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Control of Apoptosis in Epstein Barr Virus-positive Nasopharyngeal Carcinoma Cells: Opposite Effects of CD95 and CD40 Stimulation[1]

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ABSTRACT

The expression and function of CD95 and CD40 were investigated in malignant cells from EBV-positive undifferentiated nasopharyngeal carcinomas (NPCs). Large amounts of CD95 and CD40 expression were detected in 15 of 16 EBV-positive NPC specimens. In contrast, CD95 was not detected in two biopsies from patients with EBV-negative differentiated NPCs. We tested whether the CD95 apoptotic pathway was functional in NPC cells by treating two EBV-positive NPC tumor lines *in vitro* with a CD95 agonist. In both cases, NPC cells were extremely susceptible to CD95-mediated apoptosis, despite strong constitutive expression of Bcl-x. Combined CD40 and CD95 stimulation was used to investigate the possible anti-apoptotic activity mediated by CD40. The CD40 receptor was activated by incubating NPC cells with murine L cells producing CD154, the CD40 ligand. This treatment resulted in a strong inhibition of CD95-related cytotoxicity. Such an anti-apoptotic effect of CD40 is well known for B lymphocytes, but has not previously been reported for epithelial cells. These data suggest that NPC tumor-infiltrating lymphocytes, which often produce the CD40 ligand *in situ*, may increase the survival of malignant cells, thereby enhancing tumor growth in patients.

INTRODUCTION

Undifferentiated NPCs[3] are rare in most countries; however, their incidence is high in South China and North Africa. Regardless of patient origin, NPCs are consistently are associated with EBV, whose genome is contained in malignant epithelial cells. Several EBV proteins, including EBNA1, LMP1, and the BARF0 protein, are consistently expressed in NPC and probably contribute to the malignant phenotype (1, 2). Small deletions of chromosome 3p and the loss of $p16^{LNK4}$ expression by homozygous gene deletion or, more often, by abnormal gene methylation are the most frequent genetic changes reported in NPC (3, 4). In contrast to most epithelial malignancies, there is a very low frequency of p53 mutations in NPC (5). NPC primary tumors are infiltrated heavily by nonmalignant lymphocytes. Approximately 60% of NPC tumor-infiltrating leukocytes are mature $CD3^+$ T lymphocytes, 15% are CD20-positive B lymphocytes, and ~10% are cells of monocyte/macrophage lineage (6). We previously reported a series of NPC cell characteristics that are potentially important for lympho-epithelial interactions: the constitutive production of IL-1α and constitutive expression of CD40, CD54, and HLA class II molecules (7–9).

There are several lines of evidence supporting the idea that malignant cells are more prone to apoptosis in NPCs than in other head and neck carcinomas. NPCs often are more susceptible than squamous cell carcinomas to radiotherapy and chemotherapy, especially at early stages of the treatment (10, 11). In addition, despite their short doubling time *in situ*, NPC cells adapt poorly to culture *in vitro* or even to transplantation into nude or SCID mice (8, 12). This suggests that critical survival factors from the tumor microenvironment protect NPC cells from apoptosis. We therefore investigated in NPC cells the expression and function of CD95 and CD40, which are key determinants of apoptosis and cell survival in various cell types.

CD95 (also called Fas or Apo1) is an M_r 43,000 transmembrane cell surface receptor of the tumor necrosis factor receptor family. The binding to CD95 of anti-CD95 antibodies or its cognate ligand, CD95L, rapidly induces apoptosis in sensitive cells both *in vitro* and *in vivo* (13). Cell death results from a signaling cascade involving molecules such as the FADD protein and a series of cysteine proteases, particularly caspases 8 and 3 (14). The functional importance of the CD95 system was first recognized in immune systems. It plays a key role in the control of the immune response, particularly in the deletion of autoreactive clones, and is involved in the killing of antigenic target cells by cytotoxic T cells and in the maintenance of immune privilege in organs such as the eye and the testis (15, 16). Constitutive expression of CD95 and CD95L has been observed in a variety of mouse and human tissues with high rates of apoptotic cell death, suggesting that the CD95 system may also be involved in apoptotic cell death during physiological cell turnover (17, 18). Such a mechanism has been demonstrated in female reproductive organs in mice (19). Finally, there are abundant data showing that the CD95 pathway is activated and induces apoptosis in response to a wide range of cellular aggressions in both malignant and nonmalignant cells. This pathway probably is involved in the lethal response to hypoxia (20). Some anticancer drugs, such as doxorubicin, induce production of the CD95 ligand in their target cells. This molecule may then interact with CD95, resulting in autocrine cell suicide (14). In summary, the expression of the CD95 receptor coupled with a functional apoptotic signaling pathway probably significantly increases cell susceptibility to various challenges, including attack by CTLs, hypoxia, radiotherapy, and chemotherapy.

CD40 is an M_r 48,000 membrane receptor of the tumor necrosis factor receptor family. It is activated by CD154, its membrane-bound cognate ligand. CD40 was described initially as a B-lymphocyte activation antigen, but is now known to be expressed by a variety of other cell types, especially endothelial and epithelial cells (21, 22). In human B lymphocytes and dendritic cells, CD40 activation antagonizes CD95-mediated apoptosis (23, 24). We and others have reported previously that malignant NPC cells have consistent and intense membrane expression of CD40, but no precise function has been assigned to this receptor and its ligand in NPCs (8, 25, 26).

