The Effects of Malignant Transformation on Susceptibility of Human Urothelial Cells to CD40-Mediated Apoptosis

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Background: **The tumor necrosis factor (TNF) superfamily of ligands and receptors mediates immune cell survival. Some members possess a death domain, a protein motif that functions to transmit apoptotic signals, whereas others, such as CD40, do not. CD40 is expressed by both normal and malignant epithelial cells. To investigate the functional significance of this expression, we studied the effects of ligation of CD40, Fas, and TNF receptors (TNFRs) on the proliferation and survival of normal and malignant human urothelial cells and urothelial cells with disabled p53 function.** ***Methods:*** **Normal and malignant human urothelial cells were cultured with soluble TNF family agonists (CD40 ligand [CD40L], TNF-α, anti-Fas antibody, or cocultured with mouse fibroblasts stably transfected with plasmids that caused the cells to constitutively express CD40L or CD32; cell proliferation was estimated by an [^{3}H]thymidine incorporation assay, and apoptosis was determined by Annexin V staining and by a DNA fragmentation assay. Messenger RNA levels for CD40 and potential downstream effector molecules were quantified by polymerase chain reaction-based and ribonuclease protection assays, respectively, and nuclear factor (NF) κB nuclear translocation was detected by immunofluorescence. All statistical tests were two-sided.** ***Results:*** **Soluble trimeric CD40L inhibited the growth of normal and malignant urothelial cells but did not induce apoptosis. Cell surface-presented CD40L induced massive apoptosis in CD40-positive transitional cell carcinoma cells but not in normal urothelial cells. Normal cells underwent CD40L-mediated apoptosis only in the presence of other TNFR agonists. An agonistic anti-CD40 antibody presented on the surface of CD32-transfected fibroblasts also induced apoptosis in transitional cell carcinoma cells and in normal urothelial cells. Apoptotic responses of tumor (but not normal) cells to soluble agonists were enhanced by blocking protein synthesis. Karyotypically normal urothelial cells with disabled p53 function underwent apoptosis during coculture with CD40L-expressing fibroblasts alone but were not additionally sensitive to additional TNFR agonists.** ***Conclusions:*** **Susceptibility to CD40 ligation-induced apoptosis may be a novel mechanism for eliminating neoplastically transformed urothelial cells. Loss of CD40 expression may be an important adaptive mechanism for transitional cell carcinoma development and progression. [J Natl Cancer Inst 2002;94:1381–95]**

CD40 and its cognate ligand, CD40L (CD154), are central to the efficient functioning of many aspects of the adaptive immune system *(1–9).* Along with other members of the tumor necrosis factor receptor (TNFR) superfamily, CD40 regulates lymphoid cell death and survival by transmitting either apoptotic or rescue signals to B cells, according to their state of differentiation *(9–12).* However, unlike other members of the TNFR superfamily, such as the p55 TNF-α receptor (also known as TNFRI) and Fas (also known as Apo-1 or CD95), CD40 does not contain a death domain, a protein motif that functions to transmit apoptotic signals after activation by the cognate ligand. Instead, apoptotic signals may be transmitted through CD40 by interactions or "cross-talk" between the downstream elements of the different TNFR superfamily signaling pathways. For example, interactions between CD40 and CD40L or between TNFR and TNF-α both inhibit apoptosis in immature B cells while sensitizing mature B cells to apoptotic signals from Fas *(13,14).* CD40 may also recruit other members of the TNFR superfamily that function in death receptor-dependent pathways, resulting in autotropic or paratropic activation of the apoptosis machinery *(15–18).*

CD40 expression was first identified on the surface of bladder carcinoma cells and has since been demonstrated on the surfaces of a variety of normal epithelial cell types and their malignant counterparts. Although this distribution suggests a role for the binding of CD40 to CD40L (i.e., CD40 ligation) in T cell-mediated lymphoepithelial cell interactions, the functional significance of CD40 expression by normal and malignant epithelial cells is poorly understood. Thymic epithelial cells, keratinocytes, kidney proximal tubule epithelial cells, and cervical carcinoma cells all respond to CD40 ligation by increasing chemokine or cytokine secretion *(19–26).* However, whereas CD40 ligation stimulates fibroblast growth *(27,28),* it inhibits the proliferation of keratinocytes *(23,29,30)* and various carcinoma cells *(29–34).* When protein synthesis is blocked, CD40 ligation promotes apoptosis in CD40-transfected HeLa cells *(17,35)* as well as in untransfected HepG2 hepatoma cells *(36)* and untransfected ovarian carcinoma cells *(17).* Normal intrahepatic biliary epithelial cells and hepatocytes, as well as HepG2 cells, are also susceptible to CD40-mediated apoptosis, either directly *(16,18)* or, in the case of HepG2 cells, following *Cryptosporidium parvum* infection *(37).*

Such observations have led to the suggestion that CD40L may be a potential anticancer agent *(38).* Indeed, results that

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See "Notes" following "References."

support its *in vivo* efficacy against human carcinoma cells in SCID mice have been reported *(33,34,39).* CD40L has also been shown to enhance the effects of platinum-based chemotherapy in this model system *(39).* CD40 ligation has also been reported both to enhance Fas-mediated apoptosis in carcinoma cells *(16,17,29,31),* which would enhance its potential antitumor effect, and to inhibit Fas-mediated apoptosis in other carcinoma cells, which would reduce its antitumor effect *(40–44).*

It has, however, been difficult to make distinctions between cell type-specific and malignancy-associated responses to CD40 ligation, because few suitable in vitro model systems exist that would allow such distinctions to be made. One exception is our robust, and highly reproducible, system for normal human urothelial (NHU) cell culture *(45).* NHU cells grown in monoculture show a proliferative, basal-to-intermediate cell phenotype that can be maintained for up to 12 passages before the cells undergo senescence *(46).* NHU cells grown in monoculture also have the ability to re-form a histologically normal, stratified urothelium when seeded onto an appropriate stroma *(47),* which suggests that these cells have retained a full, normal response repertoire. Thus, the NHU system permits a direct comparison to be made between a normal epithelial cell type and its fully transformed malignant counterpart. The system further permits the testing of the effects of genetic alterations that predispose to malignant transformation. Because p53 mutations represent one of the two main pathways implicated in urothelial carcinogenesis *(48,49),* we compared NHU cells with human urothelial cells that were karyotypically normal but that had disabled p53 function *(50,51),* and with a panel of well-characterized transitional cell carcinoma cell lines of malignant urothelial origins *(52).* Our aims were threefold: first, to determine the normal responses of urothelial cells to ligation of CD40; second, to investigate how these responses are modulated by simultaneous ligation of the archetypal death receptors Fas and TNFRI, and finally, to determine if, and to what extent, these responses are modified during malignant transformation.

Materials and Methods

Study Design

We used NHU cell lines, cell lines established from human urothelial transitional cell carcinomas representing a spectrum of grades and stages, and NHU cells with disabled p53 function to determine the effects of ligation of CD40 on cell proliferation and survival. On the basis of results from preliminary experiments (data not shown), detailed studies concentrated on three transitional cell carcinoma cell lines: RT4 and EJ, which were used to represent well-differentiated and anaplastic CD40-positive transitional cell carcinoma cells, respectively, and RT112, a moderately differentiated cell line that was used as a CD40-negative transitional cell carcinoma control.

We also studied the effects of Fas and TNFR ligation, separately and in combination with CD40 ligation, because their cognate ligands are, like CD40, the products of activated T cells and because Fas and TNFR pathways have been implicated in CD40-mediated apoptosis in other cell systems *(15–18).* Because the mode of CD40 ligation is known to profoundly influence outcome (i.e., apoptosis versus rescue) in B cells *(53–56),* we used three different approaches to effect CD40 ligation in target cells: 1) incubation with soluble recombinant trimeric CD40 ligand (sCD40L); 2) incubation with an agonistic anti-CD40 antibody, either in solution or presented on the surface of Fcγ receptor (CD32)-transfected fibroblasts; and 3) coculture of target cells with murine fibroblasts transfected with a plasmid that constitutively expressed CD40L.

Cell Lines

NHU cell lines were newly established from urinary tract specimens obtained during urologic procedures that were performed on non-neoplastic tissues, as described elsewhere *(45,46).* We used 22 independent NHU cell lines. NHU cell lines were grown in Keratinocyte Serum-Free Medium (Gibco-BRL, Paisley, U.K.) containing 5 ng/mL recombinant epidermal growth factor, 50 μg/mL bovine pituitary extract, and 30 ng/mL cholera toxin (Sigma Chemical Co., Poole, U.K.).

NHU cells with disabled p53 function were prepared by stable incorporation of the E6 gene of human papillomavirus 16 into passage 4 cells by retroviral transduction, as previously described *(50,51).* E6-transduced human urothelial (HU-E6) cells show an extended lifespan compared with their untransduced counterparts, which undergo growth arrest at passages 12–15 *(50).* At passage 20, HU-E6 cells undergo "crisis," which is characterized by growth arrest and extensive cell death, from which populations of immortalized cells with karyotypic abnormalities eventually emerge *(50).* For this study, HU-E6 cells were used between passages 10 and 14, when no karyotypic abnormalities could be detected *(50,51).*

We studied 11 established human transitional cell carcinoma cell lines of the urinary bladder—RT4, HT1376, RT112, HT1197, COLO 232, KK47, VM-CUB-3, T24, VM-CUB-1, 253J, and EJ—that encompassed phenotypes ranging from well-differentiated to highly anaplastic *(52,57).* Transitional cell carcinoma cell lines were cultured in standard growth medium consisting of a 1 : 1 (vol/vol) mixture of RPMI 1640 and Dulbecco's modified Eagle's medium (Gibco-BRL) that contained 5% (vol/vol) fetal bovine serum (FBS; Sera-Lab Ltd., Crawley Down, U.K.) and were passaged using trypsin–EDTA.

NIH 3T3 mouse fibroblasts stably cotransfected with an expression plasmid bearing the sequences coding for CD40L and for the neomycin resistance gene (3T3CD40L cells), as well as NIH 3T3 cells stably transfected with an expression plasmid carrying the neomycin resistance gene alone (3T3neo cells), were the generous gift of J. G. Gribben (Dana-Farber Cancer Institute, Boston, MA) *(58).* For some experiments, we used murine L cells that were stably transfected with plasmids encoding CD40L (CD40L-L cells) or CD32 (CD32-L cells), as well as nontransfected L cells, as described previously *(53,54,59,60).* The transfected NIH 3T3 cells were cultured in standard growth medium (RPMI 1640/Dulbecco's modified Eagle's medium with 5% FBS) that contained 0.5 mg/mL G418 (Sigma Chemical Co.), which was omitted from culture medium in coculture experiments. Cell surface expression of CD40L was routinely monitored by flow cytometry. Human monocytic U937 cells and human T-cell leukemia Jurkat J6 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.) and were maintained in suspension culture in standard growth medium, as described above.

