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Activation of CD40 in Cervical Carcinoma Cells Facilitates CTL Responses and Augments Chemotherapy-Induced Apoptosis

Seran C. Hill,* Sarah J. Youde,† Stephen Man,‡ Glyn R. Teale,*† Amanda J. Baxendale,* Andrew Hislop,* Clare C. Davies,* David M. Luesley,§ Anna M. Blom,§ Alan B. Rickinson,* Lawrence S. Young,* and Aristides G. Eliopoulos2,3*

In this study, we describe the expression and function of CD40, a TNF receptor family member, in cervical carcinomas. CD40 was present at very low levels in normal cervical epithelium but was overexpressed in human papillomavirus-infected lesions and advanced squamous carcinomas of the cervix. The stimulation of CD40-positive cervical carcinoma cell lines with soluble CD40L (CD154) resulted in activation of the NF-κB and MAPK signaling pathways and up-regulation of cell surface markers and intracellular molecules associated with Ag processing and presentation. Concomitantly, the CD154-induced activation of CD40 in carcinoma cells was found to directly influence susceptibility to CTL-mediated killing. Thus, CD40 stimulation in cervical carcinoma cell lines expressing a TAP-dependent human papillomavirus 16 E6 Ag epitope resulted in their enhanced killing by specific CTLs. However, CD154 treatment of carcinoma cells expressing proteasome-dependent but TAP-independent Ags from the EBV-encoded BRFL1 and BMLF1 failed to increase tumor cell lysis by specific CTLs. Moreover, we demonstrate that chemotherapeutic agents that suppress protein synthesis and reverse the CD40-mediated dissociation of the translational repressor eukaryotic initiation factor 4E-binding protein from the initiation factor eukaryotic initiation factor 4E, such as 5-fluorouracil, etoposide, and quercetin, dramatically increase the susceptibility of cervical carcinoma cells to CD40L-induced apoptosis. Taken together, these observations demonstrate the functional expression of CD40 in epithelial tumors of the cervix and support the clinical exploitation of the CD40 pathway for the treatment of cervical cancer through its multiple effects on tumor cell growth, apoptosis, and immune recognition. The Journal of Immunology, 2005, 174: 41–50.

A member of the TNF receptor superfamily, CD40 is expressed on a plethora of cell types, including normal B lymphocytes, macrophages, endothelial cells, and dendritic cells, and this widespread expression is likely to account for its central role in the regulation of immunity and host defense (1). Indeed, the interaction of CD40 with its ligand (CD40L, gp39, or CD154), which is predominantly expressed in activated T cells, is critical for the survival and proliferation of normal B lymphocytes engaged in adaptive immune responses. Furthermore, the stimulation of CD40 in macrophages promotes their activation, thereby amplifying the innate immune response to both extracellular and intracellular pathogens. The key role of the CD40 and its ligand in orchestrating these processes is exemplified by clinical data from patients with X-linked hyper-IgM syndrome, a rare immune disorder caused by mutations in the CD40L gene that maps to human chromosome Xq26. In these patients, thymus-dependent responses to Ags, such as Ig class switching and Ab production, are impaired with consequent deficiency in germline center formation, recurrent infections, and high frequency of carcinomas and lymphomas (2, 3). A similar phenotype has been observed in mice in which either the CD40 or the CD40L gene has been disrupted by gene targeting (4, 5).

In addition to its expression in normal lymphoid cells, CD40 is also found in malignant hemopoietic cells, including leukemias and lymphomas. However, in marked contrast to the proliferative effects of CD40 ligation on normal B lymphocytes, CD40 stimulation in malignant lymphoid cell lines results in growth retardation both in vitro and in vivo, indicating a cell type/differentiation state-dependent response to CD40 stimulation (6–8). A similar growth-inhibitory effect has been noted in a number of carcinoma cell lines and early-passage ovarian tumor cells treated with a soluble trimeric form of CD40L (9–11). This observation, coupled with the reported overexpression of CD40 in a variety of solid tumors, such as those of the ovary, nasopharynx, liver, bladder, and breast (10–15), raise the possibility that the CD40 pathway could be exploited for cancer therapy. This is further supported by our more recent work demonstrating that CD40 ligation can deliver potent apoptotic signals to carcinoma cells, which are manifested only upon disruption of CD40-activated survival pathways, such as PI3K and ERK (16). Data shown in the present study demonstrate that chemotherapeutic agents may also disrupt CD40-induced survival signals, resulting in enhanced susceptibility of carcinoma cells to CD40L-mediated apoptosis.
In addition to the direct antiproliferative and proapoptotic effects of CD40 ligation in carcinoma cells, the influence of CD40L on immune recognition and the generation of CTL responses may provide further therapeutic opportunities. Indeed, the activation of the CD40 pathway in malignant hemopoietic cells plays a major physiological role in Ag-presenting function and immune recognition. Thus, CD40 stimulation has been shown to up-regulate Ag processing and presentation on Burkitt lymphoma cells, resulting in enhanced recognition of these cells by specific CTLs (17). In vitro observations and clinical trials in chronic lymphocytic leukemia have also demonstrated that CD40 activation of malignant B cells improves their immunogenicity and induces tumor-specific T cell immunity (18, 19). Recent evidence also suggests that the T cell help required for efficient generation of CTL responses in vivo depends on the interaction of CD40L-expressing CD4+ T cells with CD40 on dendritic cells, and this effect plays a crucial role in the generation of protective antitumor immunity (20). In agreement with this observation, CD40 ligation has been shown to augment antitumor immune responses via its ability to induce dendritic cell maturation and independent of the expression of CD40 on tumor cells (21, 22). However, it is currently unknown whether the activation of CD40 in carcinomas may directly enhance their immunogenicity. In this study, we have investigated the functional consequences of CD40 activation in cervical carcinoma and provided the first evidence of the ability of the CD40 pathway to directly induce Ag presentation in carcinoma cell lines, resulting in their enhanced recognition by specific CTLs. Therefore, the CD40 pathway provides an important opportunity for cervical cancer therapy through multiple effects on tumor cell growth, apoptosis, and immune recognition.

