201 cells were pretreated with IKK inhibitor (PS1145), JNK inhibitor (SP600125), or p38 inhibitor (SB203580) prior to TNFα/H9251 treatment. PS1145 completely blocked TNF-induced reduction of Rb, phospho-Rb, E2F1, and Cdk4 levels (Fig. 10A, lanes 5 and 6). JNK and p38 inhibitors did not alter the TNF-induced reduction of these proteins (Fig. 10A, lanes 7–10). Thus, IKK mediates the TNFα signal for regulation of Rb, E2F1, and Cdk4.

TNFα did not cause G0-G1 arrest in HeLa, COS-7, and HEK293 cells (Fig. 5). We compared the effect of TNFα on Rb, E2F1, and Cdk4 in these cell lines with p35-colo201 cells. Although the basal levels of Rb, E2F1, Cdk4, and Cdk6 are different in these cells, after a 48-h treatment with TNFα, these levels did not change (Fig. 10B). As shown above and here again, TNFα down-regulated Rb, E2F1, and Cdk4 in p35-colo201, but not in parental colo201 cells (Fig. 10B).

To examine whether TNFα regulates transcription of Rb, E2F1, and Cdk, we determined the steady-state levels of their mRNAs in p35-colo201 cells. The mRNA of Rb, family, p130, Rb, and p107 was determined by S1-nuclease protection assay (Fig 10C). TNFα did not reduce the mRNA of Rb family members. As determined by semiquantitative RT-PCR, the mRNA of Cdk4 and E2F1 were reduced in response to TNFα in p35-colo201, but did not change significantly in parental colo201 (Fig. 10D). This indicates that transcription of E2F1 and Cdk4 are suppressed by TNFα in p35-colo201 cells. Together, the data establish a connection between TNFα, IKK, and regulation of Rb, E2F1, and Cdk4 that leads to cell cycle arrest before S-phase and is specific to p35-colo201 drug-resistant cells.

**DISCUSSION**

In this study, we show that the cytostatic function of TNFα protects a small population of colon tumor cells from cytotoxicity of purine and pyrimidine analogues. Repeated treatment with FdUrd and recovery resulted in selection of a TNF-dependent drug-resistant colo201 cell population. The mechanism of TNF-induced resistance to FdUrd includes cell cycle arrest at G0-G1, which explains the resistance of p35-colo201 cells to a number of other antimetabolites such as 5-fluorouracil, methotrexate, Ara-C, ribavirin, 3-deazauridine, and hydroxyurea in a TNF-dependent manner. This suggests that TNF-induced drug resistance may apply to a broad range of antimetabolites used in cancer therapy.

An important question is whether this cell culture mechanism for development of drug resistance applies to the *in vivo* situation. First is the question of *in vivo* availability of TNFα in tumor microenvironments; TNFα is produced in tumor microenvironments by infiltrating macrophages and T cells and/or by some tumors (37). In patients with advanced and metastasized tumors, systemic TNFα levels are often high, which is also the cause of muscle wasting (38). Thus, *in vivo* availability of TNFα for TNF-dependent development of drug resistance in cancer is quite plausible.

Second is the criticism that many mechanisms of drug re-
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resistance discovered using cell culture systems have been found to be unique to in vitro conditions. This is also a valid criticism to the model system we have developed here. Often protocols used to select for drug-resistant cells in vitro do not mimic the in vivo treatment of cancer. Drug-resistant cells in vitro are selected by continuous exposure of cells to drugs for a long period. Clinically, however, patients are treated with multiple rounds for short periods followed by recovery. The method we used here mimics such protocols. Thus, there is a reasonable likelihood that the drug resistance mechanism we describe here bears substantial relevance to the in vivo situation.

Mechanistically, a necessary function of TNFα in protecting colo201 cells from purine and pyrimidine analogues is the cell cycle arrest at the G₀-G₁ phases. In general, TNFα is not known to induce cell cycle arrest, but it does so in a number of cases reported (13–17, 39). In the cell types tested here including the parental colo201, the great majority of cells did not arrest at the G₀-G₁ phases by TNFα (Fig. 4). The fact that only in a very small fraction of cells TNFα prevented S-phase entry indicates that this is not a normal function for TNFα. This function of TNFα may be part of its pathology. Whether this pathology is unique to cancer cells is currently not known.

Are there differences between TNF signaling in cells that arrest in G₀-G₁ versus those that do not? The majority of cells originating from a colony that survived the first round ofFdUrd treatment in a TNF-dependent manner were not able to utilize TNFα to survive the next round ofFdUrd treatment. This indicates that the initial genetic changes causing a rewiring of TNF signaling are weakly penetrant. However, they become dominant under selection pressure, possibly after acquiring additional mutations.

Given the time and selection pressure, the TNF-dependent drug-resistant cell population increased exponentially (Fig. 2). From these conditional drug-resistant cells, TNF-independent drug-resistant cells also emerged (Fig. 2A). Thus, TNF-dependent survival of cancer cells during treatment can also be considered a mechanism to provide time and survival conditions for the emergence of new mutations leading to TNF-independent drug-resistant cells. TNFα is known to produce reactive oxygen species, which by themselves or in combination with drugs can cause mutations in DNA (40).