Pretreatment of Urothelial Cells

In order to determine the effects of archetypal type 1 and type 2 cytokines on expression of CD40, urothelial cells were cultured in the presence of 0–1000 U/mL interleukin 4 (IL-4),

TNF-α (both obtained from R&D Systems, Abingdon, U.K.), or interferon gamma (IFN γ) (Amersham, Slough, U.K.) in 24-well plates (Falcon, BD Biosciences, Cowley, U.K.). Replicate cultures were established at 10^5 cells/well and were harvested at 0, 24, 48, and 72 hours after cytokine addition for analysis by flow cytometry or were used after harvest at those times to determine the effects of cytokine pretreatment on subsequent effects of ligation of CD40, Fas, and TNFRs as described below.

In experiments in which we wished to inhibit protein synthesis, cycloheximide (Sigma Chemical Co.) was added to transitional cell carcinoma-derived cell lines (at 1 μg/mL) and to NHU cells (at 0.01 μg/mL) 30 minutes before exposing them to soluble TNFR agonists.

Ligation of TNFR Superfamily Members

CD40 ligation. sCD40L, prepared as described previously *(61),* was added to cell cultures at final concentrations of 1–10 μg/mL. The sCD40L-FLAG-tag fusion protein (Alexis Corp., Nottingham, U.K.) was added to cell cultures at final concentrations of 1–20 μg/mL, in the presence and absence of the cross-linking "enhancer" reagent that was supplied with the fusion protein, at the manufacturer's recommended concentrations.

The effects of cell surface-presented CD40L were also investigated in coculture experiments using urothelial cells and CD40L-transfected fibroblasts (3T3CD40L cells and CD40L-L cells). 3T3neo cells or untransfected L cells were included in each experiment as controls. Fibroblasts were treated with 10 μg/mL mitomycin C for 2 hours, washed, and then seeded at 10^4 cells/well or 10^5 cells/well in 96- or 24-well plates, respectively (Falcon). CD40L expression before and after mitomycin C treatment was monitored by flow cytometry. After the mitomycin C-treated fibroblasts had attached to the substrate, urothelial cells were seeded onto the fibroblasts at a ratio of 0.9 urothelial cells to 1 fibroblast.

We also used the G28–5 agonistic anti-CD40 antibody *(53–55)* in native form, cross-linked with secondary antibody or presented on the surface of L cells transfected with a plasmid bearing the coding sequence for CD32 (Fcγ receptor II) (CD32-L cells) to examine the effects of ligand cross-linking *(54).* Native G28–5 antibody was purified from culture supernatants of the G28–5 hybridoma cell line (American Type Culture Collection, obtained *via* LGC Promochem, Teddington, U.K.) and was added to cultured cells at a final concentration of 1 μg/mL. In some experiments, the antibody was cross-linked with affinity-purified human serum protein-adsorbed goat anti-mouse immunoglobulin G (IgG; Sigma Chemical Co.), which was added at 5 μg/mL to the cell culture medium after the cells had been incubated for 1 hour with G28–5 or isotype-matched control antibody. To present the antibody on the surface of fibroblasts, CD32-L cells were treated with mitomycin C, washed, and seeded onto 96- or 24-well plates, as described above. They were then incubated with G28–5 antibody, with goat anti-mouse IgG followed by G28–5, or with isotype-matched control antibody before urothelial cells were added, as described above.

To determine whether apoptosis induced by cell surface-presentation of CD40L in EJ cells was *via* the Fas/Fas ligand (FasL) pathway, EJ cells were harvested at various time-points from cocultures with CD40L-transfected fibroblasts and assessed for expression of Fas and FasL by flow cytometry. In addition, EJ cells were cocultured with CD40L-transfected fibroblasts in the presence of the Fas-blocking antibody NOK-1 (5 μg/mL; BD PharMingen, Cowley, U.K.).

Fas and TNFR ligation. Fas was ligated by treating urothelial cells with Apo-1 agonistic antibody (Alexis Corp.) at 0.5 μg/mL and with a cross-linking goat anti-mouse IgG at 10 μg/mL, as described above. Jurkat J6 cells were used as a positive control for Fas-mediated apoptosis *(62).* In experiments that examined effects of combinations of ligands, cells were treated with Apo-1 antibody (at 0.5 μg/mL) that was cross-linked with goat anti-mouse IgG (at 10 μg/mL), TNF-α (at 300 U/mL), and sCD40L (at 10 μg/mL).

Flow Cytometric Quantitation of Cell Surface Antigen Expression

Expression of CD40, Fas, and FasL on urothelial cells and CD40L on mitomycin C-treated 3T3CD40L and CD40L-L fibroblasts was determined by flow cytometry. Fas expression was assessed by using a fluorescein isothiocyanate (FITC)-conjugated anti-human CD95 antibody (Immunotech, supplied by Coulter Electronics, Luton, U.K.); FasL expression was determined with biotinylated NOK-1 antibody (BD PharMingen), followed by incubation with phycoerythrin-conjugated streptavidin (BD PharMingen); and expression of CD40 and CD40L was determined by incubation of cells with unconjugated specific primary antibodies (Serotec, Oxford, U.K.), followed by phycoerythrin-conjugated goat anti-mouse Ig ($F[ab]'_2$ fragment) (Southern Biotechnology Associates, supplied by Eurogenetics, Hampton, U.K.). All antibodies were titrated before use and diluted in 0.2 μm-filtered phosphate-buffered saline (PBS) containing 1% FBS and 0.1% NaN_3. Cells were harvested, and antibody incubations were carried out as described previously *(63,64).* Cells were analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences). At least 3000 events (i.e., cells) were acquired from each sample. The baseline median fluorescence channel was established for each cell line with the use of control cells that were incubated with either an irrelevant antibody to glucose oxidase from *Aspergillus niger* (Dako Ltd., High Wycombe, U.K.) or no antibody.

Analysis of TNFR Expression

TNFR expression was assessed by competition binding of ^{125}I-TNF-α (Amersham) to cells in the presence or absence of the htr-9 antibody to TNF p55 (also known as TNFRI or CD120a) and the utr-1 antibody to TNF p75 (also known as TNFRII or CD120b), exactly as described previously *(65).* The htr-9 and utr-1 antibodies were the generous gift of Manfred Brockhaus (Hoffmann-La Roche, Basel, Switzerland). Experiments were performed on four independent NHU cell lines as well as on the RT112 and EJ cell lines. U937 cells were used as a positive control for TNFR expression.

Reverse Transcription–Polymerase Chain Reaction Analysis of CD40 Expression and Sequencing of CD40

CD40 messenger RNA (mRNA) expression levels were determined by reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was isolated from approximately 5×10^6 urothelial cells with the use of TRIzol™ reagent, according to the manufacturer's instructions (Life Technologies, Paisley U.K.). Complementary DNA (cDNA) was prepared from total RNA by using a RETROscript™ First Strand Synthesis kit (Ambion, Huntingdon, U.K.). The full-length CD40 coding sequence

(834 bp) was amplified from cDNA by using oligonucleotide primers 5′-ATGGTTCGTCTGCCTCTGCAGTGCGTCCTC-3′ (sense) and 5′-TCACTGTCTCTCCTGCACTGAGATGC GACT-3′ (antisense) and PLATINUM *Pfx* DNA polymerase (Life Technologies). As a control, a 314-bp segment of β-actin was amplified from the same RNA preparation by using oligonucleotide primers 5′-ATCATGTTTGAGACCTTCAA-3′ (sense) and 5′-CATCTCTTGCTCGAAGTCCA-3′ (antisense). All primers were purchased from MWG-Biotech (Milton Keynes, U.K.). CD40 mRNA was amplified by PCR, which consisted of 25 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 64 °C for 30 seconds, and DNA synthesis at 72 °C for 1 minute, and ending with a final extension at 72 °C for 10 minutes. β-actin mRNA was amplified by 25 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, and ending with a final extension at 72 °C for 10 minutes. RT–PCR products were separated by electrophoresis through 1.5% agarose gels that contained ethidium bromide. Control PCR assays were performed in the absence of reverse transcriptase to confirm the absence of genomic DNA contamination in the total RNA samples.

To confirm that the CD40 mRNA expressed by urothelial cells was identical to that sequenced from B cells, we PCR-amplified the full-length coding region of CD40 from a NHU cDNA library *(66)* with the use of a high-fidelity DNA polymerase (Advantage HF-PCR Kit; Clontech, BD Biosciences). The 834-bp product was cloned into the pTRE vector (Clontech, BD Biosciences), five clones were isolated, and their DNA was sequenced. All five clones contained a sequence identical to the published human CD40 coding sequence (GenBank accession No. X60592).

Cell Proliferation Assay

Urothelial cells were seeded at 9×10^3 cells/well in 96-well plates. sCD40L (concentration range = 0–10 μg/mL) or TNF-α (concentration range = 0–800 U/mL) in culture medium was added to sextuplicate wells, and the cells were maintained in culture for 72 hours. [^{3}H]Thymidine (0.5 μCi; Amersham) was added to each well for the final 18 hours of the incubation period. The cells were then incubated in 0.1% (w/vol) EDTA in PBS for 2 hours and harvested onto glass fiber filters (Pharmacia Wallac U.K. Ltd., Milton Keynes) with an automated cell harvester, as previously described *(67)*. [^{3}H]Thymidine incorporation was measured on a Betaplate liquid scintillation spectrometer (Wallac). Results were expressed as (T × 100)/C, where T = cpm recovered from cells treated with sCD40L or TNF-α, and C = cpm recovered from untreated control cells after background subtraction.

Assessment of Apoptosis

NHU cells cultured in the presence of 0.1 μg/mL G418 (Sigma Chemical Co.) were used as a positive control for apoptosis. Apoptosis was assessed by the following three methods.

Nuclear morphology. We examined the nuclear morphology of cells stained with Mayer's hematoxylin or 10 μg/mL acridine orange, as described previously *(67),* using the criterion of nuclear condensation and fragmentation to identify apoptotic cells.

Flow cytometry of Annexin V- and propidium iodide-stained cells. We used flow cytometry to analyze populations of cells for the physical changes in cell size and granularity *(68)* that accompany apoptosis. We also performed flow cytometry on Annexin V-stained cells to detect early apoptotic cells that expressed extracellular phosphatidylserine *(69)* and on propidium iodide (PI)-stained cells to detect late apoptotic and dead cells with disrupted plasma and nuclear membranes, as described previously *(50,70).* Culture supernatants were collected and centrifuged to concentrate detached cells, and adherent cells were harvested after brief trypsinization. Adherent and detached cell fractions were combined and resuspended at 2×10^6 cells/mL in RPMI 1640 medium that contained 10 m*M* HEPES (pH 7.6), 1 μg/mL PI, and 1 μL of FITC-conjugated Annexin-V-FLUOS (Boehringer Mannheim, Lewes, U.K.). Cells were incubated on ice for 30 minutes and analyzed by flow cytometry. We acquired 5000 events per sample.