Materials and Methods

**Cell culture, treatments, and apoptosis assays**

HeLa cervical carcinoma cells stably expressing CD40 were generated and maintained as previously described (23). MS751 and CaSkii cell lines were cultured in DMEM with HEPES, 10% FCS, 2 mM glutamine, and 1% penicillin-streptomycin. LCL lines were cultured in RPMI 1640, 8% FCS, 2 mM glutamine, and 1% penicillin-streptomycin. MS751 were transfected with pcDNA3-CD40 (23) using electroporation at 260 V and 1050 μF. Selection for stable clones was performed by culturing in the presence of 1 μg/ml 418 (Invitrogen Life Technologies). HLA typing of cervical cell lines was performed. MS751 carry HLA-A2, -A24, -B35, and -B60; CaSkii are HLA-A2, -A3, -B7, and -B18; and HeLa are HLA-A68, -B1503, -B6, and -C1203. For induction of apoptosis, cells were plated in triplicate on a 96-well plate at 8000 cells per well in 0.2 ml of medium. The following day, cells were washed with serum-free medium and serum-starved for 10 h. MS751 were then suspended in serum-free medium, and 500 μM of etoposide (ETO), quercetin (QC), or doxorubicin (DOX), and subsequently cocultured with 1 μg/ml trimeric recombinant soluble CD40L (rsCD40L) for 36–48 h. Cells were then trypsinized, pooled into a small volume of FCS, and centrifuged, and fixed in 1% paraformaldehyde. Apoptotic cells were identified following staining with propidium iodide (5 μg/ml; Sigma-Aldrich) and visualization under UV light on the basis of nuclear condensation and degradation. At least 300 cells were counted in each experiment. In some experiments, cell death was determined using a commercially available Cell Death ELISA (Roche), which detects mono- and oligonucleosomes in the cytoplasmic fraction of cells undergoing apoptosis.

**Immunohistochemical staining**

Archived formalin-fixed paraffin-embedded specimens were obtained from patients who had undergone treatment for cervical carcinoma by large loop excision of the transformation zone. The specimens were analyzed and graded as follows: 10 had normal histology; 10 showed human papillomavirus (HPV); changes only (koilocytosis and HPV PCR-positive); 10 were cervical intraepithelial neoplasia (CIN) grade 1; 10 were CIN grade 2; 20 were CIN grade 3; and 10 samples showed invasive squamous carcinoma. Ethical approval had been obtained for the collection of these specimens. Five-micrometer sections were cut and mounted on coated slides; one section was stained with H&E for confirmation of histological grade, and another was immunostained for CD40. The sections were deparaffinized in xylene and washed in ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Ag retrieval was performed in 0.01 M citrate buffer (pH 6) using a 650-W microwave on full power as optimized in preliminary studies. Slides were then suspended in 0.01 M PBS (pH 7.6) and incubated for 30 min with goat serum diluted 1/5 with PBS. A polyclonal primary Ab to CD40 (Santa Cruz N-16, sc774; Santa Cruz Biotechnology) was applied at 1/1000 dilution and incubated in a humidified chamber at 4°C for 24 h. Ab binding was revealed using biotinylated secondary Ab (1/100 dilution for 10 min; DakoCytomation) followed by avidin-peroxidase (1/100 dilution for 10 min; DakoCytomation) and diaminobenzidine substrate. Thorugh 5-min wash steps were performed in between each stage. Slides were counterstained in hematoxylin and mounted in aqueous mountant (Thermo Electron). Tonicular tissue sections were used as a positive control for each staining run. Negative controls were obtained by substitution of the primary Ab for PBS. Isotype-matched controls were used as anti-CD3 and anti-IRBA Ab (both originating in goat). Evaluation of staining was performed independently by two histopathologists using a graded scoring system for both the percentage of stained cells and the intensity of the staining within a representative area. Absence of CD40-positive cells was scored as 0. Staining of the basal epithelial layer or of 1–10% of all cells was given a score of 1. Staining that was confined to a third of the stratified epithelium was given a score of 2, and staining of 11–33% of all cells was given score of 2. Immunostaining of two-thirds of the stratified epithelium or of ≥33–66% of all cells was scored as 3, and the presence of CD40-specific staining throughout the epithelium or in >66% of the cells was given a score of 4. Likewise, a subjective score of 0, 1, 2, or 3 (nil, mild, moderate, and strong, respectively) was given for the intensity of staining within the representative chosen area. The combined scores for intensity and percentage of stained cells were used in a Kruskall-Wallis one-way ANOVA by ranks. Results are presented as medians, ranges, and interquartile ranges.