At this point, a genetic cause for the rewiring of the TNFα signaling pathway downstream of IKK is not known, and it appears to be complex. Nevertheless, we have determined that the IKK pathway, but not JNK, p38, or ERK, plays an essential role in transducing the TNF signal eventually to prevent cells entering DNA synthesis. In many cell types, NF-κB activation by IKK has been linked with accelerated cell cycle progression through induction of D cyclins (41). In parental and p35-colo201 cells, mRNA and protein expression of D cyclins were not up-regulated by TNFα (data not shown). Rather, TNFα down-regulates Rb, E2F1, and Cdk4 in an IKK-dependent manner in cells that developed resistance (Fig. 10). Hypophosphorylation of Rb as a result of TNF treatment of cells has also been reported in A375-C6 melanoma cells, which also arrest in G₀-G₁ (23). The fact that TNFα does not up-regulate D cyclins in p35-colo201 but rather down-regulates transcription of E2F1 and Cdk4 (Fig. 10) suggests that the molecular mechanisms downstream of IKK/NF-κB that regulate cell cycle events are changed, most likely at the level of transcription. In epidermis, activation of NF-κB has also been associated with growth arrest, although the mechanism is not known (42).

Our strongest evidence for involvement of IKK and NF-κB is the reversal effect of PS1145 on the TNF-induced colony survival, cell cycle arrest, and restoration of Rb, E2F1, and Cdk4 expressions. The siRNAs for IKKα, IKKβ, and RelA had a significant reversal effect on TNF-induced colony survival. The reversal effects of siRNA experiments were not as strong as the IKK inhibitor effect. This is most likely due to incomplete knockdown of these proteins by siRNA. It is also possible that PS1145 inhibits other kinases in addition to IKK, in which case the IKK/NF-κB pathway, although required, is not the sole mediator of TNF action in p35-colo201 cells.

The effective reversal of TNF-induced drug resistance by PS1145 provides a reasonable rationale for potential combination of IKK small molecule inhibitors with purine and pyrimidine analogues in cancer therapy. The IKK inhibitor, PS1145, by itself did not result in cellular toxicity, which is an advantage over cytotoxic agents used currently in combination therapy in cancer.

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REFERENCES

1. Pinedo, H. M., and Giaccone, G. (1997) *Lancet* **349**, Suppl. 2, 7–9
2. Pinedo, H. M. (1996) *Int. J. Cancer* **65**, 561–566
3. Kinsella, A. R., Smith, D., and Pickard, M. (1997) *Br. J. Cancer* **75**, 935–945
4. Banerjee, D., Mayer-Kuckuk, P., Cagius, G., Budak-Alpdogan, T., Gorlick, R., and Bertino, J. R. (2002) *Biochim. Biophys. Acta* **1587**, 164–173
5. Mader, R. M., Muller, M., and Steger, G. G. (1998) *Gen. Pharmacol.* **31**, 661–666
6. Gottesman, M. M. (2002) *Annu. Rev. Med.* **53**, 615–627
7. Chu, E., Johnston, P. G., Grem, J. L., Takimoto, C. H., Van Groeningen, C., Chabner, B. A., and Allegra, C. J. (1994) *Cancer Chemother. Biol. Response Modifiers* **15**, 1–31
8. Spears, C. P. (1995) *Hematol. Oncol. Clin. N. Am.* **9**, 397–413
9. Skeel, R. T. (2003) *Handbook of Cancer Chemotherapy*, 6th Ed., Lippincott Williams & Wilkins, Philadelphia, PA
10. Aggarwal, B. B. (2003) *Nat. Rev. Immunol.* **3**, 745–756
11. MacEwan, D. J. (2002) *British J. Pharmacol.* **135**, 855–875
12. Nalca, A., and Rangnekar, V. M. (1998) *J. Biol. Chem.* **273**, 30517–30523
13. Jeoung, D. I., Tang, B., and Sonenberg, M. (1995) *J. Biol. Chem.* **270**, 18367–18373
14. Merli, M., Benassi, M. S., Gamberi, G., Ragazzini, P., Sollazzo, M. R., Molendini, L., Magagnoli, G., Ferrari, C., Maltarello, M. C., and Picci, P. (1999) *Int. J. Oncol.* **14**, 1117–1121
15. Prewitt, T. W., Matthews, W., Chaudhri, G., Pogrebniak, H. W., and Pass, H. I. (1994) *J. Thoracic Cardiovasc. Surg.* **107**, 43–49
16. Miwa, M., Kojima, T., and Naruse, T. (2001) *Cancer Biother. Radiopharm.* **16**, 317–322
17. Borrelli, E., Roux-Lombard, P., Grau, G. E., Girardin, E., Ricou, B., Dayer, J., and Suter, P. M. (1996) *Crit. Care Med.* **24**, 392–397
18. Morinaga, Y., Suzuki, H., Takatsuki, F., Akiyama, Y., Taniyama, T., Matsushima, K., and Oonoki, K. (1989) *J. Immunol.* **143**, 3538–3542
19. Muthukkumar, S., Sells, S. F., Crist, S. A., and Rangnekar, V. M. (1996) *J. Biol. Chem.* **271**, 5753–5760
20. Nelson, J. W., and Balkwill, F. R. (2000) *Biochim. Biophys. Acta* **1470**, 1–12
21. Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Olinger, J. D., and Helin, K. (2000) *Cancer Chemother. Pharmacol.* **43**, 45–49
22. Negus, R. P., and Balkwill, F. R. (1996) *World J. Urol.* **14**, 157–165
23. Tisdale, M. J. (1999) *J. Nutrition* **129**, Suppl. 1S, 243–246
24. Cheng, K., Sawamura, Y., Sakuma, S., Tada, M., Subo, M., Aida, T., and Abe, H. (1994) *Neurology* **44**, 274–278
25. Inlay, J. A., and Lin, S. (1988) *Science* **240**, 1302–1309
26. Cao, Y., Bonizzi, G., Seagroves, T. N., Greten, F. R., Johnson, R., Schmidt, E. V., and Karin, M. (2001) *Cell* **107**, 763–775
27. Takao, J., Yudate, T., Das, A., Shikano, S., Benkobara, M., Arizumori, K., and Cruz, P. D. (2003) *Br. J. Dermatol.* **148**, 680–688