We report here that malignant NPC cells strongly express the CD95 receptor and that both CD95 and CD40 are functional in short-term *in vitro* assays in EBV-positive NPC tumor lines. The stimulation of CD95 induces rapid apoptosis. However, prior stimulation of CD40 results in a dramatic reduction of NPC cell apoptosis following CD95 activation. This is the first report of CD40-ligand activation protecting malignant cells of epithelial origin against CD95-mediated apoptosis.

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; FDA, fluorescein diacetate.

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MATERIALS AND METHODS

NPC Tumor Lines. Four EBV-positive undifferentiated NPC tumor lines, C15, C17, C18, and C19, were propagated by s.c. passage in nude mice (8, 9). C15 was established from the primary nasopharyngeal tumor of a 13-year-old girl. The other tumor lines were derived from cutaneous (C17) and cervical lymph node (C18 and C19) metastases. C17 and C18 were established from recurrent lesions after irradiation and several courses of chemotherapy.

NPC Biopsies. Frozen biopsies from 14 NPCs collected at the Institut Gustave Roussy (Villejuif, France) were included in this study. Patients were mainly from Algeria, Italy, and France (Table 1). The WHO type was determined in each case by examination of H&E-stained sections. EBV DNA was detected by Southern blotting of tumor DNA for eight specimens (using the *Bam*HI W fragment of the EBV genome as a probe; data not shown). No EBV DNA was detected in biopsies 9 and 10, which were well-differentiated NPCs (WHO type I and II) from French patients. It was not possible to perform a Southern blot for biopsies 4–7. However these patients had typical EBV serological markers and undifferentiated type III NPC. Therefore, these biopsies were classified as EBV-positive.

Antibodies. CD95 and CD40 were detected by flow cytometry or staining of tissue sections with the purified monoclonal antibodies UB2 and MAB 89, respectively (both IgG1; Immunotech, Marseille, France). Tumor-infiltrating leukocytes were stained with an anti-CD45 (Leukocyte Common Antigen, Dako-LC; Dako, Trappes, France). 7C11 (IgM; Immunotech) was used as a CD95 agonist with strong apoptosis-inducing activity. Surface HLA class I molecules were stained with the ATCC W6–32 antibody. An anti-Bcl-x polyclonal antibody (S-18) from Santa Cruz Biotechnology (Santa Cruz, CA) and an anti-Bcl-2 monoclonal antibody from Dako (clone 124) were used for Western blotting.

Immunohistology. Specimens were sectioned at 5 μm and placed on SuperFrost/plus slides (Menzel-Glaser, Braunschweig, Germany) without any fixative. Cryostat sections were incubated with anti-CD95 (UB2), anti-CD40 (MAB 89), or anti-CD45 (Dako-LC) at 2, 2, and 4 μg/ml, respectively, for 1 h at 37°C. Immunoreactivity was detected using a biotin-conjugated antimouse goat antibody followed by an avidin-peroxidase complex (Biostain kit; Biomeda, Foster City, CA). Peroxidase was revealed with the chromogen AEC (3-amino-9-ethyl carbazole), and sections were counterstained with Harris's hematoxylin.

NPC Cell Dispersion and Flow Cytometry Analysis. C15 and C17 were cut into 1 mm^3 fragments and incubated with 8 mg/g tumor collagenase (Worthington, Freehold, NJ) and 20 μg/g tumor DNase I (Sigma, Saint Quentin Fallavier, France), in RPMI containing 20% FCS at 37°C for 3 h. Collagenase-digested fragments were homogenized, and the resulting cell suspension was filtered through a nylon cell strainer with 70 μm pores to remove cell aggregates. Cells were stained indirectly with the appropriate primary antibodies and FITC-conjugated goat antimouse $F(ab')_2$ (Jackson ImmunoResearch, West Grove, PA) and then analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Table 1 *CD95 and CD40 staining on tissue sections*

Biopsy[a]	Origin	WHO type	EBV DNA	CD95[b]	CD40[b]
1	Algeria	III	+	+	+
2	Algeria	III	+	+	+
3	Algeria	III	+	++	+
4	Italy	III	ND[c]	++	+
5	France	III	ND	+	ND
6	Bulgaria	III	ND	+	ND
7	Italy	III	ND	+	+
8	Algeria	III	+	+	+
9	Algeria	II	−	−	+
10	France	I	−	+/−	+
11	Italy	III	+	++	+
12	Algeria	III	+	++	+
13	Algeria	III	+	++	+
14	France	II	+	+	+/−
Nude Mouse Tumor Lines					
C15	Morocco	III	+	+	+
C17	France	III	+	++	+
C18	Algeria	III	+	−	−
C19	Italy	III	+	++	+

[a] Patients 4–7 had typical EBV serological markers.
[b] ++, strong plasma membrane staining of all malignant cells; +, moderate staining of the majority of malignant cells; +/−, faint staining of a minority of malignant cells.
[c] ND, not determined.

Short-Term Culture of NPC Cells. NPC cells derived from tumor xenografts were seeded at 5×10^5 cells/well in positively charged 24-well plates (Primaria; Becton Dickinson). They were incubated in RPMI containing 10% FCS, at 37°C in a 5% CO_2 atmosphere. When these experimental conditions were used, >90% of the NPC cells were attached on the plastic support after 1–2 h incubation, with ~60% confluence. Most cells were round. There was no significant cell proliferation; the percentage of cells in $S+G_2$ phases dropped from 15 to 7% in 12 h (data not shown). During the first 24 h, NPC cell loss was <10% and fibroblast contamination remained minimal. However NPC cell survival decreased significantly beyond 24 h, and it was not possible to make additional passages *in vitro*, in part because of major contamination by mouse fibroblasts. All of the experimental procedures reported here were performed within 18 h after tumor cell dispersion.