**Flow cytometry**

Cells were lightly trypsinized and left to recover in complete medium for 30 min, then washed and counted. Cells were aliquoted into wells of a 96-well plate (2 × 10^4) well, washed, and resuspended in PBS supplemented with 10% heat-inactivated goat serum (HINGS). Relevant diluted Abs were added, and the plate was incubated at 4°C for 1 h. Following this incubation, the cells were washed three times with 10% HINGS, the secondary Ab was added, and cells were incubated in the dark for 1 h. The cells were then washed three times as before, resuspended in 50 μl of PBS, and added to 45 μl of 1% paraformaldehyde for fixing, before flow-cytometric analysis. MHC class I-FITC-conjugated Ab (Sigma-Aldrich), CD54-FITC (Serotec), and CD80-PE-conjugated Ab (Serotec) were used at 1/1000 dilution for 10 min. Appropriate FITC- or PE-conjugated Abs were used to measure background staining.

**Abs and immunoblotting**

Phosphospecific Abs were diluted in 5% BSA in TBS plus 0.1% v/v Tween 20 (TBST), whereas all other Abs were diluted in 5% milk in TBST. Phosphotyrosine Ab (Ser(104)), phospho-p42/p44 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and the corresponding Ab that recognize “total,” i.e., both the phosphorylated and nonphosphorylated forms, were purchased from Cell Signaling Technology. The eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) Ab (1/1000) was kindly provided by Dr. R. Denton (University of Bristol, Bristol, U.K.), and the eukaryotic initiation factor 4E (eIF4E) Ab (1/1000) was purchased from Cell Signaling Technology. The TAP1 Ab was purchased from Calbiochem and used at 1/1000 dilution, and the TAP2 and LMP7 Abs were a kind gift from Dr. S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY) and used at 5 μg/ml. Anti-rabbit IgG HRP (1/2000) was purchased from Cell Signaling Technology, and anti-mouse IgG HRP (1/1000) was from Sigma-Aldrich. For immunoblotting, 15–30 μg of protein was separated by SDS-PAGE, transferred onto polyvinylidene fluoride membrane (0.45 μm; Millipore), and blocked for 45 min at room temperature with 5% nonfat milk dissolved in TBST. Following three washes with TBST, membranes were incubated overnight.
at 4°C with primary Ab and for 1 h at room temperature with the appropriate secondary Ab followed by ECL (Amerham Biosciences).

**Viruses, infections, and chromium release assays**

Recombinant vaccinia viruses or HPV16 and 18 E6/E7 fusion gene products (TA-HPV) were generated as previously described (24–26). HLA-A2-restricted peptides originating from the viral proteins were pulsed onto the cells for 1 h at 37°C to act as a positive control for peptide-specific CTL-mediated killing. The peptides for BRLF1 and BMLF1 were YVLDHLIVV and GLCTLVAML, respectively, and for HPV (HPV16 E6,20,33) was THDILIECV. For infections with viruses, cells were trypsinized, washed in serum-free medium, and counted. Reombinant vaccinia virus was added to 1 million cells at a multiplicity of infection of 15 and incubated at 37°C for 1 h with agitation every 20 min. Complete medium was then added, and the cells were plated out in a six-well plate overnight. Cells were then trypsinized, washed, and used in chromuium-51 release assays as previously described (24–26).