Assessment of DNA fragmentation. DNA fragmentation was assessed by means of the so-called JAM test *(71),* as described previously *(70).* Briefly, exponentially growing target cells were labeled with 5 μCi/mL [^{3}H]thymidine for at least 6 hours and then washed, harvested, and plated alone, with sCD40L, TNF-α, cross-linked agonistic anti-Fas antibody (as detailed above), or onto mitomycin C-treated fibroblasts, as described above. After 48 or 72 hours of culture or coculture, respectively, cells were harvested and collected onto glass fiber filters with an automated cell harvester as described above, with the consequence that only intact (i.e., nonfragmented) DNA was retained on the filters. The amount of intact DNA retained on the filter was quantified by using a Betaplate liquid scintillation spectrometer. The percentage of DNA fragmentation was calculated as a function of spontaneous fragmentation, according to the formula (S – E × 100)/S, where S = cpm recovered from control cultures (urothelial cells cultured alone, with 3T3neo cells or with untransfected L cells, or with CD32-transfected L cells in the absence of antibody), and E = cpm recovered from test cultures (urothelial cells incubated with soluble agonist [sCD40L, TNF-α, or cross-linked agonistic anti-Fas antibody] or cocultured with CD40L-transfected fibroblasts or with CD32-transfected L cells in the presence of G28-5 anti-CD40 antibody), after background subtraction.

Western Blot Analysis

Expression of apoptosis-associated proteins of the Bcl family was assessed by western blotting. Urothelial cells were cultured for 24 hours in 100-mm dishes (Falcon) with and without mitomycin C-treated 3T3neo or 3T3CD40L fibroblasts, as described above. Cell lysates were prepared by the addition of electrophoresis sample buffer to cell cultures, as described previously *(50),* and a volume of lysate that was equivalent to approximately 2×10^5 cells was loaded into each well. Because some of the lysates were prepared from cocultures of human urothelial cells and murine fibroblasts, we conducted titration experiments with antibodies to cytokeratins 8 and 18 (CK8 and CK18) to adjust loadings to ensure that equivalent numbers of urothelial cells were loaded in all lanes. Lysates were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were transferred to nitrocellulose membranes (Amersham) by electroblotting, and the membranes were probed with human-specific antibodies against CK8 (Zymed Laboratories, Cambridge BioScience, Cambridge, U.K.) and CK18 (Sigma), as well as with antibodies specific for human Bcl-2, Bax, and Bak (R&D Systems). Lysates from mitomycin C-treated fibroblasts alone were

included as controls. Antibody binding was detected by enhanced chemiluminescence (Amersham), according to the manufacturer's instructions.

Ribonuclease Protection Assays

Expression of specific mRNA species associated with apoptosis was quantified by ribonuclease protection assays with the use of Apo 2, Apo 3, and Apo 5 multiprobe kits obtained from BD PharMingen. Two femtomoles of each labeled probe was mixed with 5 μg of total RNA isolated from the test cells or 5 μg of total yeast RNA provided with the kit. Hybridizations and ribonuclease digestions were performed with an RPA II™ Ribonuclease Protection Assay Kit (Ambion), according to the manufacturer's instructions. The resulting hybrids were ethanol precipitated and subjected to electrophoresis on 5% denaturing polyacrylamide gels (Sequagel; Flowgen Instruments Ltd., Lichfield, U.K.). Protected probe fragments were visualized by autoradiography and quantified by phosphorimaging analysis.

Nuclear Factor κB Activation Assays

Baseline levels of nuclear factor κB (NFκB) expression were estimated by western blotting as described above, and nuclear translocation of NFκB was determined by indirect immunofluorescence. To examine the effects of soluble TNFR agonists on urothelial cells, cells were plated onto sterile 12-spot Multiwell slides (Hendley, Essex, U.K.) at 10^4 cells/spot, incubated overnight, and then incubated with sCD40L or TNF-α or cocultured with CD40L-transfected fibroblasts, as described above, for 30 minutes to 5 hours. The slides were washed, fixed in methanol/acetone (vol/vol), and air-dried. To determine the effects of CD40 ligation by cell-surface-presented ligand, mitomycin C-treated 3T3neo or 3T3CD40L fibroblasts were seeded at 1.5×10^4 cells/spot and incubated overnight before the addition of 10^4 urothelial cells/spot. Following coculture for 2–5 hours, slides were washed and fixed as above.

Single- and double-label indirect immunofluorescence were then performed, as described elsewhere *(72).* NFκB was localized by using a rabbit polyclonal antibody (NFκB p65; Santa Cruz, Insight Biotechnology, London, U.K.). We used a mouse monoclonal antibody to CK8 (Zymed) to identify urothelial cells and an anti-CD40L mouse monoclonal antibody (Serotec) to identify 3T3CD40L fibroblasts. All antibodies were used at pretitrated optimal dilutions. Goat anti-rabbit Ig–Texas Red and anti-mouse–FITC conjugates (Southern Biotechnology Associates) were used to visualize the immunolabeling patterns. As positive controls for NFκB translocation to the nucleus, human foreskin-derived fibroblasts were treated with TNF-α and processed for immunofluorescence localization of NFκB.

To further determine the involvement of NFκB in CD40-mediated apoptosis, urothelial cells were treated for 30 minutes with BAY 11–7082 (10 μg/mL), an inhibitor of cytokine-induced IκB-α protein phosphorylation, or with SN50 (50 μg/mL), a cell-permeable peptide that inhibits nuclear translocation of the NFκB inhibitory complex (Biomol Research Laboratories, Affinity, Exeter, U.K.). The cells were then seeded onto monolayers of 3T3neo cells or 3T3CD40L cells and assessed for apoptosis by flow cytometry or by the JAM test, as described above.

Statistical Analyses

Means and 95% confidence intervals (CIs) were used for descriptive statistics. Unless otherwise stated, either the two-tailed Student *t* test with the Welch correction or analysis of variance with the Tukey–Kramer multiple comparisons test, applied as appropriate, was used to evaluate statistical significance of differences between treatment effects. The alpha value for statistical significance was $P<.01$. All statistical tests were two-sided and were performed with GraphPad InStat software version 3.01 (GraphPad Software, San Diego, CA).

RESULTS

Expression of CD40, Fas, and TNFRs by Urothelial Cells

We screened 22 independent NHU cell lines by immunofluorescence (five cell lines) and/or flow cytometry (17 cell lines; mean median fluorescence channel value = 40.95, 95% CI = 38.57 to 43.33) for CD40 expression and found that all expressed cell-surface CD40. Of the 11 established human transitional cell carcinoma cell lines of the urinary bladder that we screened for expression of CD40, only RT4, T24, 253J, and EJ cells expressed cell-surface CD40 by flow cytometry (median fluorescence channel value = 21.1, 79.5, 29.8, and 112.8, respectively; n = 2–7 experiments per cell line); cell-surface expression of CD40 was undetectable in the other seven cell lines. On the basis of these results, we selected three transitional cell carcinoma cell lines for detailed investigation: RT4 and EJ cells were used to represent well-differentiated and anaplastic CD40-positive transitional cell carcinoma cell, respectively, and the RT112 cells were used as a CD40-negative transitional cell carcinoma control.

By flow cytometry, NHU cells expressed cell-surface Fas at low density (seven independent cell lines, mean median fluorescence channel value = 11.7, 95% CI = 8.46 to 14.94). The selected transitional cell carcinoma cell lines expressed Fas at low to moderate densities (n = 2–5 experiments per cell line, mean median fluorescence channel values were 17.8 for EJ cells, 10.05 for RT112 cells, and 11.14 for RT4 cells). Fas mRNA expression in these cell lines was confirmed by ribonuclease protection assays (data not shown). All of the NHU cell lines, as well as EJ and RT112 cells, were found to express both the p55 and p75 TNFRs by ribonuclease protection assays and by competition binding assays that used htr-9 antibody to detect p55 and utr-1 antibody to detect p75 (data not shown).

Regulation of CD40 Expression by Cytokines

Using flow cytometry, we found that, in agreement with previous reports *(28,73),* CD40 expression on all NHU cell lines and CD40-positive transitional cell carcinoma cell lines was increased when the cells were treated with IFN γ or TNF-α. IFN γ was more efficient than TNF-α at mediating an increase in CD40 expression in all the CD40-positive cell lines (Fig. 1, A). These results were confirmed by RT–PCR; CD40 mRNA was readily detectable in the CD40-positive EJ cells and showed further increases in expression after the cells were pretreated with IFN γ, TNF-α, or a combination of both agents (Fig. 1, B). By contrast, treatment with IL-4 had no effect on CD40 expression in either normal urothelial cells or in RT4, RT112, and EJ carcinoma cell lines (data not shown).

Treatment with TNF-α, IFN γ, or IL-4 did not induce CD40 protein expression in any of the CD40-negative cell lines. Furthermore, CD40 mRNA transcripts were undetectable by RT–PCR in the CD40-negative RT112 cell line. No *de novo* induc-