Anti-CD95 Antibody-mediated Apoptosis. The cells were incubated for 2 h in culture conditions, and the purified 7C11 antibody was added to the treated cell wells. Purified nonspecific mouse IgM was used as a negative control. Cells were incubated with antibodies for 10–14 h, and were then collected by aspiration of the culture medium and trypsin treatment. Viable cells were counted by trypan blue exclusion. CD95-specific cytotoxicity was calculated as the percentage decrease in viable cells in treated wells *versus* nontreated wells. NPC cells were also collected for apoptotic DNA fragment and oligonucleosome assays.

Detection of Apoptotic DNA Fragments and Oligonucleosomes. We detected apoptotic DNA fragments by extracting DNA using the salting out procedure (27). Briefly, 2×10^6 cells were incubated overnight at 37°C in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 400 mM NaCl, 0.6% SDS, and 166 μg/ml proteinase K. After digestion, the salt concentration was raised to 1 M for NaCl and 10 mM for $MgCl_2$ and the tubes were shaken vigorously. Samples were centrifuged at 7000 g for 20 min at room temperature, the supernatants were collected, 2.5 volumes of ethanol were added, and DNA was precipitated overnight at −20°C. The DNA was resuspended in Tris-EDTA buffer, treated by DNase-free RNase, and subjected to electrophoresis in a 2% agarose gel. Oligonucleosomes were detected by a sandwich ELISA with antihistone and anti-DNA monoclonal antibodies according to the manufacturer's recommendations (Cell Death Detection ELISA plus; Boehringer, Meylan, France).

Assessment of Viable NPC Cells under CD40 and CD95 Stimulation. CD40 stimulation of NPC cells was achieved by cocultivation with murine L-cells transfected stably with the CD40 ligand gene (kindly provided by Dr. Francine Brière, Schering-Plough, Dardilly, France; Ref. 28). The presence of the CD40 ligand at the cell surface was checked by flow cytometry (data not shown). The tumor was dispersed, and NPC cells were mixed with CD40L-transfected or control untransfected L cells in 24-well plates (2.5×10^5 L-cells and 5×10^5 NPC cells per well). Cocultivated cells were incubated for 6–8 h prior to CD95 stimulation, and then treated with the 7C11 antibody for 10–14 h. At the end of the incubation, the following procedure, based on flow cytometry analysis, was used to count viable human NPC cells. All cells were harvested by aspiration of the culture medium and trypsin treatment. The resulting cell suspension was incubated with the anti-HLA I antibody W6–32 and Cy5-labeled goat antimouse IgG as a secondary antibody (Jackson Immunochemicals). W6–32 did not react with the murine H2 antigen; therefore, only cells of human origin were labeled with Cy5. Viable cells were labeled specifically by activation of FDA before flow cytometry (5 min incubation with 2 mg/ml FDA). Cells labeled with both Cy5 and activated FDA were counted using a FACScalibur flow cytometer (Becton Dickinson). A red diode emitting at 535 nm and a laser argon beam emitting at 480 nm were used to excite Cy5 and fluorescein, respectively. A minimum of 100,000 events was collected for each sample. The sample volumes used for cytometry were determined so that an absolute count of viable NPC cells could be obtained for each sample.

Assessment of Apoptotic NPC Cells under CD40 and CD95 Stimulation. The above-described procedure used for counting viable cells was modified to count cells undergoing apoptosis. Two changes were introduced: (a) cells were collected earlier after the beginning of CD95 stimulation (~8 h later); and (b) in the final step, instead of FDA, cells were stained with 5 μM YO-PRO-1 iodide (quinolinium, 4-{(3-methyl-2(3H)-benzoxazolylidene)-

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methyl)}-1{(3(trimethyl-ammonio) propyl}-, diiodide; Molecular Probes, Eugene, OR). YO-PRO-1 is a nucleic acid stain that emits green fluorescence, which passes through the plasma membranes of apoptotic cells even when they are still viable, *i.e.*, when they have undergone changes in membrane permeability but no major membrane breaks (29, 30). Excitation by the red diode (535 nm) and the laser argon beam (480 nm) was used for Cy5 and YO-PRO-1 iodide, respectively. Cells with reduced forward scatter or strong side scatter (dead cells) and negative W6–32 staining (murine cells) were gated out. The remaining YO-PRO-1-positive cells were assumed to be apoptotic, but still viable, NPC cells.

Western Blot Analysis of Bcl-2 and Bcl-x Expression. NPC tumor pieces were disrupted in lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% NP40, 0.5 mM Pefabloc] at 4°C, using a conical pestle glass grinder. The lysate was further homogenized by sonication with a microtip probe and clarified by centrifugation for 15 min at 10,000 × *g*. A sample of this protein extract (40 μg) was electrophoresed in a 10% polyacrylamide gel. Western blotting was performed on PVDF membranes (Immobilon P; Millipore, Saint Quentin en Yvelines, France) according to standard protocols. The enhanced chemiluminescence system was used to visualize bound peroxidase-conjugated secondary antibodies, according to the manufacturer's recommendations (Amersham, Les Ulis, France).