**m7-GTP-Sepharose affinity chromatography and [35S]methionine labeling**

To analyze the dissociation of 4E-BP1 from eIF4E, m7-GTP-Sepharose affinity chromatography was used. HeLa/CD40 cells were stimulated with m7-lysis buffer (20 mM Tris (pH 7.5), 100 mM KCl, 0.5% Triton X-100, 0.5% Nonidet P-40, 20 mM β-glycero-phosphate (pH 7.4), 10 mM NaF, 1 mM EGTA, 1 mM DTT, 250 μM sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupetin, and 1 μg/ml aprotinin). eIF4E was immunoprecipitated from 250 μg of total protein by incubating with 15 μl of m7-GTP-Sepharose 4B beads (1:1 slurry; Pharmacia) in a total volume of 500 μl of m7-lysis buffer. After 2-h incubation at 4°C, unbound proteins were removed by two 500-μl washes with lysis buffer, and beads were resuspended in SDS-PAGE loading buffer. Following denaturation, immunoprecipitates were run on a 15% SDS-PAGE gel and subjected to standard Western blotting for eIF4E and 4E-BP1. Protein synthesis was measured as previously described (16).

**Results**

**Expression of CD40 in normal and malignant human cervical epithelium**

We examined the expression of CD40 in normal human cervical epithelium, in specimens from different stages of premalignant CIN and in advanced squamous carcinomas of the cervix using immunohistochemistry. In 6 of 10 normal cervical specimens examined, CD40 expression was restricted to the basal epithelial layer (Fig. 1A). In the rest of these samples, no CD40-specific staining was detected. Interestingly, elevated levels of CD40 throughout the differentiating epithelium were observed in all HPV-positive but otherwise normal cervical tissue specimens analyzed (Fig. 1B). Likewise, all but one of the CIN grade 1 lesions stained positive for CD40 throughout the epithelium (Fig. 1C). In CIN grade 2 specimens, staining was noted in at least 33–66% of the cells, with 6 of 10 lesions showing CD40 expression in the upper one-third of the epithelium (Fig. 1D). The majority of the 20 CIN stage 3 specimens examined showed significant CD40 levels, although poorly differentiated areas had somewhat reduced expression (Fig. 1E). Moreover, in three of these specimens, CD40 was expressed in <33% of the cells, and in one case, there was only weak staining in the otherwise severely dysplastic epithelium. Ten cases of invasive cervical carcinoma were also examined. These specimens showed high levels of CD40 expression that was uniform in intensity and distribution (Fig. 1F).

A semiquantitation of these immunohistochemical data was performed. CD40 expression was graded from 0 to 4 on the basis of the proportion and from 0 to 3 on the basis of the intensity of staining, as described in detail in Materials and Methods, and combined scores were obtained. As shown in Table I, HPV-positive lesions and preneoplastic and malignant specimens expressed higher levels of CD40 than normal cervical epithelium. These results suggest that CD40 is up-regulated in the hyperplastic and dysplastic cervix and could potentially serve as a therapeutic target for this type of cancer.

**A rsCD40L (CD154) activates CD40 expressed in cervical carcinoma cell lines**

The expression of CD40 in primary cervical epithelial tissue prompted us to examine the ability of CD40L (CD154) to deliver biological signals to cervical carcinoma cell lines. For these experiments, three established cervical carcinoma cell lines were used. CaSki is an HPV16-transformed line that naturally expresses CD40 (Fig. 2A). MS751 and HeLa are HPV18 positive, but do not express CD40. These cells were stably transfected with a CD40 expression vector, and selected clones (Fig. 2A) were used for subsequent experiments.

In other cell backgrounds, CD40 ligation has been shown to engage the NF-κB and the JNK and ERK MAPKs (1, 16, 27). The activation of NF-κB by CD40L involves the phosphorylation and degradation of IκBα, a cytoplasmic inhibitor of NF-κB. Following stimulation of MS751-CD40, CaSki, and HeLa-CD40 with 1 μg/ml trimeric rsCD40L (CD40L/CD154), cell lysates were immunoblotted for detection of IκBα phosphorylation and degradation as a surrogate for NF-κB activation. In addition, lysates were analyzed for MAPK activation using Abs that recognize the phosphorylated, active forms of JNK or ERK or Abs that recognize these kinases independently of their phosphorylation status. The results showed that CD40 ligation induces significant activation of the NF-κB, JNK, or ERK pathways in cervical carcinoma cells (Fig. 2B). As a control, stimulation of parental, CD40-negative MS751 cells with CD40L failed to induce any of these activities. These data demonstrate that CD40 expressed in cervical carcinoma cell lines is functional.