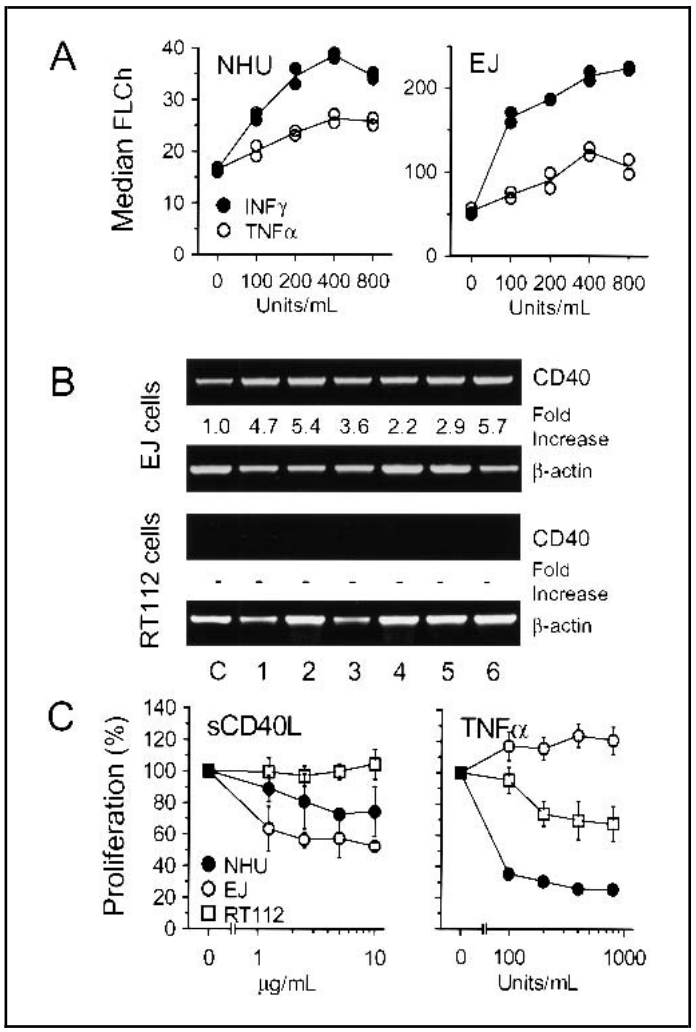

Fig. 1. CD40 expression in urothelial cells and effects of CD40 ligation and tumor necrosis factor-α (TNF-α) on proliferation. **A**) Normal human urothelial (NHU) cells and transitional cell carcinoma (TCC)-derived EJ cells (both CD40-positive) were seeded in replicate cultures at 10^5 cells/well and cultured for 72 hours with TNF-α or interferon gamma (IFN γ) at 0–800 U/mL in 24-well plates. Cell-surface expression of CD40 was measured by flow cytometry in duplicate runs, and results are expressed as median fluorescence channel (FLCh). **B**) CD40-positive EJ cells and CD40-negative RT112 cells were cultured for 48 hours with 200 or 800 U/mL of IFN γ (**lanes 1** and **2,** respectively), 200 or 800 U/mL of TNF-α (**lanes 3** and **4,** respectively), or with both cytokines together at 100 or 400 U/mL each (**lanes 5** and **6,** respectively). Control cells were cultured without cytokines (**lane C**). Messenger RNA was isolated from each culture and used to prepare complementary DNAs, which were used as templates for polymerase chain reaction (PCR) amplification of the CD40 and β-actin coding sequences that used gene-specific oligonucleotide primers. PCR products were resolved and visualized on agarose gels. Gel bands were quantified by densitometry, and results are expressed as fold induction relative to the intensity of the bands obtained for β-actin. "–" indicates no increase in CD40 mRNA compared with β-actin mRNA. **C**) NHU, EJ, and RT112 cells were seeded into 96-well plates at 10^4 cells/well and exposed to soluble trimeric CD40 ligand (sCD40L; range = 0–10 μg/mL) or TNF-α (range = 0–800 U/mL) in sextuplicate wells for 72 hours. [^{3}H]Thymidine (0.5 μCi) was added to each well for the final 18-hour incubation period. The cells were then harvested onto glass fiber filters, and [^{3}H]thymidine incorporation into DNA was quantitated by scintillation spectrometry. The results are expressed as means of percentages of the "no ligand" controls for each cell line. **Error bars** correspond to 95% confidence intervals.

tion of CD40 mRNA expression was detected in any cell line after pretreatment with TNF-α, and only minimal levels of CD40 mRNA were apparent following exposure to IFN γ (Fig. 1, B).

Effects of CD40L on Urothelial Cell Proliferation and Survival

Presentation of CD40L as soluble ligand. sCD40L had a pronounced growth inhibitory effect on both NHU cells and CD40-positive EJ cells (Fig. 1, C, left), in agreement with previous reports *(23,30–32).* However, sCD40L alone did not statistically significantly induce apoptosis in any cell line tested (*see* Figs. 4, A, and 5, B). sCD40L had no effect on proliferation (Fig. 1, C) or apoptosis (*see* Figs. 4, A, and 5, B) in the CD40-negative RT112 cell line.

Presentation of CD40L by coculture with CD40L-transfected fibroblasts. By contrast to the results obtained when CD40L was presented as a soluble ligand, when EJ cells were presented with CD40L on the cell surface (e.g., by coculturing with 3T3CD40L cells), they underwent extensive apoptosis compared with EJ cells cocultured with control (3T3neo) cells (Figs. 2 and 3). In all experiments, the percentage of cells undergoing apoptosis at 48 hours was always at least 70%–80%, as assessed by flow cytometry. Because the presence of cocultured 3T3 cells was a potential confounding factor in the flow cytometric analysis, we confirmed these results in the same two types of cocultures by using the JAM test, which allowed us to estimate DNA fragmentation specifically in the prelabeled target cell population (Fig. 3, A). We further confirmed these results by using another independent CD40L-transfected murine fibroblast cell line (CD40L-L cells; Fig. 3, A) which, as assessed by flow cytometric analyses, expresses CD40L at densities comparable to that of the 3T3CD40L cell line.

All of the CD40-positive transitional cell carcinoma cell lines similarly underwent extensive apoptosis when they were cocultured with 3T3CD40L cells. The degree of apoptosis was not statistically significantly increased by pretreating the cells for up to 72 hours with IFN γ or TNF-α (data not shown), despite the ability of these cytokines to increase CD40 expression, implying that even a low surface density of CD40 was adequate to trigger a maximal apoptotic response. Pretreatment of the CD40-negative RT112 cells with IFN γ or TNF-α did not render them susceptible to CD40-mediated apoptosis (data not shown).

None of the NHU cell lines tested showed susceptibility to apoptosis when they were cocultured with 3T3CD40L cells (Fig. 2), even though all expressed CD40 on their surfaces at densities comparable to those in the CD40-positive tumor cell lines. Pretreatment of NHU cells with IFN γ or TNF-α also did not result in any CD40L-mediated apoptosis (data not shown). CD40-negative RT112 cells were unaffected in terms of survival by coculture with 3T3CD40L cells (Fig. 2, B).

CD40 cross-linking experiments. Because sCD40L and surface-presented CD40L appeared to induce different responses in tumor cells, we tested whether these differences were due to cross-linking of CD40. The FLAG-tagged sCD40L construct, with and without the cross-linking enhancer, did not induce any statistically significant apoptosis compared with untreated cells (data not shown). Similarly, the G28–5 agonistic anti-CD40 antibody did not induce any statistically significant apoptosis either used alone or in conjunction with a goat anti-mouse IgG cross-linking secondary antibody (Fig. 3, B). Nevertheless, the anti-CD40 antibody was functionally capable of inducing apoptosis in EJ cells when it was presented on the surface of L cell fibroblasts transfected with the Fcγ receptor CD32 (Fig. 3, B). These findings demonstrate that soluble CD40 agonists, even if

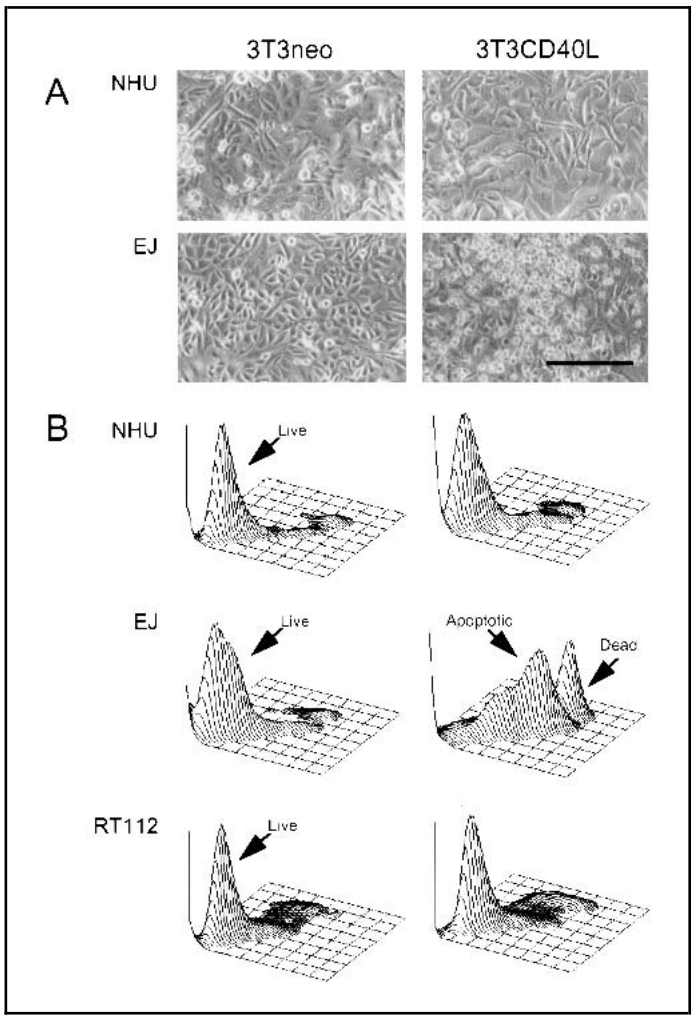

Fig. 2. Induction of apoptosis in malignant urothelial cells by coculture with CD40 ligand (CD40L)-transfected 3T3 fibroblasts. Mitomycin C-treated 3T3 fibroblasts expressing either the neomycin resistance gene alone (3T3neo, **left column**) or the CD40L gene (3T3CD40L, **right column**) were seeded at 10^5 cells/well in 24-well plates. After the fibroblasts had attached to the substrate, normal human urothelial (NHU) cells or transitional cell carcinoma-derived EJ or RT112 bladder tumor cells were seeded on top of them at 9×10^4 cells/well. **A**) Phase–contrast micrographs of CD40-positive NHU and EJ cells taken after 72 hours of coculture. Scale bar = 100 μm. **B**) Nonadherent and adherent cells harvested after 72 hours of coculture of transfected fibroblasts with NHU, EJ, or RT112 cells were incubated in suspension with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI) and then analyzed by flow cytometry. The results are plotted as three-dimensional density plots of mean cell number on the ***z*-axis** versus Annexin V fluorescence on the ***x*-axis** and PI fluorescence on the ***y*-axis.** Cells that did not stain for either dye were considered "live," Annexin V-positive cells that stained medium-low with PI were considered apoptotic (i.e., in the process of undergoing apoptosis), and Annexin V-positive cells that stained brightly with PI were considered as overtly dead cells (i.e., cells with disrupted membrane integrity), as indicated by the **arrows.**

extensively cross-linked, cannot transmit apoptotic signals to tumor-derived urothelial cells, because cell death occurred only when the ligating receptor agonist was presented on the surface of cells. Western blots of EJ cells induced to undergo apoptosis by coculture with 3T3CD40L cells showed that levels of the anti-apoptotic protein Bcl-2 decreased markedly. By contrast, in these same cocultures, levels of the pro-apoptotic Bax protein increased, as did those of Bak, although to a lesser extent (Fig. 3, C).

Effects of TNF-α and Fas Ligation on Urothelial Cell Proliferation and Survival

To determine the effects of ligation of other members of the TNFR superfamily, we used TNF-α and an agonistic anti-Fas antibody. TNF-α inhibited the growth of NHU cells (Fig. 1, C). However, TNF-α had only minor pro-apoptotic effects on normal cells: In nine of 11 independent NHU cell lines tested, the percentage of apoptotic and/or dead cells was increased slightly in TNF-α-treated cells compared with untreated cells (Fig. 4, A). The other two NHU cell lines did not undergo TNF-α-mediated apoptosis. TNF-α had a small but statistically significant stimulatory effect on growth in EJ cells (Fig. 1, C) but did not promote apoptosis in these cells (Fig. 4, A). By contrast, RT4 and RT112 cells were highly sensitive to TNF-α-mediated apoptosis, with TNF-α exposure typically resulting in 60%–70% dead cells after 48 hours (Fig. 4, A).

NHU cells showed modest sensitivity to Fas-mediated apoptosis following surface ligation of Fas with anti-Fas antibody cross-linked with a goat anti-mouse IgG secondary antibody (Fig. 4, A). We observed, by using acridine orange staining, that NHU cell cultures contained a few apoptotic bodies at 24 hours after incubation with antibody. By 48 hours, the percentage of apoptotic and/or dead cells had approximately doubled in all nine independent NHU cell lines tested.