RESULTS

CD95 and CD40 Surface Expression on Transplanted NPC Cells. CD95 surface expression was demonstrated in NPC tumor lines by flow cytometry after tumor cell dispersion (C15 and C17) and by immunohistochemistry (C15, C17, C18, and C19). CD95 labeling was very intense on C15 and, to a lesser extent, on C17 cells (Fig. 1). The labeling of C15 and C17 cells was consistently brighter than that of Jurkat (T-cell leukemia) and A431 (squamous cell carcinoma) cell lines. Malignant cells of the C19 tumor were labeled for CD95 by immunohistochemistry. In contrast, the C18 tumor was unstained for CD95 (Table 1). Similar results were obtained for CD40. It was detected in C15, C17, and C19 cells, but not in C18 cells (Table 1 and Fig. 1).

CD95 and CD40 Expression in NPC Biopsies. CD95 expression was analyzed on tissue sections from 14 biopsies. Twelve of these biopsies were derived from typical EBV-associated undifferentiated or poorly differentiated NPCs (WHO type III or II; EBV detected in tumor tissue and/or typical EBV serological markers). All these biopsies tested positive for CD95 (Table 1 and Fig. 2). In contrast, CD95 staining was negative or very weak for two EBV-negative, more differentiated NPCs (WHO type I or II; Table 1, *biopsies 9* and *10*). In the biopsies testing positive for CD95, most of the malignant epithelial cells were stained. In contrast, there was no significant staining of tumor-infiltrating lymphocytes. The expression of CD40 was investigated in 12 biopsies from the same series; malignant cells were labeled strongly in all but one case (*biopsy 14*), including the two EBV-negative carcinomas (Table 1). Some tumor-infiltrating lymphocytes were also positive. These results are consistent with those of previous studies (8, 25, 26).

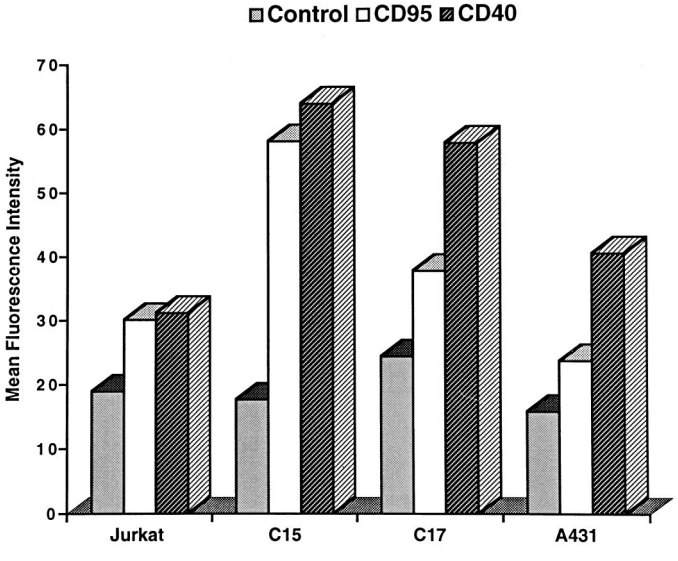

Fig. 1. Flow cytometry analysis of CD95 and CD40 expression on cells derived from the C15 and C17 NPC tumor lines. Negative control samples were incubated with purified mouse IgG1 (10 μg/ml; Sigma, France). CD95 and CD40 molecules were stained with the UB2 (10 μg/ml) and the MAB 89 (5 μg/ml) antibodies. Fluorescence intensities were determined after subtraction of the dead cells stained with propidium iodide. Similar results were obtained in three independent experiments.