**Effects of CD40 ligation on surface cervical carcinoma cell phenotype associated with Ag presentation**

Given the ability of trimeric rsCD40L to induce signal activation in CD40-expressing cervical carcinoma cell lines, we examined the effects of CD40 cross-linking on cell surface marker expression associated with Ag presentation. To this end, CaSki, MS751-CD40, and HeLa-CD40 cultures were treated with CD40L/CD154 for 24 h and analyzed for expression of MHC class I, MHC class II, and CD80 by flow cytometry. The levels of CD54 (ICAM-1), a cell adhesion molecule that interacts with LFA-1 on the surface of CTLs, and of CD40 were also examined. Representative profiles from these assays are shown in Fig. 3. The results showed significant basal levels of CD54 expression, which were further induced by CD40L treatment. Thus, the mean fluorescence intensity of these cell surface markers increased by 80–150%, with the majority of the cell population expressing the Ag (Fig. 3). The majority of the cells in these cultures also expressed high levels of MHC class I, which were only modestly up-regulated upon CD40 ligation. These increases were sustained for at least 48 h post-stimulation (data not shown). Unlike CD54 and MHC class I, the levels of CD40 were not influenced by CD40 ligation. CD80 was expressed at very low levels in unstimulated cells and was marginally up-regulated following CD40 stimulation (Fig. 3). Moreover, we observed very low levels of MHC class II in MS751-CD40 cells, which remained unaffected upon CD40 ligation (data not shown). Thus, the CD154-mediated activation of CD40 in cervical carcinoma cells results in modest up-regulation of certain molecules important for Ag presentation and immune recognition.

**CD40 ligation facilitates CTL killing in cervical carcinoma cells**

The processing and presentation of most endogenous viral Ags to CD8+ CTL critically depends on the expression of the TAP and on
a large multicatalytic protease complex, the proteasome. We examined the expression levels of TAP1, TAP2, and the immunoproteasome component LMP7 in CD40L-stimulated CaSki and MS751-CD40 cells and their untreated controls. Immunoblot analysis of lysates from these cultures was performed and demonstrated a significant induction of TAP1 expression in both cell lines following CD40 ligation (Fig. 4A). A small up-regulation of TAP2 but not of LMP7 was also observed in response to CD40 activation (data not shown).

These findings raised the possibility that CD40 stimulation of cervical carcinoma cells may facilitate the recognition of TAP-dependent Ag epitopes by CTLs. To confirm or refute this hypothesis, the HLA-A2-positive CaSki and MS751-CD40 cell lines were stimulated with CD40L for 24 h to allow for up-regulation of TAP1 and then infected with a recombinant vaccinia virus expressing HPV16 and HPV18 E6/E7 proteins (TA-HPV; Ref. 25). Carcinoma cells were then labeled with $^{51}$Cr and cultured in the presence of a CTL clone that recognizes the HLA-A2-restricted TAP-dependent epitope TIHDIIILECV (aa 29–38) of HPV16 E6 (25). This CTL clone was unable to kill HPV16 E6-transfected CaSki and MS751 cells (25). However, pretreatment of CaSki cultures with CD40L promoted a dramatic increase in specific lysis by CTLs, compared with untreated controls (Fig. 4B). Thus, >10-fold enhancement in the percentage of specific lysis (from 5 to 50%) occurred at an E:T ratio of 12:1 as a consequence of CD40 ligation. Uninfected CaSki cells were not killed by these HPV-specific CTLs, and CD40 ligation did not induce apoptosis in uninfected cultures (data not shown; Ref. 25). The ability of CD40 ligation to augment CTL-mediated cervical tumor cell killing was reproduced in TA-HPV-infected MS751-CD40 cells where a significant increase in CTL-mediated lysis was also observed (Fig. 4C). This effect was specific for CD40, because treatment of parental control MS751 cells with CD40L failed to augment CTL responses (data not shown). Control experiments were also performed to demonstrate that CD40 ligation does not affect the susceptibility of the cells to vaccinia infection. For this purpose, cervical carcinoma cells were treated with CD40L or left untreated and then infected with vaccinia virus expressing the EBV-encoded protein EBNA3A.
Table I. Combined results for proportion and intensity of CD40 staining for each grade of histology analyzed

<table>
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<tr>
<th>Grade</th>
<th>Median</th>
<th>Range</th>
<th>IQR*</th>
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<tr>
<td>Normal</td>
<td>2±</td>
<td>0–4</td>
<td>0–3</td>
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<tr>
<td>HPV</td>
<td>5</td>
<td>3–7</td>
<td>4.5–6</td>
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<tr>
<td>CIN1</td>
<td>5</td>
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<tr>
<td>Invasive</td>
<td>6</td>
<td>3–7</td>
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* IQR, Interquartile range.

Differences between normal and other pathological grades differ significantly (p < 0.05).

Chemotherapeutic agents synergize with CD40L to promote apoptosis in cervical carcinoma cells

We investigated the hypothesis that, in addition to its ability to enhance CTL responses, CD40 ligation may also augment chemotherapy-induced apoptosis in cervical carcinoma cells. The rationale behind this hypothesis is based on earlier studies demonstrating that CD40 ligation induces apoptosis in carcinomas only in the presence of the protein synthesis inhibitor cycloheximide (CHX) (30, 31). This observation suggests that CD40 transduces potent survival signals capable of masking its proapoptotic effects. Indeed, our recent studies demonstrated that the survival arm of this balance is activated by PI3K and ERK MAPK signals that converge to the regulation of the protein synthesis machinery and the de novo production of labile antiapoptotic proteins (16). Thus, the specific inhibition of these signaling pathways mimics the effects of CHX treatment and sensitizes HeLa-CD40 cells to CD40L-induced apoptosis (16). On the basis of these published data, we asked whether anticancer drugs could mimic the effects of CHX or of PI3K and ERK MAPK inhibition on protein synthesis and sensitize cervical carcinoma cells to CD40L-induced apoptosis.