After 48 hours of incubation with cross-linked anti-Fas antibody, RT112 and RT4 cells displayed a 30%–40% increase in apoptosis compared with control (i.e., untreated) cells (Fig. 4, A). By contrast, EJ cells were completely resistant to Fas-mediated apoptosis and showed no increase in the percentage of apoptotic cells following incubation with anti-Fas antibody, independent of cross-linking with secondary antibody (Fig. 3). In control experiments, the efficacy of the cross-linked anti-Fas antibody was demonstrated by using the Fas-sensitive Jurkat T lymphoblastoid cell line, in which up to 96% of cells underwent apoptosis by 48 hours of incubation with the antibody (data not shown).

Effects of Multiple Receptor Ligation on Urothelial Cell Survival

Although NHU cells did not undergo apoptosis when they were cocultured with 3T3CD40L cells alone (Fig. 2), addition of either TNF-α or anti-CD95 antibody to such cocultures resulted in extensive apoptosis (Fig. 4, B). However, cocultures of NHU cells with 3T3neo cells did not confer any additional apoptotic sensitivity over that induced by exposing NHU cells to TNF-α (Fig. 4, B), anti-Fas antibody (Fig. 4, B), or sCD40L (data not shown). By contrast, addition of soluble ligands to EJ cells cocultured with 3T3CD40L or 3T3neo cells did not increase either the very high apoptotic rate seen with ligand-transfected fibroblasts or the low spontaneous rates, respectively, seen in control cultures (Fig. 4, B).

To examine whether apoptotic responses in NHU cells could be elicited by other ligands of the TNF family, we tested the effects of combinations of sCD40L with TNF-α, sCD40L with anti-Fas antibody, or TNF-α with anti-Fas antibody. All three combinations showed synergistic enhancement of apoptosis in NHU cells over the amounts of apoptosis induced by the single effectors alone, typically inducing 60%–80% apoptosis. By contrast, tumor-derived RT4 and RT112 cells showed no synergistic

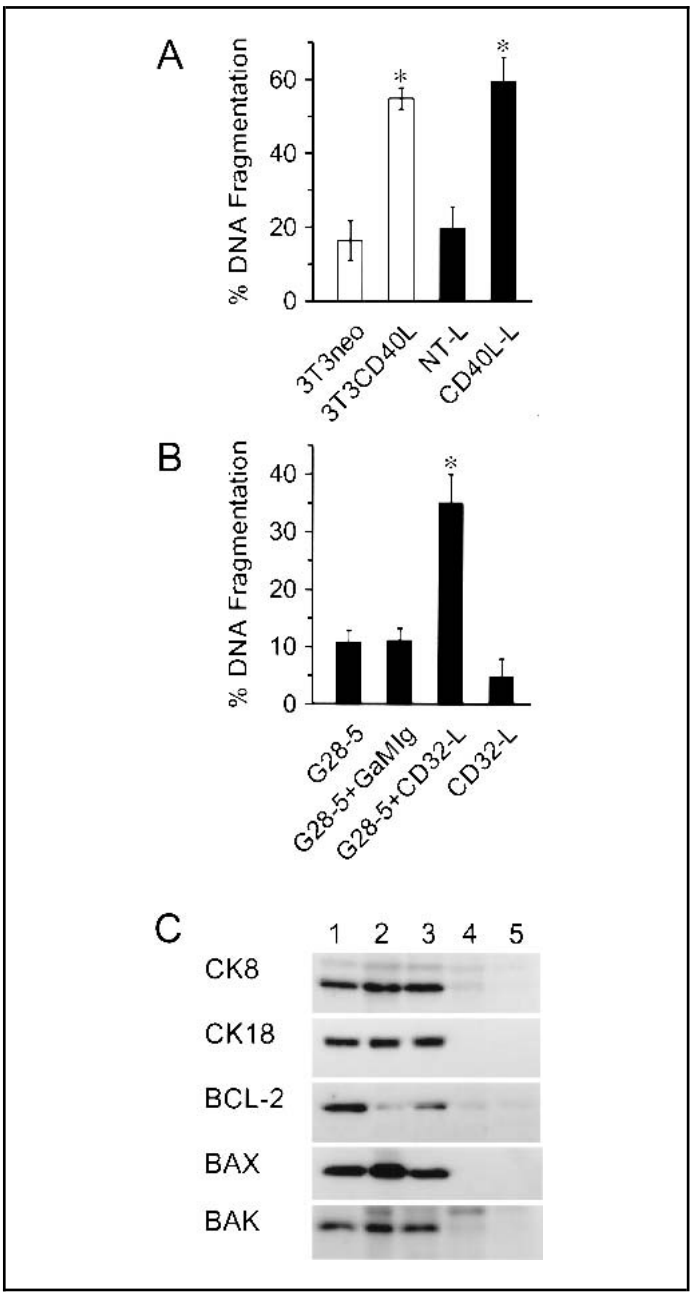

Fig. 3. Analysis of apoptosis induced in malignant bladder epithelial cells by CD40 ligation. **A**) EJ cells were cocultured with 3T3 cells or L fibroblast cells that were transfected with the coding sequence for CD40 ligand (CD40L) (3T3CD40L cells and CD40L-L cells, respectively) and with control-transfected 3T3neo cells or untransfected (NT-L) fibroblasts. The fibroblasts were treated with mitomycin C, plated at 10^4 cells/well in 96-well plates, and allowed to attach overnight. The EJ cells were prelabeled while they were in the exponential growth phase with [^{3}H]thymidine overnight and then harvested, washed, and plated onto the fibroblasts at 9×10^3 cells/well. After 48 hours of coculture, cell contents were harvested onto glass fiber filters, and the amount of ^{3}H-labeled nuclear DNA retained was estimated by liquid scintillation spectrometry. The percentage of nuclear DNA fragmentation was calculated according to the formula $(S - E \times 100)/S$, where S = cpm recovered from control cultures and E = cpm recovered from test cultures, after background subtraction. Results are means of six replicates; **error bars** correspond to 95% confidence intervals. * indicates a statistically significant difference ($P<.001$; two-tailed Student's *t* test) in the percentage of DNA fragmentation between EJ cell–3T3neo cell cocultures and EJ cell–3T3CD40L cell cocultures and between EJ cell–NT-L cell cocultures and EJ cell–CD40L-L cell cocultures. Bars in (**A**) are colored for clarity and contrast. **B**) EJ cells prelabeled with [^{3}H]thymidine were plated out as in **A** and incubated with native G28-5 agonistic anti-CD40 antibody (G28-5), G28-5 that was cross-linked with affinity-isolated human serum protein-adsorbed goat anti-mouse immunoglobulin (G28-5 + GaMIg), or G28-5 that was presented on the surface of L cells transfected with CD32 (G28-5 + CD32-L cells). CD32-L cells were used as a control. To present the antibody on fibroblasts, CD32-L cells were pretreated with mitomycin C, washed and plated out as in **A** above, and then incubated with G28-5 antibody or isotype-matched control before addition of epithelial cells as in **A.** Results are means of six replicates; **error bars** correspond to 95% confidence intervals. * indicates a statistically significant difference ($P<.001$; two-tailed Student's *t* test) in the percentage of DNA fragmentation between EJ cells that were exposed to G28-5 and CD32-L cells and EJ cells that were exposed to any of the other experimental conditions. **C**) Western blotting was performed on lysates of 24-hour cocultures of EJ cells and 3T3CD40L or 3T3neo fibroblasts. Cell lysates were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with human-specific antibodies against cytokeratins 8 and 18 (CK8 and CK18), and against Bcl-2, Bax, and Bak. Lysates from fibroblasts cultured alone were included as controls. Track loading was adjusted for equal amounts of epithelial cell protein on the basis of immunoreactivity with antibodies to CK8 and CK18 in western blots. Antibody binding was detected by enhanced chemiluminescence. **Lane 1,** EJ cells only (control); **lane 2,** EJ cells cocultured with 3T3CD40L cells; **lane 3,** EJ cells cocultured with 3T3neo cells; **lane 4,** 3T3CD40L cells only; **lane 5,** 3T3neo cells only.

or even additive responses when exposed to multiple soluble ligands. Thus, EJ cells remained refractory to all combinations of soluble ligands, whereas in RT4 and RT112 cells, the apoptotic response to combinations of soluble ligands did not exceed that of the most potent single soluble ligand (TNF-α) (Fig. 4, A).

These results demonstrate that receptor cooperation is necessary for the efficient induction of apoptosis in normal bladder epithelial cells, whereas responses of their carcinoma-derived counterparts were dependent solely on the individual receptors. This concept was further supported by two additional observations (data not shown): first, that expression of Fas was not statistically significantly increased, as determined by flow cytometry, on EJ cells undergoing apoptosis induced by coculture with 3T3CD40L cells; and second, that addition of the Fas-blocking NOK-1 antibody had no effect on 3T3CD40L cell-induced apoptosis.

Effect of Protein Synthesis Inhibition on Apoptosis

In the majority of studies of the effects of CD40 ligation on carcinoma cells *(17,35,36),* apoptosis is evident only if protein synthesis is blocked by treating the cells with cycloheximide. We therefore sought to determine whether cycloheximide would affect apoptotic responses in our urothelial cell model. Cycloheximide had no effect on apoptosis in NHU cells or in EJ cells cocultured with 3T3CD40L cells beyond that caused by toxicity of the cycloheximide alone (Fig. 5, A). Similarly, pretreatment of NHU cells with cycloheximide did not affect the levels of apoptosis induced by sCD40L, TNF-α, or CD95 ligation (Fig. 5, B). By contrast, pretreatment of EJ cells with cycloheximide rendered them sensitive to all three soluble effectors. In RT4 and RT112 cells, apoptotic responses were augmented following cycloheximide treatment, exceeding 95% killing in TNF-α-treated