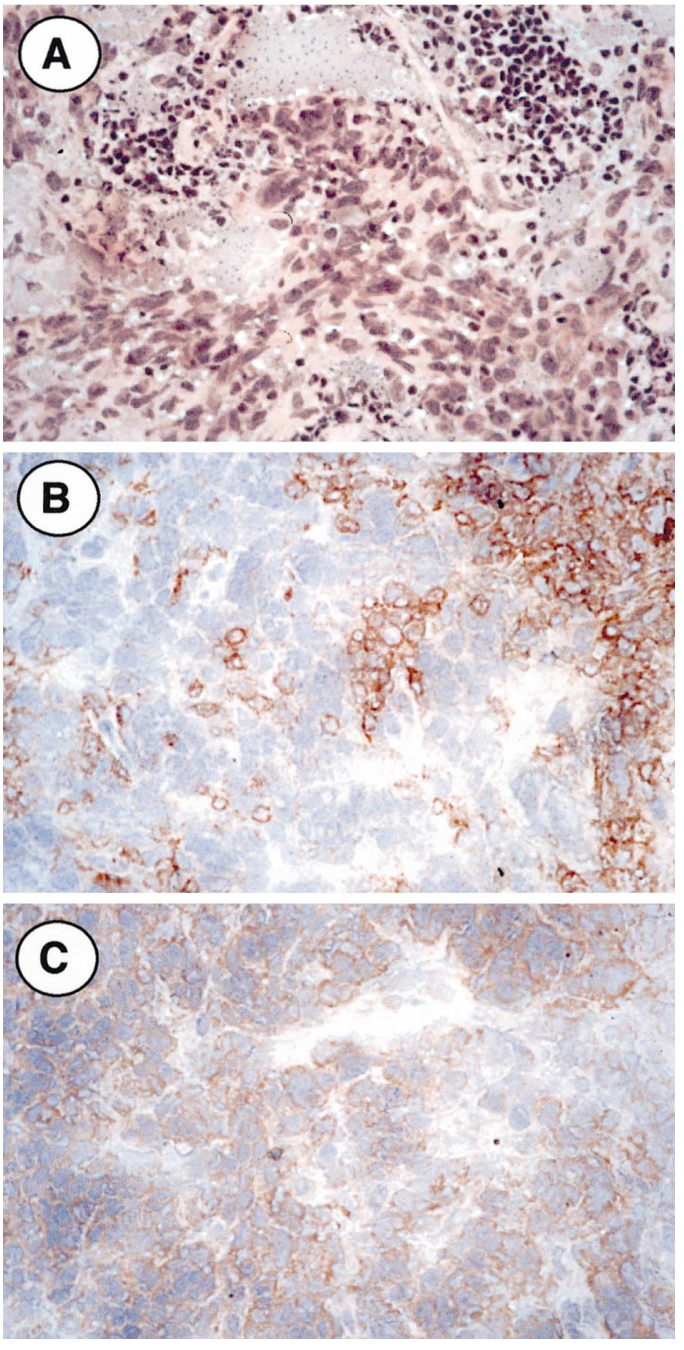

Fig. 2. CD95 staining on tissue sections of NPC biopsies. Photomicrographs of three cryostat sections of the biopsy of patient 2. *A*, stained with H&E. *B*, stained with anti-CD45 (Leukocyte Common Antigen), showing the distribution of tumor-infiltrating lymphocytes. *C*, anti-CD95 (UB2) which stains membranes of malignant epithelial cells. Magnification: *A* and *B*, ×200; *C*, ×400.

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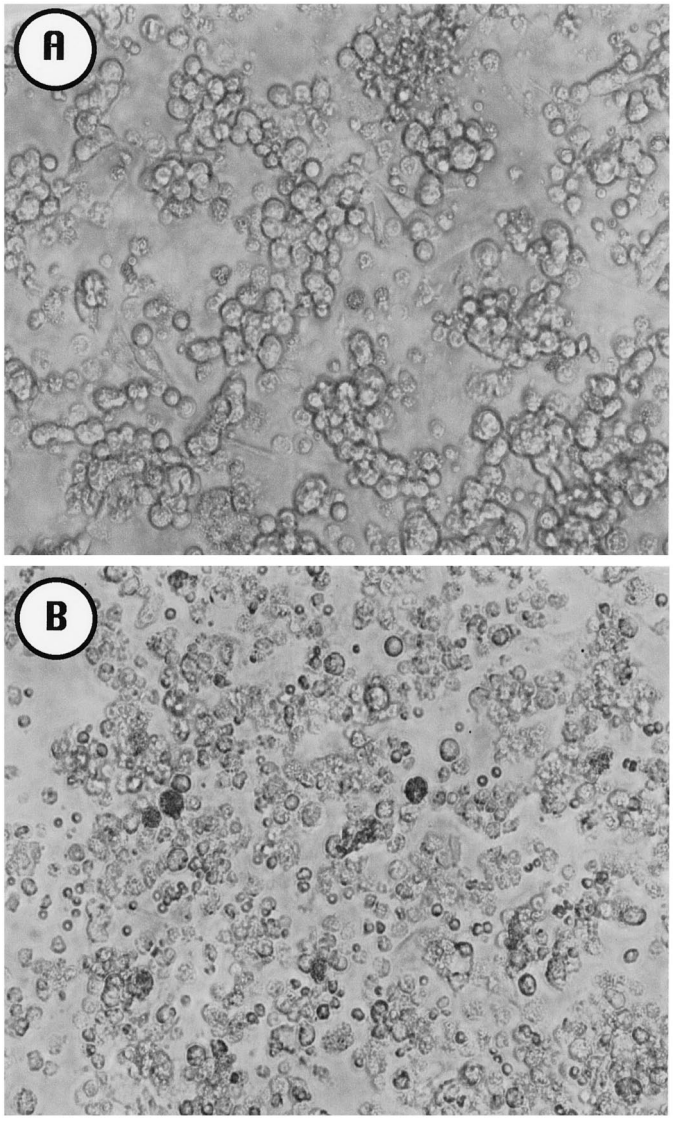

Fig. 3. Phase-contrast microscopy of C15 cells cultured *in vitro*, with or without treatment by a CD95 agonist (7C11). *A*, C15 cells (5×10^5 cells/well in a 24-well plate) incubated for 14 h with control IgM; *B*, C15 cells incubated for 14 h with the 7C11 Ab (100 ng/ml). Magnification: ×125.

CD95-mediated Apoptosis in NPC Tumor Lines. It has not been possible to derive permanent *in vitro* cell lines from the transplanted NPC tumor lines. However, cell suspensions derived from the C15 and C17 tumors can be used for short-term cultures and biological assays. This type of short-term culture was sufficient for assessment of CD95-mediated cytotoxicity that was morphologically visible within 10–12 h. Treatment of C15 and C17 cells with the 7C11 monoclonal antibody, a CD95 agonist, was highly cytotoxic (Figs. 3 and 4). This cytotoxic effect was readily detected with doses as low as 5 ng/ml. The ED_{50} was ~50 ng for C15 and 100 ng for C17 cells, in the same range as that for Jurkat cells, a classic target model for CD95 agonists. In contrast, the 7C11 monoclonal antibody had no significant effect on A431 cells obtained by nude mouse tumor dispersion (Fig. 4). To check that the toxicity of the 7C11 antibody to NPC cells was associated with typical apoptotic DNA fragmentation, agarose gel electrophoresis and oligonucleosome ELISA were performed on C15 cells incubated for 14 h with the 7C11 antibody or a control IgM (100 ng/ml; Fig. 5). Jurkat cells were processed as part of the same experiment. For Jurkat cells, DNA fragmentation and oligonucleosomes were detected only when they were treated with the CD95 agonist. In contrast, a background level of DNA fragmentation was detected with the control IgM for the C15 cells, probably due to apoptosis caused by artificial tumor cell dispersion. However, there was much more DNA and oligonucleosome fragmentation in the presence of the CD95 agonist 7C11 than with the control (more than twice as much, as measured by ELISA). Therefore, it is highly probable that the 7C11 toxicity was due to a potent and rapid apoptotic process.