To this end, we assessed the ability of clinically relevant concentrations of 5FU, ETO, and QC to modulate protein synthesis and apoptosis in the presence or absence of CD40L. The antime tabolite 5FU is commonly used for the treatment of many types of cancer, including those of the cervix, and its mode of action has been attributed to inhibition of thymidylate and, more recently, RNA synthesis (32). The natural flavonoid QC, which provided the basis for the generation of the PI3K inhibitor LY294002 (33), can inhibit various tyrosine kinases, including PI3K (34, 35), and has entered phase I clinical trials in our institute with promising results (36). Moreover, a recent study has demonstrated that ETO, a topo isomerase II inhibitor, suppresses protein synthesis before induction of cell death (37). Thus, despite the differences in their known primary targets, ETO, 5FU, and QC can all inhibit protein synthesis. This was confirmed in HeLa-CD40 cells by measuring the incorporation of [35S]methionine into protein during a 16-h treatment with these anticancer drugs. It was found that ETO, 5FU, and QC, but not clinically relevant concentrations of the DNA-intercalating agent DOX, induced a substantial 40% decrease in the overall rates of protein synthesis, compared with untreated control cultures (Fig. 6A). This inhibition of protein synthesis occurred in the absence of an effect on viability (data not shown).

The initiation of protein synthesis by CD40 is key to its antiapoptotic effects and involves the dissociation of the translational repressor 4E-BP1 from the initiation factor eIF4E (16), which drives the translation of 5'-cap (7-methylguanosine; mG) mRNAs (38). Therefore, we asked whether chemotherapeutic drugs that suppress protein synthesis also inhibit the CD40L-induced dissociation of 4E-BP1 from eIF4E. Following pretreatment with 5FU or QC, HeLa-CD40 cervical carcinoma cells were stimulated with 1 μg/ml CD40L for 1 h or left untreated and lysates were subjected to mGTP affinity chromatography, which allows the precipitation of eIF4E and interacting proteins. The levels of eIF4E and 4E-BP1 bound to mGTP-Sepharose beads were then examined by immunoblot analysis. The results showed that the CD40L-induced dissociation of 4E-BP1 from eIF4E is inhibited by pretreatment with 5FU or QC, suggesting that these anticancer agents can efficiently suppress the CD40L-mediated initiation of translation (Fig. 6B). The long-term consequences of this effect on the ability of CD40 ligation to trigger apoptosis were addressed. To this end, HeLa-CD40 cells were treated for 16 h with 5FU, ETO, DOX, or QC, and then cocultured with 1 μg/ml CD40L for an additional 48-h time period. Cell death was quantitated using propidium iodide...
staining and fluorescence microscopy (Fig. 6C) or an ELISA-based assay that detects nucleosome enrichment in apoptotic cell extracts (D). In agreement with previous studies (9, 31), CD40L alone failed to induce cell death (Fig. 6C). Interestingly, although treatment with 5FU, QC, and ETO alone had a relatively small apoptotic effect on HeLa-CD40 cells, combination of either of these agents with CD40L dramatically increased apoptosis to 40–60% (Fig. 6C). This enhancement in the percentage of apoptotic cells is mirrored by the relative levels of nucleosome enrichment in parallel cultures (Fig. 6D). DOX, which at clinically relevant concentrations failed to suppress protein synthesis, did not sensitize these cells to CD40L-induced apoptosis (Fig. 6, C and D). Moreover, this was a CD40-dependent phenomenon, because no synergy was noted in parental HeLa control cultures (data not shown). We conclude that chemotherapeutic agents that suppress protein synthesis synergize with CD40L to augment carcinoma cell death.

**Discussion**

The induction of Ag processing and presentation through the activation of CD40 represents a promising approach for immunotherapy of B cell malignancies, including non-Hodgkin’s lymphoma and chronic lymphocytic leukemia (19, 39, 40). Indeed, CD40 ligation has been shown to promote Ag processing and presentation in lymphoma cells, resulting in their recognition by specific CTLs (17, 41). Moreover, a growing number of reports demonstrate that CD40 ligation activates the normal host APC function which enables antitumor responses in CD40-negative carcinomas (22, 42, 43).