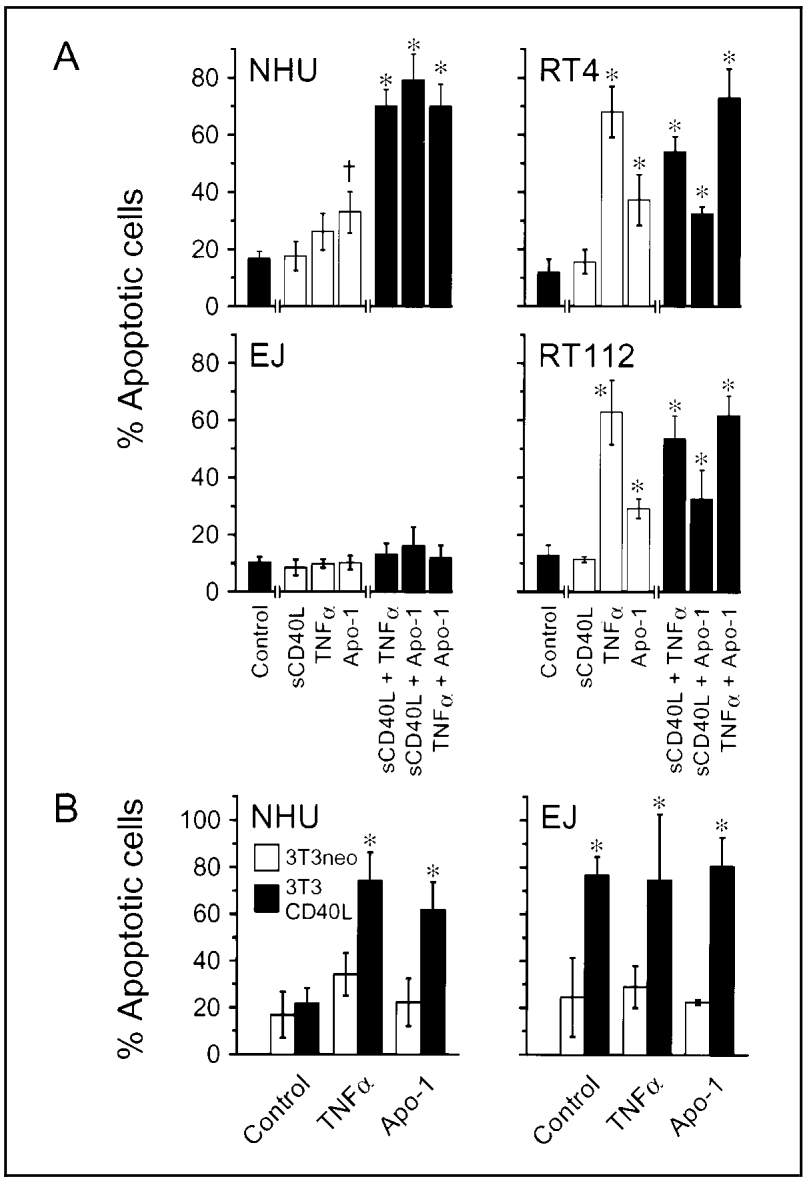

Fig. 4. Apoptotic responses to ligation with other receptors, both singly and in combination. **A**) Normal human urothelial (NHU) cells and the transitional cell carcinoma-derived cell lines RT4 (well-differentiated, CD40-positive), RT112 (moderately-differentiated, CD40-negative), and EJ (anaplastic, CD40-positive) were plated in replicates at 10^5 cells/well in 24-well plates and incubated for 72 hours with soluble receptor agonists alone or in combination. Soluble trimeric CD40 ligand (sCD40L) was used at 10 μg/mL, tumor necrosis factor-α (TNF-α) was used at 300 U/mL, and agonistic anti-Fas antibody (Apo-1) was used at 0.5 μg/mL with a cross-linking goat anti-mouse immunoglobulin (Ig) at 10 μg/mL. The contents of each well were harvested, labeled in suspension with Annexin V fluoroconjugate and propidium iodide, and analyzed by flow cytometry. Gates were set to detect viable and apoptotic cells (compare Fig. 2), and the percentage of apoptotic cells in each culture was plotted. **Bars** represent mean values for 11 independent NHU cell lines and for at least six independent determinations for each transitional cell carcinoma-derived cell line; **error bars** represent 95% confidence intervals. For NHU cells, * indicates a statistically significant difference ($P<.001$) in the percentage of apoptotic cells between cultures treated with combinations of agonists and control cultures or cultures treated with single agonists, and † indicates a statistically significant difference ($P<.01$) in the percentage of apoptotic cells between cells treated with Apo-1 antibody and control cultures. For RT4, EJ, and RT112 cell cultures, * indicates a statistically significant difference ($P<.001$) in the percentage of apoptotic cells between cells treated with TNF-α, Apo-1, or any combination of agonists and control cultures. Bars in (**A**) are colored for clarity and contrast. **B**) Mitomycin-C pretreated control- or CD40L-transfected fibroblasts (3T3neo and 3T3CD40L cells, respectively) were plated at 10^5 cells/well and cocultured with 9×10^4 NHU or EJ cells, in the presence or absence of TNF-α or Apo-1 antibody for 72 hours. The cells were harvested and labeled with Annexin V and PI, and the percentage of apoptotic cells in each culture was assessed by flow cytometry. **Bars** represent mean values for six replicates; **error bars** represent 95% confidence intervals. * indicates a statistically significant difference in the percentage of apoptotic cells between NHU or EJ cells that were cocultured with 3T3neo cells and NHU or EJ cells that were cocultured with 3T3CD40L cells for each treatment ($P<.001$). All statistical significance was determined by analysis of variance using the Tukey–Kramer multiple comparisons test.

RT4 cells. Cycloheximide did not induce sensitivity to sCD40L in CD40-negative RT112 cells (Fig. 5, B).

These findings suggest that tumor cells rely on the synthesis of apoptosis-inhibitory proteins to escape cell death induced by soluble TNF superfamily ligands, whereas normal cells do not. This dependence on synthesis of apoptosis inhibitory proteins appears to be particularly the case for EJ cells. The inability of these cells to resist 3T3CD40L-mediated apoptosis, even when protein synthesis is allowed, further suggests that the two modes of receptor ligation resulted in different downstream consequences.

Expression of Genes Associated With Apoptotic and TNF Signaling Pathways

Multi-probe ribonuclease protection assays were used to identify differences in gene expression between normal and malignant bladder epithelial cells that might account for the differential responses of tumor-derived versus normal urothelial cells to ligation of CD40. NHU cells and transitional cell carcinoma-derived cell lines expressed mRNA transcripts for TNF family receptors (data not shown) and intracellular interacting proteins involved in the transduction of apoptotic signals. The abundance of mRNA species that encode proteins involved in death receptor signaling, such as Fas-associated death domain-containing protein (FADD), Fas-associated protein factor 1 (FAF1), and receptor interacting protein (RIP), was lower in NHU cells and the Fas-resistant EJ cells than in Fas-sensitive RT4 or RT112 cells. TNF-α-resistant EJ cells exhibited relatively low levels of TNFR1-associated protein (TRADD) mRNA compared with RT4 and RT112 cells, which were susceptible to TNF-α-mediated apoptosis (Table 1). Expression of transcripts for Bcl-2 family members was similar between cell types (data not shown).

We also examined the abundance of mRNA transcripts for proteins reported to inhibit apoptosis mediated by TNF family members. All cell lines studied expressed variable amounts of mRNA for members of the TNF receptor-associated factor (TRAF) family (TRAF1, TRAF2, and TRAF3) and inhibitor of apoptosis protein (IAP) family (IAP1 and X-linked IAP [XIAP]). IAP2 mRNA was expressed at high abundance in EJ cells compared with the other cell types and was undetectable in NHU cells and RT112 cells (Table 1).

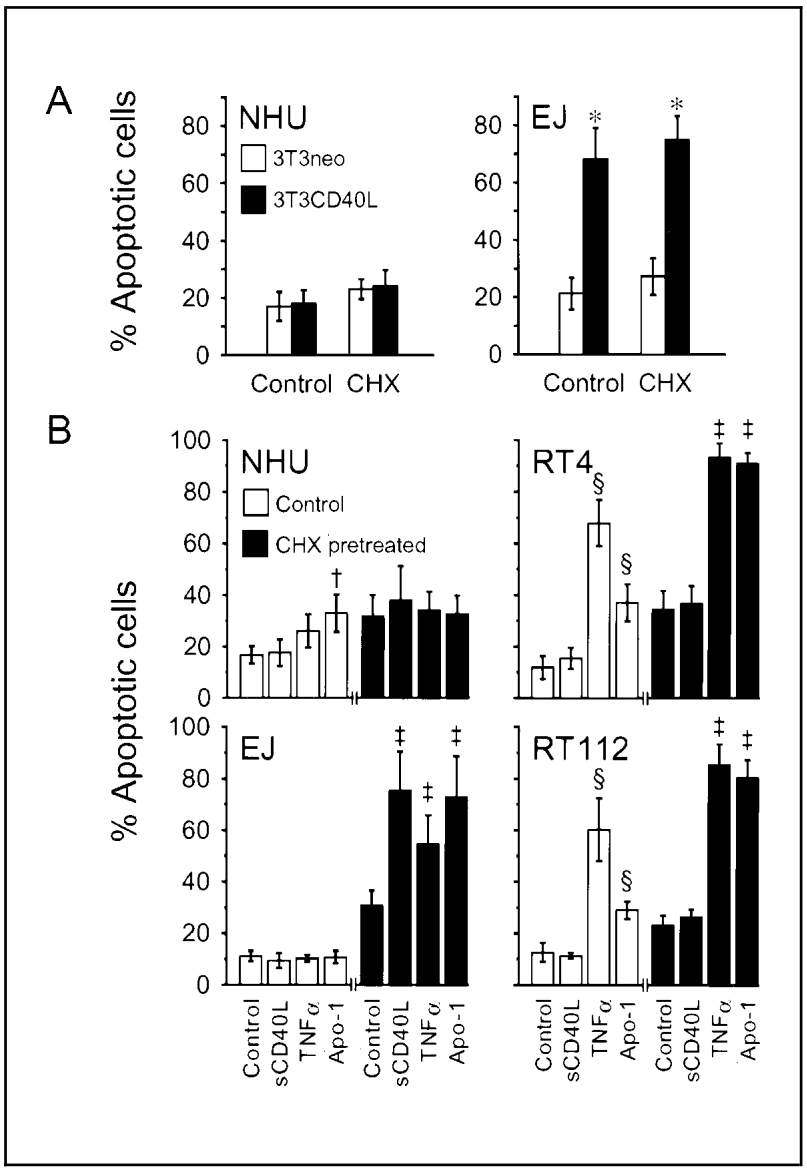

Fig. 5. Effects of blocking protein synthesis on apoptotic responses. The effects of blocking protein synthesis was assessed by pretreating normal human urothelial (NHU) cells with 0.01 μg/mL cycloheximide (CHX), and the transitional cell carcinoma-derived RT4 (well-differentiated, CD40-positive), EJ (anaplastic, CD40-positive), and RT112 (moderately-differentiated, CD40-negative) cell lines with 1 μg/mL cycloheximide for 30 minutes, after which agonists were added to the cells, which were cultured in the continued presence of cycloheximide. Control cultures were performed in the absence of cycloheximide. **A**) Mitomycin C pretreated control- or CD40 ligand (CD40L)-transfected fibroblasts (3T3neo and 3T3CD40L cells, respectively) were plated at 10^5 cells/well and cocultured with 9×10^4 NHU or the transitional cell carcinoma EJ cells for 72 hours. The cells were harvested and labeled with Annexin V and propidium iodide (PI), and the percentage of apoptotic cells in each culture was assessed by flow cytometry. **Bars** represent mean values for four replicates; **error bars** represent 95% confidence intervals. * indicates a statistically significant difference ($P<.001$) in the percentage of apoptotic cells between EJ cells that were cocultured with 3T3neo cells and EJ cells that were cocultured with 3T3CD40L cells, regardless of CHX treatment. **B**) NHU cells and the transitional cell carcinoma-derived cell lines RT4, RT112, and EJ were plated in replicates at 10^5 cells/well in 24-well plates and incubated for 72 hours with soluble receptor agonists. Soluble trimeric CD40 ligand (sCD40L) was used at 10 μg/mL, tumor necrosis factor-α (TNF-α) was used at 300 U/mL, and Apo-1 agonistic anti-Fas antibody was used at 0.5 μg/mL with a cross-linking goat anti-mouse immunoglobulin (Ig) at 10 μg/mL. The contents of each well were harvested, labeled in suspension with Annexin V fluoroconjugate and PI, and analyzed by flow cytometry. Gates were set to detect viable and apoptotic cells (compare Fig. 2), and the percentage of apoptotic cells in each culture was plotted. **Bars** represent mean values of 4–17 replicates; **error bars** represent 95% confidence intervals. † indicates a statistically significant difference ($P<.01$) in the percentage of apoptotic cells between control cells and cells treated with Apo-1 in the absence of CHX; § indicates a statistically significant difference ($P<.001$) in the percentage of apoptotic cells between control cells and cells treated with TNF-α or Apo-1 in the absence of CHX; ‡ indicates a statistically significant difference in the percentage of apoptotic cells ($P<.001$) between control cells and treated cells in cultures that were pretreated with CHX. The statistical significance of all differences was determined by analysis of variance using the Tukey–Kramer multiple comparisons test.