CD40-mediated Increase in NPC Cell Survival. CD95 was expressed strongly on NPC cells and was extremely efficient at signaling apoptosis. We therefore investigated mechanisms that might protect NPC cells within the tumor microenvironment. Protection by CD40 activation was one attractive hypothesis, because lymphocytes infiltrating NPC consistently produce CD154, the CD40 ligand (26). To

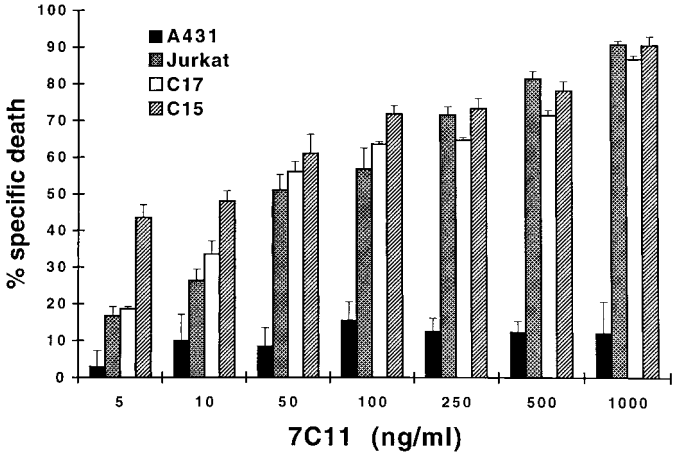

Fig. 4. Assessment of the cytotoxicity of a CD95 agonist (7C11) to the C15 and C17 cells. Viable NPC cells were counted by trypan blue dye exclusion. The data are representative of three independent experiments and the values are presented as the mean of triplicate determinations; *bars*, SD.

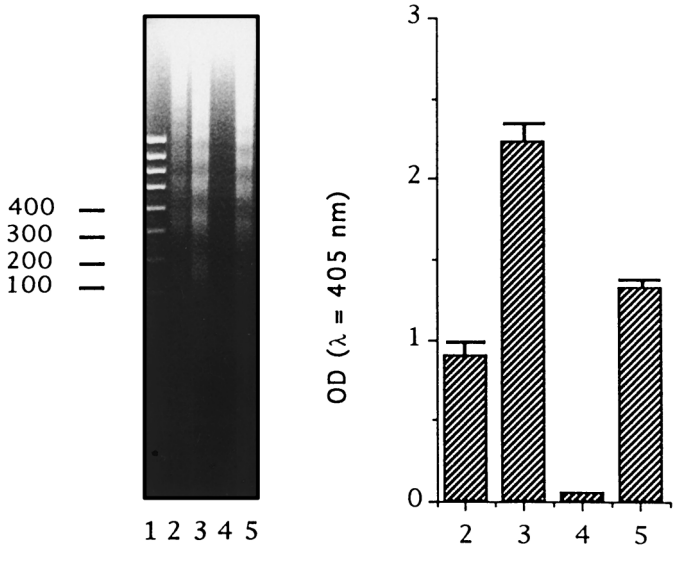

Fig. 5. Assessment of DNA fragmentation in C15 cells treated with a CD95 agonist (7C11). *Left panel*, agarose gel separation of apoptotic DNA fragments. *Right panel*, detection of oligonucleosomes by an ELISA (absorbance of the peroxidase substrate was read at 405 nm); the values are presented as the mean of triplicate determinations; *bars*, SD. *Lane 1*, DNA molecular weight markers; *Lane 2* and *column 2*, C15 cells (5×10^5/well in a 24-well plate) in short-term culture for 14 h with control IgM; *Lane 3* and *column 3*, C15 cells in short-term culture for 14 h with the CD95 agonist, 7C11 (100 ng/ml); *Lane 4* and *column 4*, Jurkat cells incubated in the same conditions with control IgM; *Lane 5* and *column 5*, Jurkat cells incubated with the 7C11 antibody (100 ng/ml).