In this study, we provide the first evidence that engagement of the CD40 pathway by CD40L/CD154 directly influences immune responses in carcinoma cells. Thus, we show that CD40 ligation up-regulates the expression of cell surface markers and intracellular molecules associated with cell-to-cell contact and Ag processing and presentation, and dramatically enhances CTL-mediated killing of cervical carcinoma cells expressing a TAP-dependent HPV16 E6 Ag epitope (Fig. 4). Interestingly, CD40 ligation failed to elicit similar CTL responses against proteasome-dependent but TAP-independent epitopes (Fig. 5, A and B). This observation suggests that the induction of the Ag-processing machinery by CD40 significantly contributes to the generation of CTL responses in carcinoma cells. One of the components of this machinery is TAP1, which is up-regulated in CD40L-stimulated cervical carcinoma cell lines (Fig. 4A). This effect is likely to be under the control of CD40-transduced NF-κB signals, because NF-κB is activated in cervical carcinoma cells treated with CD40L (Fig. 2B), and TAP1 promoter activity critically depends on NF-κB binding (44). Although the levels of TAP2 were only modestly induced by CD40 ligation, it is possible that other components of the TAP...
complex, such as tapasin and calreticulin, are also under the control of CD40 and contribute to Ag processing. Indeed, our recent unpublished gene expression microarray analyses reveal a significant induction of tapasin mRNA in CD40L-treated HeLa-CD40 cells (S. C. Hill, L. S. Young, and A. G. Eliopoulos, unpublished observations).

Moreover, the inability of CD40 ligation to enhance CTL responses against TAP-independent epitopes argues against CD40 simply sensitizing target cells to killing by any CTLs, for example, through the Fas pathway. This is further supported by the observation that CD40 ligation does not enhance the susceptibility of HPV16 E6 Ag peptide-loaded CaSki cells to CTL-mediated lysis in a standard 4-h $^{51}$Cr release assay (Fig. 5C). In addition, CaSki cells naturally express high levels of Fas, which are only modestly induced by CD40 ligation (mean fluorescence intensities compared with isotype control Ab, 15.5 and 17.8, respectively). Furthermore, previous studies in mice deficient in perforin, an effector of CD8+/H11001 MHC class I-restricted T cell killing, have demonstrated its major contribution to tumor surveillance and regression of established tumors and suggested a rather minor role for the Fas pathway (45, 46). However, other studies have highlighted a significant contribution of Fas/Fas ligand (FasL) to CTL-mediated killing of carcinoma cells (47). These differences may be partly explained by the fact that tumor cells often overexpress antiapoptotic proteins, such as cellular FLIP (cFLIP), which render them resistant to Fas-mediated killing. Indeed, a previous study showed that control and cFLIP-transfected tumor cells are lysed equally well in vitro by CTLs using granule-mediated lytic pathways, whereas the cFLIP transfectants are much more resistant to FasL-induced lysis (48). This observation is of particular relevance to our work, because CD40 ligation up-regulates cFLIP in cervical carcinoma cells (16) and may therefore promote resistance to Fas-mediated apoptosis (49). However, we cannot formally exclude a role for the Fas/FasL pathway in cytotoxicity induced by CTLs following a prolonged coculture with CD40-activated cervical carcinoma cell lines, and we are currently investigating this possibility.

The presented data contribute to our understanding of the function of CD40 in carcinomas and may have implications for the immunotherapy of cervical and, perhaps, other types of carcinoma. However, the in vivo efficiency of this direct immunostimulatory effect of CD40L/CD154 is likely to be hindered by the loss of HLA class I and TAP1 in a significant number of cervical cancers. Thus, although CD40 is overexpressed in the majority of cervical tumors (Fig. 1 and Table I), coordinated loss of TAP1 and MHC class I occurs in $\sim$35% of these cases (50). Interestingly, these defects are more prevalent in metastatic rather than primary cervical tumors (51), indicating that a CD40-based immunotherapy may be more efficient at the early stages of the disease where up-regulation of MHC class I and TAP1 expression by CD40L/CD154 can be achieved.

Our recently published work has demonstrated that CD40 ligation can deliver apoptotic signals to carcinoma cells that are manifested only upon disruption of CD40-activated survival pathways, such as PI3K and ERK. These signaling pathways converge on the regulation of the translation machinery, which is critical for the synthesis and maintenance of labile antiapoptotic proteins (16)
Thus, the CD40L/CD154-mediated induction of c-FLIP short (cFLIPS), but not of other apoptosis regulators or structural proteins, such as Bax, Bcl-2, and actin, was affected by the inhibition of the PI3K pathway (16). On the basis of these observations, we examined the possibility that anticancer drugs could mimic the sensitizing effects of the protein synthesis inhibitor CHX or of PI3K inhibition on CD40L-induced apoptosis. Most chemotherapeutic agents in clinical use lack specificity and affect a plethora of cellular components. This property may be beneficial when targeting tumor cells that possess multiple genetic and molecular abnormalities. A recent report has demonstrated that anticancer drugs that cause DNA damage in distinct manner, such as ETO, cis-platin, and mitomycin C, partially suppress protein synthesis in fibroblasts before the onset of apoptosis (37). Data shown in the present study using carcinoma cells confirm that ETO induces protein synthesis inhibition. Furthermore, we have found that clinically relevant concentrations of 5FU and QC, but not DOX, also inhibit protein synthesis in the absence of significant cytotoxicity and reverse the CD40-mediated dissociation of the translational repressor 4E-BP1 from the initiation factor eIF4E. Importantly, pretreatment of cervical carcinoma cells with 5FU, ETO, and QC dramatically enhances the proapoptotic properties of CD40 ligation (Fig. 6). A recent report by Bugajska et al. (52) has demonstrated that mitomycin C-treated fibroblasts carrying CD40L are