Involvement of NFκB in Responses to CD40, Fas, and TNFR Ligation

Because NFκB has been implicated in influencing cell-survival outcomes mediated by members of the TNFR superfamily *(74,75),* we studied its involvement in signaling mediated by CD40, TNFR, and Fas in our urothelial cell system. All normal and cancer-derived urothelial cell lines showed comparable constitutive expression of NFκB by immunoblotting (data not shown). Indirect immunofluorescence microscopy revealed that NFκB was localized to the cytoplasm in both normal and malignant urothelial cells; incubation of those cells with sCD40L, TNF-α, or anti-CD95 antibody did not induce the nuclear translocation of NFκB. By contrast, in human foreskin-derived fibroblasts, which were used as a positive control, NFκB translocated to the nucleus within 10 minutes of exposing the cells to 400 U/mL TNF-α, where it remained for up to 120 minutes (data not shown).

Ligation of the CD40 receptor in EJ cells following coculture with 3T3CD40L cells resulted in NFκB translocation to the nucleus, whereas, in EJ cells cocultured with 3T3neo cells, it remained in the cytoplasm (Fig. 6). By contrast, coculturing CD40-positive NHU and RT4 cells and CD40-negative RT112 cells with 3T3CD40L cells did not trigger NFκB translocation. Moreover, the NFκB inhibitors BAY 11–7082 and SN50 did not inhibit apoptosis of EJ cells cocultured with 3T3CD40L cells (data not shown). These data suggest that, despite the ability of NFκB to translocate to the nucleus in EJ cells, NFκB activation is not involved in the execution phase of apoptosis induced by 3T3CD40L fibroblasts in CD40-expressing tumor cells.

Responses of p53-Disabled Urothelial Cells to TNF Family Signaling

Because loss of p53 function is a key genetic event for malignant progression in bladder cancer *(48,49),* we investigated whether deletion of p53 function in otherwise normal urothelial cells would alter their responses to CD40, Fas, and TNFR ligation. We found that NHU cells with disabled p53 function (HU-E6 cells) expressed TNFR and CD95 at levels comparable to those in parental NHU cells; CD40 expression was slightly higher in HU-E6 cells than in the parental NHU cells (data not shown).

Table 1. Expression of mRNA for apoptosis-associated proteins in urothelial cells*

Protein	Cell lines				
	NHU	HU-E6	RT4	RT112	EJ
Adaptors					
FADD	9.90	23.73	82.54	29.49	25.43
FAF-1	10.21	5.63	86.88	32.97	30.40
FAP-1	52.29	7.33	82.30	60.00	18.65
RIP	5.60	6.58	35.55	42.73	7.56
TRAF1	5.70	3.72	7.55	9.83	5.20
TRAF2	16.90	11.86	6.75	19.48	10.80
TRAF3	19.39	7.50	11.28	5.00	13.10
TRADD	32.28	18.4	31.61	29.95	7.05
Apoptosis inhibitors					
IAP1 (IAP-C)	23.46	12.89	8.85	15.5	7.05
IAP2 (IAP-B)	—	10.19	4.05	—	53.00
XIAP	26.69	13.50	26.91	23.84	20.13

*Messenger RNA (mRNA) was quantitated by ribonuclease protection assays; values are expressed as a percentage of the mRNA for the glyceraldehyde-3-phosphate dehydrogenase in each cell line. NHU = normal human urothelial; FADD = Fas-associated death domain-containing protein; FAF = Fas-associated protein factor; FAP = Fas-associated phosphatase 1; RIP = receptor interacting protein; TRAF = tumor necrosis factor receptor-associated factor; TRADD = tumor necrosis factor receptor 1-associated protein; IAP = inhibitor of apoptosis protein; XIAP = X-linked inhibitor of apoptosis protein; — = a value too low to quantitate.

HU-E6 cells, despite showing no karyologic abnormalities, showed the same overall pattern of sensitivity to TNF family ligands as fully malignant (i.e., tumor-derived) cells rather than that shown by the untransfected NHU parental cells. HU-E6 cells were highly sensitive to apoptosis induced by coculture with 3T3CD40L cells (Fig. 7, A) but were not sensitive to apoptosis induced by exposure to any soluble ligand (e.g., sCD40L, TNF-α, or anti-Fas), either alone or in combination (Fig. 7, B). No nuclear translocation of NFκB was seen following coculture of HU-E6 cells with either 3T3CD40L cells or 3T3neo cells (data not shown), and no major differences were detected in the expression of apoptosis-related mRNA species between HU-E6 cells and NHU cells (data not shown). These results suggest that even the early genetic events of malignant transformation, typified by loss of p53 function *(48,49),* profoundly alter the apoptotic susceptibility and response patterns of human urothelial cells to signaling by members of the TNF family.

Discussion

Results from our study reveal three novel aspects of CD40 biology in urothelial cells: 1) that the effects of CD40 ligation, either alone or in conjunction with ligation of other members of the TNFR family, are context-specific, in that outcome depends on additional factors; 2) that the mode of CD40 ligation is critical to the outcome; and 3) that genetic changes that lead to malignant transformation alter the responses of urothelial cells to TNF family signaling.

Our data were obtained by using established cell lines and therefore may not be entirely applicable to the situation *in vivo.* Nevertheless, the established transitional cell carcinoma-derived cell lines that we used have been shown, both *in vitro (76)* and *in vivo (52),* to faithfully recapitulate the grade and stage of the originating tumor. The ability of NHU cell lines to re-form stratified transitional epithelia *(47)* also supports the biologic relevance of our model system. The findings of antitumor effects of CD40L in SCID mice inoculated with established breast carcinoma cell lines *(33,34)* has recently been extended to xenografts of surgical specimens of ovarian adenocarcinoma *(39),* thereby supporting the use of cell lines as indicators of CD40 sensitivity. These and other studies have also shown that carcinoma cells from a number of epithelial origins are susceptible to the antitumor effects of CD40 ligation.

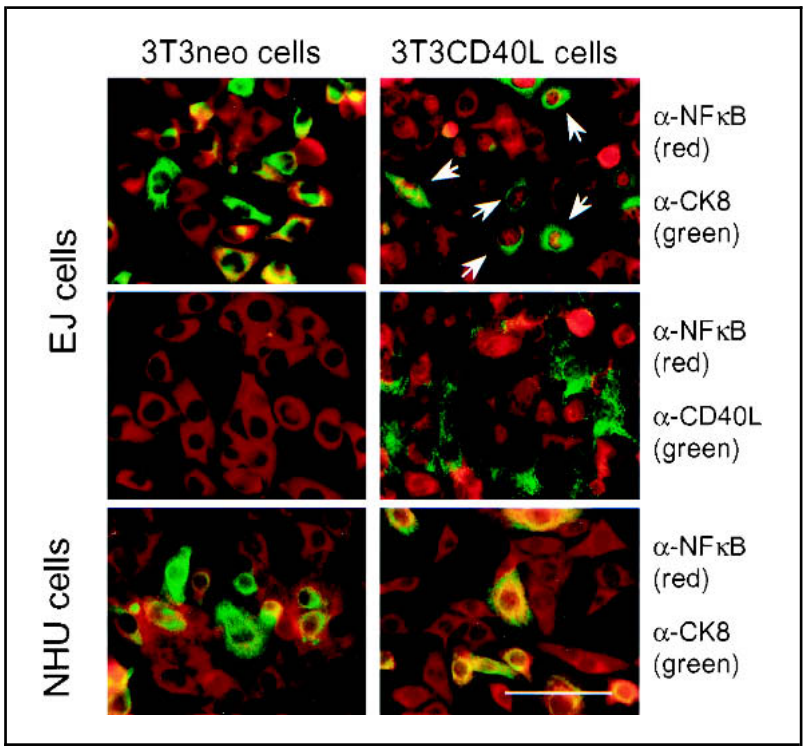

Fig. 6. Nuclear translocation of nuclear factor-κB (NFκB) in response to CD40 signaling. Mitomycin C-treated 3T3neo or 3T3CD40L fibroblasts were seeded at 1.5×10^4 cells/spot on 12-spot glass slides and incubated overnight before addition of 10^4 normal human urothelial (NHU) cells or transitional cell carcinoma-derived EJ cells per spot. The cells were cocultured for 2 hours, then washed, fixed in a 1 : 1 mixture of methanol/acetone, air-dried, and processed for two-color indirect immunofluorescence. NFκB (**red**) was detected with a rabbit anti-NFκB p65 antibody and visualized with goat anti-rabbit immunoglobulin (Ig)–Texas Red conjugate. Monoclonal antibodies specific for cytokeratin 8 (CK8) or for CD40 ligand (CD40L) were used to detect epithelial cells and CD40L-transfected 3T3 fibroblasts, respectively. Monoclonal antibodies were visualized by using a fluorescein isothiocyanate-conjugated goat anti-mouse Ig (**green**). **Yellow** indicates colocalization of **red**- and **green**-tagged antibodies. **Arrows** indicate cells that displayed nuclear translocation of NFκB. Scale bar = 50 μm.