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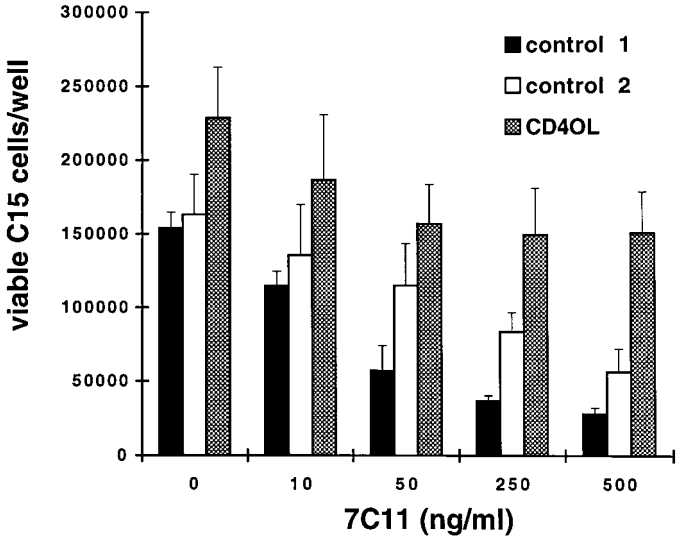

Fig. 6. Protection against Fas-mediated apoptosis by engagement of CD40. Assessment of viable NPC cells. CD40 stimulation was provided by co-cultivation with transfected murine L cells expressing the CD40 ligand (CD154). Viable C15 cells were identified among co-cultivated cells by flow cytometry analysis based on their positive HLA I staining (to test their human origin) and activation of intracellular FDA (to test their viability). *Y*-axis values are the number of viable C15 cells/well in 24-well plates. This histogram was constructed from the mean values obtained from three independent experiments. *Control 1*, C15 cells incubated without control or stimulating cells (5×10^5 cells/well); *control 2*, C15 cells incubated with nontransfected L cells (5×10^5 C15 cells + 2.5×10^5 L cells/well); *CD40L*, C15 cells incubated with L cells producing CD154 (same ratio of C15 and L cells).

test this hypothesis, we stimulated CD40 on C15 cells with transfected murine L cells having intense artificial expression of CD154. C15 and murine L cells producing CD154 were mixed, in a proportion of 2 to 1. They were incubated for 6–8 h, and the 7C11 antibody was then added for 10–14 h. Viable C15 cells were counted using a procedure based on flow cytometry analysis (Fig. 6). A much higher proportion of NPC cells survived if they were cocultivated with CD154-positive L cells prior to CD95 stimulation. Control L cells also had a nonspecific protective effect, but it was much weaker than the CD40-specific effect. With a maximum dose of CD95 agonist (500 ng/ml), there were almost three times as many viable cells if cells were mixed with CD154-producing cells rather than with control L cells. A small but consistent increase in NPC cell survival due to CD154-positive L cells was also observed in basal conditions in the absence of CD95 stimulation. Additional flow cytometry experiments were performed to confirm that the increased number of surviving NPC cells was due to a decrease in the number of apoptotic cells. Apoptotic C15 cells were identified directly by the uptake of YO-PRO-1, a nucleic acid stain that penetrates apoptotic cells even when they are still viable (28, 29). After an 8-h incubation with 250 ng/ml CD95 agonist, the fraction of viable C15 cells that was YO-PRO-1-positive was significantly lower when the cells were cocultivated with murine cells expressing CD154 (36%) compared with coculture with murine control cells (54%) or C15 cells alone (74%; Fig. 7). This is consistent with the data obtained in the previous experiment (Fig. 6). In contrast, in the absence of the CD95 agonist, the protective effect of CD40 stimulation was less clear than with the NPC cell survival assay. This is probably due to the fact that this assay recapitulates a difference in the rate of apoptosis over a longer period of time and is therefore more sensitive.

Detection of Bcl-2 and Bcl-x in NPC Tumor Lines. High concentrations of the Bcl-2 protein have been reported in about 80% of NPCs (31). Depending on the cell type, Bcl-2 and Bcl-x may interfere with CD95-mediated apoptosis (32). Therefore, we investigated the status of both proteins in our NPC tumor lines (Fig. 8). Bcl-2 was readily detected by Western blotting in C17 and C18 tumors. It was also detected, but at a low level, in the C15 tumor. The M_r 26,000 large form of Bcl-x (Bcl-x_L) was abundant in all three tumors. The amounts of Bcl-2 and Bcl-x were similar in C15 and C17 cells after tumor dissociation and 18 h of short-term culture; the amount of Bcl-x was not increased by CD40 stimulation (data not shown).

DISCUSSION

According to many reports, the level of CD95 expression is often lower in carcinoma cells than in their nonmalignant counterparts. This has been demonstrated clearly for malignant cells from colon, breast, esophagus, and cutaneous basal carcinomas (33–37). Not surprisingly, low levels or lack of CD95 cell surface expression are generally associated with a phenotype of resistance to CD95-mediated apoptosis

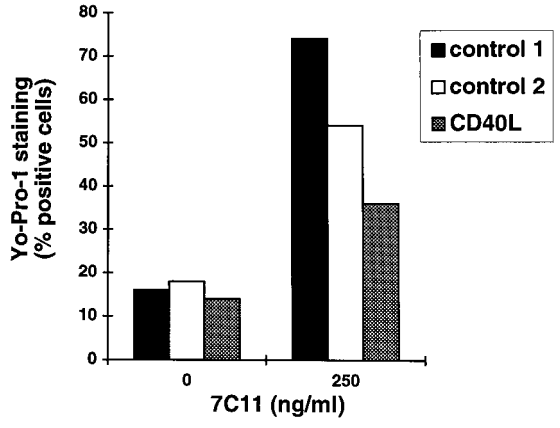

Fig. 7. Protection against Fas-mediated apoptosis by engagement of CD40. Assessment of apoptotic NPC cells. As in Fig. 6, CD40 stimulation was provided by co-cultivation with transfected murine L cells expressing the CD40 ligand. Apoptotic C15 cells were identified by flow cytometry analysis based on the uptake of YO-PRO-1, a fluorescent nucleic acid stain. Background YO-PRO-1 staining was determined on a suspension of viable L cells processed in the same experiment. *Y*-axis values are percentages of YO-PRO-1-positive cells among all cells gated out of HLA I-negative staining, reduced forward scatter, and strong side scatter (to eliminate murine cells and dead cells). This is one of three similar experiments. *Control 1*, C15 cells incubated without control or stimulating cells (5×10^5 cells/well); *control 2*, C15 cells incubated with nontransfected L cells (5×10^5 C15 cells + 2.5×10^5 L cells/well); *CD40L*, C15 cells incubated with L cells producing CD154 (same ratio of C15 and L cells).