FIGURE 4. CD40 ligation increases the susceptibility of cervical carcinoma cells expressing a TAP-dependent HPV16 E6 epitope to CTL-mediated killing. A. Immunoblot analysis of TAP1 and β-actin expression before and 24 h after stimulation of cervical carcinoma cell lines with CD40L. Lysates from lymphoblastoid cells (LCLs) serve as a positive control for TAP1 detection. The increase in TAP1 expression was sustained for at least 48 h poststimulation (data not shown). B and C, CD40 ligation augments CTL-mediated killing of CaSki (B) and MS751-CD40 (C) cervical carcinoma cells expressing the HLA-A2-restricted TAP-dependent epitope TIIHDEEVC of HPV16 E6. Carcinoma cells were treated with CD40L for 24–30 h (+L) or left untreated (−L) and then infected with a vaccinia virus expressing the HPV16 E6 protein. These cells (targets) were labeled with 51Cr and mixed with epitope-specific CTLs (effectors) at increasing E:T ratios (1:1.5, 1:3, 1:6, and 1:12). Data depict the percent lysis of the target cells and represent the mean of triplicate determinations from at least three independent experiments in CaSki cells (±SD) and from one representative experiment performed in MS751-CD40 cells, where the SD of percent lysis was <7% of the mean of triplicate determinations.

FIGURE 5. A and B, CD40 ligation does not increase CTL responses in cervical carcinoma cells expressing TAP-independent epitopes. CaSki (A) and MS751-CD40 (B) cervical carcinoma cells were treated with CD40L for 24–30 h (+L) or left untreated (−L) and then infected with a vaccinia virus expressing the EBV-encoded BMLF1. These cells (targets) were labeled with 51Cr and mixed with CTLs (effectors) that recognize the HLA-A2-restricted, TAP-independent epitope GLCTLVAML of BMLF1. Data depict the percent lysis of the target cells and represent the mean (±SD) from at least three independent experiments in CaSki (±SD) and from a representative experiment performed in MS751-CD40 cells, where the SD of percent lysis was <9% of the mean of triplicate determinations. C, CD40 ligation does not increase CTL responses in HPV16 E6 peptide-pulsed cervical carcinoma cells. CaSki cells were stimulated with CD40L for 24–30 h (+L) or left untreated (−L) and then pulsed for 1 h with 1 μM HPV16 E6 peptide TIIHDEEVC before being analyzed for susceptibility to peptide-specific CTL lysis by 51Cr release assays.
Synergistic effects of CD40 ligation and chemotherapy on cervical carcinoma cell death. A. Inhibition of protein synthesis by chemotherapy agents. HeLa-CD40 cells were treated with 750 μM 5FU, 50 μM QC, 50 μM ETO, 0.5 μM DOX, or, as a control, 10 μg/ml CHX for 12 h and labeled with [35S]methionine during the last 60 min of treatment. The incorporation of the radiolabeled into protein was measured for each treatment and expressed as percentage relative to untreated controls, which were given the arbitrary value of 100%. B. Chemotherapeutic agents that inhibit protein synthesis reverse the CD40L-induced dissociation of the translational repressor 4E-BP1 from the translation initiation factor eIF4E. HeLa-CD40 cells were treated with 5FU or QC as described in A, and then stimulated for 1 h with 1 μg/ml CD40L or left untreated. Lysates were subjected to m7-GTP-Sepharose chromatography and immunoblotted for 4E-BP1 and eIF4E. C and D. Chemotherapeutic agents that inhibit protein synthesis augment CD40L-induced apoptosis. HeLa-CD40 cells were treated with 5FU, QC, ETO, or DOX as described in A, and then stimulated with 1 μg/ml rsCD40L or left untreated. Apoptosis was measured 36–48 h later by propidium iodide staining and cell counting (C) or by a cell death ELISA which detects mono- and oligonucleosomes in apoptotic cells (D). The nucleosome enrichment factor in D represents the fold increase in nucleosome enrichment relative to untreated HeLa-CD40 cultures. Mean values (±SD) from at least three independent experiments are shown.

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References