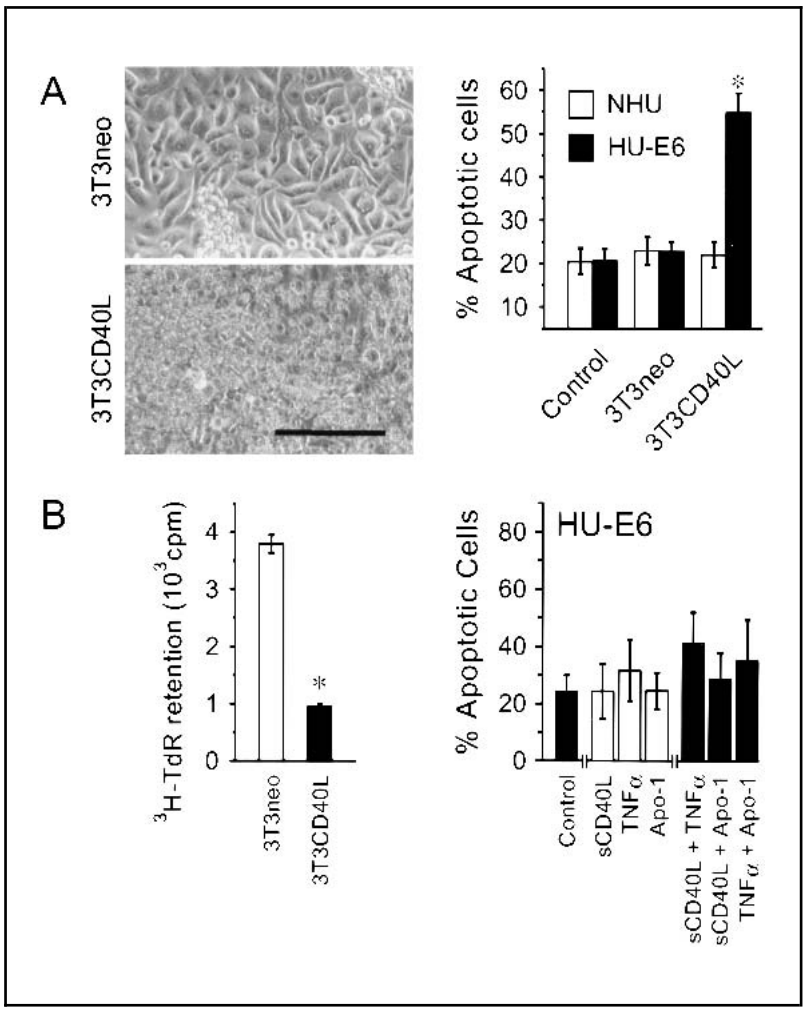

Fig. 7. Responses of p53-disabled Human Urothelial (HU-E6) cells to CD40, tumor necrosis factor receptor (TNFR), and Fas ligation. **A**) Mitomycin C-treated 3T3 fibroblasts expressing the neomycin resistance gene alone (3T3neo cells) or with the gene for CD40 ligand (CD40L) (3T3CD40L cells) were seeded at 10^5 cells/well in 24-well plates. Following fibroblast attachment, untransfected normal human urothelial (NHU) cells or urothelial cells with disabled p53 function (HU-E6 cells) were seeded on top of the fibroblasts at 9×10^4 cells/well. **Left panel** shows a phase–contrast micrograph of HU-E6 cells after 72 hours of coculture with 3T3neo cells (**top**) or with 3T3CD40L cells (**bottom**). Scale bar = 100 μm. Cells harvested from the 72-hour cocultures were labeled in suspension with Annexin V fluoroconjugate and propidium iodide and analyzed by flow cytometry to determine percentages of viable and apoptotic cells (**right panel**). Results are expressed as mean value of 4–9 replicate determinations; **error bars** represent 95% confidence intervals. * indicates a statistically significant difference in the percentage of apoptotic cells between HU-E6:3T3CD40L cocultures and HU-E6:3T3neo cocultures ($P<.001$; two-tailed Student's t test). **B**) Mitomycin C-treated 3T3neo or 3T3CD40L fibroblasts were seeded at 10^4 cells/well in 96-well plates and cocultured for 72 hours with 9×10^3 HU-E6 cells that were previously labeled with [^{3}H]thymidine (^{3}H-TdR). The cells were then harvested onto glass fiber filters, and the amount of DNA retained was estimated by measuring ^{3}H-TdR using liquid scintillation spectrometry (**left panel**). Results are expressed as the mean value of sextuplicate determinations; **error bars** represent 95% confidence intervals. * indicates a statistically significant difference in the amount of ^{3}H-TdR retained by 3T3CD40L cocultures and the amount retained by 3T3neo cocultures ($P<.001$; two-tailed Student's t test). HU-E6 cells were also plated alone (**right panel**) at 9×10^4 cells/well and cultured in the presence of soluble trimeric CD40 ligand (sCD40L) at 10 μg/mL, tumor necrosis factor-α (TNF-α) at 300 U/mL, and agonistic anti-Fas antibody (Apo-1) at 0.5 μg/mL with cross-linking goat anti-mouse immunoglobulin at 10 μg/mL, alone or in combination. Adherent and nonadherent cells were harvested at 72 hours, labeled with Annexin V and propidium iodide, and analyzed by flow cytometry. Bars in (**B**) are colored for clarity and contrast.

Context-specific responses to CD40 signaling have been well established in B cells and their malignant counterparts, where, depending on endogenous and external conditions, CD40 ligation may either induce apoptosis or promote survival *(2,8,10–12,77,78).* Our study is the first to demonstrate that a similar situation may apply in urothelial cells. Importantly, our data demonstrate that urothelial cell survival is highly dependent on the mode of CD40 ligation. Thus, sCD40L inhibited the growth of both normal and malignant urothelial cells but did not induce apoptosis, whereas surface-presented CD40L, while having no substantial inhibitory effects on growth (data not shown), specifically induced apoptosis in transformed cells but not in normal cells. Our findings with soluble ligands are consistent with the findings of others who have shown that growth inhibition is a consequence of CD40 ligation in normal keratinocytes *(23,29,30)* and in carcinoma cells *in vitro (29–32)* and *in vivo (33,34,39).*

It is well established that in B cells, responses to CD40 depend on the mode of ligation. Factors such as epitope specificity and degree of cross-linking of the ligating CD40 antibody *(53,54),* the use of agonistic antibody versus cell-surface presentation of CD40L *(55),* and even the density of CD40L expression on transfected fibroblasts in a coculture system *(56)* can critically affect functional outcome [reviewed in *(9)*]. Thus, it would appear that in B cells and epithelial cells, the precise nature of the response to CD40 ligation, be it survival or death, depends on the nature of the ligating signal, probably as a function of the quality of receptor cross-linking. A close analogy is provided by the Fas/FasL system, where killing or protection from killing depends on whether FasL is presented in soluble or membrane-bound form *(79,80).*

Our studies using CD40L-transfected fibroblasts also reveal that CD40 ligation can induce a dramatic apoptotic response in malignant, and even in "premalignant" (i.e., normal cells with disabled p53 function) urothelial cells, while sparing their normal counterparts. These novel observations are supported by previous reports of CD40-dependent apoptotic responses in other tumor cell systems, such as CD40-transfected HeLa cells after cycloheximide treatment *(17,35)* and cycloheximide-treated hepatocellular and ovarian carcinoma cells *(17,36).*

Although a wealth of information has been gathered about the downstream effectors involved in CD40 signaling, particularly those in B cells, the mechanisms by which these effectors cooperate with those mechanisms elicited by other members of the TNFR family to transmit apoptotic versus survival signals is far from clear. Although CD40 has no intrinsic death domain of its own, its ability to transmit cell death signals by recruiting members of the TRAF family of adaptor molecules may allow it to interact with the apoptotic pathways known to be associated with other TNFR superfamily molecules in addition to directly activating NFκB and/or stress kinases *(8,75,78,81,82).* For example, CD40 ligation has been reported to promote *(16,29,31)* and to inhibit *(40–44)* Fas-mediated apoptosis. Recently, CD40 has been shown to induce autotropic cell death *via* other mem-

bers of the TNFR superfamily *(15–18).* Our findings demonstrate direct interactions between downstream receptor signaling pathways in normal urothelial cells, in that extensive apoptosis was only induced by downstream signals through combinations of TNFR superfamily molecules. This finding suggests that signaling by TNFR superfamily members may play a fundamental role in the maintenance of epithelial tissue homeodynamics. Furthermore, our results argue against autotropic Fas- or TNFR-mediated mechanisms of cell death, because we found that ligation of either of these receptors alone failed to transmit a substantive pro-apoptotic signal in normal cells, whereas CD40-mediated killing was not Fas-dependent in tumor cells, and the addition of exogenous TNF-α did not enhance CD40-mediated effects.

Genetic changes that lead to malignant transformation appear to dramatically alter the susceptibilities of urothelial cells to CD40 and to ligation of multiple receptors. For example, the simple loss of p53 function in otherwise normal urothelial cells was sufficient to confer susceptibility to CD40-mediated apoptosis on those cells. Loss of p53 function is believed to represent a major pathway of transformation toward an invasive phenotype in urothelial cells *(48,49)* and hence may be regarded as a key premalignant change. Although loss of p53 function has been previously associated with changes in a cell's receptiveness to Fas-mediated killing, the data in this regard are conflicting *(80).* We have shown that loss of p53 function destroys the ability of NHU cells to induce p21 expression in response to genotoxic insult *(51),* and there is evidence that p21 may be an important mediator of sensitization to CD95-induced apoptosis *(83).* However, an increase in CD40 susceptibility as a consequence of p53 loss and malignancy has not been previously reported.

At present, the molecular basis for CD40-dependent sensitization to apoptosis in either B cells or carcinoma cells is not understood. The differences we observed in the levels of transcripts for a variety of genes implicated in TNFR-family mediated phenomena suggest that the loss of sensitivity to FasL and TNF-α in highly-anaplastic EJ cells could be due to high levels of IAP2 expression. However, the high levels of IAP2 expression by EJ cells, as demonstrated by ribonuclease protection assays (Table 1), did not appear to be sufficient to protect them from killing mediated by cell surface-presented CD40L. These findings suggest that in malignant cells, the downstream apoptotic mechanisms mediated by Fas and TNFR are distinct from those mediated by CD40.

In conclusion, our data show that genetic changes that lead to malignant transformation in human urothelial cells can affect the way in which the cells respond to signals delivered by members of the TNFR superfamily. The need to trigger more than one member of the TNFR family to induce extensive apoptosis in normal urothelial cells suggests that this mechanism protects cells against adventitious apoptosis due to bystander effects, such as diffusion of TNF-α from an adjacent site of inflammation. The need for multiple receptor ligation may reflect an adaptive response by the urothelium to provide a high threshold to apoptosis to maintain urinary barrier function. However, the susceptibility to multiple receptor ligation may be lost early in the malignant transformation pathway of urothelial cells, perhaps providing one important means by which these cells could escape eradication through immune surveillance. Paradoxically, malignant transformation appears to confer on urothelial cells *de novo* apoptotic susceptibility to cell surface-presented CD40L. Thus, loss of CD40 expression could, in turn, represent a second major mechanism of immune escape. This hypothesis predicts that such loss would be associated with a worse prognosis and with more aggressive tumor progression. Supportive evidence for this hypothesis is provided by the strong association between loss of CD40 expression by bladder tumors with increased stage and grade *(84).*

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U. Bugajska received an Overseas Research Scholarship award and was supported by a Tetley-Lupton Scholarship from the University of Leeds. J. Southgate is a research professor supported by York Against Cancer, and J. Gordon is a nonclinical research professor supported by a programme grant from the Medical Research Council.

We would like to thank Mark W. C. East for help in cDNA cloning; Professor N. Maitland (University of York) and Dr. Eva Pitt for help with the preparation of E6 transfectants; and Wendy Kennedy, Diana Quinn, and Ruth Morton for unrivalled technical assistance.

Manuscript received September 24, 2001; revised July 15, 2002; accepted July 17, 2002.