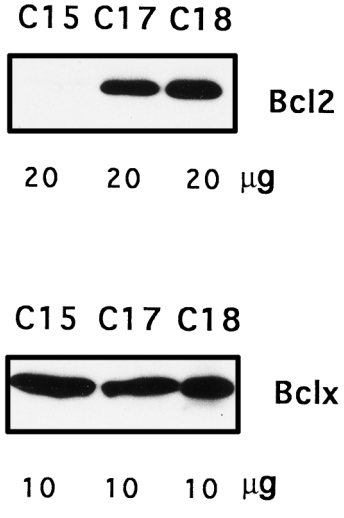

Fig. 8. Bcl-2 and Bcl-x synthesis in NPC tumor lines. The polyclonal antibody used to detect *Bcl-x* recognized both the large and the small forms of the protein. Only the large form (Bcl-x_L) was detected. On a longer exposure, a faint *Bcl-2* band was detected in *C15* material.

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(34). However, resistance to CD95-mediated apoptosis is also observed frequently in carcinoma cells that have high levels of CD95 expression; such cells include prostatic, pancreatic, and some colonic carcinoma cells (38–40). In most cases, the molecular basis of this resistance is unclear. For example, the resistance of prostatic carcinoma cell lines does not correlate with the expression of Bcl-2, Bak, and Bcl-x (38).

Regardless of the mechanisms involved, the consistency of the alterations affecting the CD95 pathway suggests that these changes are important elements of tumor progression in epithelial malignancies. In this regard, EBV-positive malignant NPC cells seem to have a special status. On the basis of our present results, they appear to constitutively produce large amounts of the CD95 receptor and to remain highly susceptible to CD95-mediated apoptosis. Thus, NPC cells are probably not selected for intrinsic alterations of the CD95 receptor and its signaling pathway during tumor progression. This may be in connection with the fact that they are protected for a long time against CD95-mediated apoptosis by factors produced in the tumor microenvironment. The CD40 ligand, CD154, which is produced consistently *in situ* by tumor- infiltrating lymphocytes, is probably one such factor (26).

The functions of the CD40 receptor and its ligand have been investigated mostly in B lymphocytes. The interaction of the CD40 ligand on T cells with the CD40 receptor on B cells leads to the clonal expansion of antigen-responsive B cells and allows them to escape CD95-mediated apoptosis (23). The CD40/CD154 system has long been suspected to have a positive role in NPC tumor growth (26). However, this notion apparently conflicted with data obtained *in vitro* with non-NPC epithelial cells. CD40 stimulation has been reported to inhibit proliferation of both normal keratinocytes and several non-NPC carcinoma cell lines (41, 42). In a bladder carcinoma cell line, EJ, CD40 stimulation even potentiated the cytotoxicity of an anti-CD95 antibody (42). In contrast, we found that CD40-ligand activation protected C15 cells against CD95-mediated apoptosis. Moreover, in one of our assays, stimulation of CD40 also appeared to protect C15 cells against the background apoptosis that occurred spontaneously after tumor cell dispersion (Fig. 6). Preliminary data showed that the C17 tumor line was also protected against CD95-mediated apoptosis by stimulation of CD40 (data not shown). This is the first demonstration of an antiapoptotic effect mediated by CD40 in epithelial cells. The differences in results obtained with non-NPC cell lines, especially the EJ carcinoma, are probably due to differences in cell lineage and stage of maturation. There was no significant proliferation of NPC cells in our experimental system; therefore, we cannot exclude the possibility that stimulation of CD40 inhibits the proliferation of NPC cells. Even if this is true, the anti-apoptotic effect mediated by CD40 may favor tumor growth on the long term, despite a slower rate of proliferation. Future investigations should aim to identify other factors, such as growth factors and extracellular matrix components, that probably combine *in vivo* with the CD40 ligand to increase both cell survival and proliferation synergistically.

Efforts should also be made to determine the mechanism of the anti-apoptotic effect of CD40 stimulation. In some lymphoid cell lines, CD40 stimulation increases Bcl-x expression (43, 44). However, large amounts of Bcl-x were present in C15 and C17 cells in basal conditions, and the amount of Bcl-x was not increased by CD40 stimulation (Fig. 8 and data not shown). Other proteins thought to be involved in the control of apoptosis have been reported to be up-regulated in epithelial cells by CD40 overexpression or ligand activation: the A20 anti-apoptotic protein, the EGF receptor, and interleukin 6 (45–47). These molecules are also up-regulated by the EBV-encoded LMP1, which is expressed constitutively in C15 cells (45, 46). CD40 stimulation may act in synergy with LMP1 signals to increase the production of one or more of these molecules to provide optimal protection. Alternatively, other effector molecules might be involved in the antiapoptotic effect of CD40 stimulation in NPC cells. Their identification would be of major interest.

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