

Surface Membrane-expressed CD40 Is Present on Tumor Cells from Squamous Cell Cancer of the Head and Neck *in Vitro* and *in Vivo* and Regulates Cell Growth in Tumor Cell Lines¹

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ABSTRACT

Because regional spread to lymph nodes without systemic spread is a relatively common event in squamous cell cancer of the head and neck (SCCHN), it is possible that lymphoid-related receptors or cytokines might directly impact the growth of these tumors. In the present study, we have shown by flow cytometry and Western blotting that the central lymphoid regulatory molecule, CD40, is expressed on the surface of all seven SCCHN tumor cell lines studied. Tumor cell lines also expressed epidermal growth factor (EGF) receptor, MHC class I, and CD95 (Fas) but did not uniformly express other important lymphoid regulatory molecules such as CD80, CD86, or interleukin (IL) 2 receptor components. CD40 ligation by trimeric CD40 ligand (CD40L) resulted in a 20–45% inhibition of tumor cell growth in three of seven cell lines tested. The cytokines IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-11, and IL-15 neither inhibited nor stimulated growth in any of the cell lines tested. EGF had pleiotropic effects on cell growth; it inhibited growth in two cell lines, stimulated growth in one cell line, and had no effect in four cell lines. When coligation by EGF and CD40L was studied, additive or supra-additive

growth inhibition was seen in four cell lines. Three cell lines were unaffected by EGF, CD40, or coligation with both reagents. Examination of tumor tissues from 12 previously untreated patients representing a broad spectrum of patients presenting with SCCHN demonstrated CD40 expression in all 12 tumor specimens. This study supports the notion that CD40 is a regulatory molecule for the growth of SCCHN. The important role of CD40-CD40L interactions in the regulation of immune cells in the lymph node and the unique high-level expression of CD40L by these immune cells lend support to the hypothesis that this ligand/receptor pair is an important mediator of cell growth in SCCHN.

INTRODUCTION

SCCHN³ is primarily a local-regional disease. Advanced disease and recurrences tend to be confined to the head and neck region. Fewer than 25% of advanced cases will eventually develop distant metastases. Distant metastases also tend to occur late (1–4). This contrasts with the biological behavior of other tumors of the upper aerodigestive tract associated with environmental carcinogens as well as tumors found in other sites such as melanoma, breast, bladder, stomach, and soft tissues. It is in fact extraordinary that the majority of fatal recurrences in SCCHN are local-regional. The unique propensity of SCCHN to be limited to local-regional spread implies basic biological differences in the growth regulation of SCCHN compared to other upper aerodigestive tract and epithelial tumors that are systemically more aggressive.

The general limitation of SCCHN to local and regional sites, principally draining lymph nodes, implies important interactions between tumor cells and local and regional environments. Lymph nodes are composed of B and T lymphocytes, dendritic cells, and macrophages, as well as other supporting stromal cells and vascular and lymphatic endothelium. It is within this array of different immune cells that effectors of SCCHN metastases are likely to reside. The regulatory control of the immune system requires both the secretion of regulatory molecules and the cognate (cell-cell) interactions of different populations of cells. The restriction of SCCHN metastases to the regional lymph nodes implies that the specific cytokine and/or regulatory microenvironment of the lymph node has a direct, unique effect on clonal populations of SCCHN tumor cells that determines the local-regional growth of these cells.

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³ The abbreviations used are: SCCHN, squamous cell cancer of the head and neck; IL, interleukin; CD40L, CD40 ligand; EGF, epidermal growth factor; EGFR, EGF receptor; MFI, mean fluorescent intensity; TGF, transforming growth factor; Ab, antibody; IL-2R, IL 2 receptor; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mAb, monoclonal antibody.

Table 1 Origin of cell lines

Cell line	Origin	Stage	Prior therapy
SCC9	Base of tongue	T ₂ N ₁	None
SCC15	Tongue	T ₂ N ₁	None
SCC25	Base of tongue	T ₂ N ₁	None
SCC66	Floor of mouth	T ₄ N ₀	None
SCC68	Tongue	T ₁ N ₀	None
UMC22A/22B (primary/ node)	Piriform sinus	T ₂ N ₁	None

In the present study, we investigated the role of a number of lymphoid-associated regulatory molecules for effects on the growth of SCCHN. Through these studies, we determined that CD40 is expressed on the surface of SCCHN cell lines and may have a functional role in SCCHN. CD40 was originally identified on the surface of B cells and has been found to have an important regulatory role in normal B-cell growth and differentiation (5–8). CD40 is a M_r 45,000–50,000 glycoprotein member of the TNF receptor family and is now known to be expressed on the surface of a variety of cells associated with the immune system (7, 9). CD40 ligation by its ligand (CD40L) via cognate (cell-cell) interactions induces a number of changes in CD40-bearing B cells, monocytes, and dendritic cells.

Whereas it is apparent that CD40 is a central cell surface molecule in lymphocyte growth regulation, its role in the regulation of normal epithelial cell growth and epithelial tumor growth remains to be determined. CD40 is normally expressed in the basal keratinocyte layer of skin and is lost during the differentiation of keratinocytes (10). CD40 is also expressed on some epithelial tumor cell lines *in vitro* including a subset of breast, ovarian, and lung tumors (11–13). As we will describe, CD40 is expressed on all SCCHN tumor cell lines we have tested *in vitro* and has functional effects on SCCHN cell lines. CD40 is also expressed on tumor cells *in vivo*. Our data in SCCHN tumor cell lines and data from others studying CD40 in keratinocytes and other epithelial tumors suggest an important role for CD40 in SCCHN. The central role of CD40-CD40L interactions in the regulation of immune cells in the lymph node and the unique high-level expression of CD40L by these cells lend support to the hypothesis for a role of this ligand/receptor pair in the regulation of growth of SCCHN.

MATERIALS AND METHODS

Cells and Cell Lines. The SCCHN cell lines SCC9, SCC15, SCC25, SCC66, and SCC68 were established within the Head and Neck Oncology Program and used for these studies. The paired cell lines UMC22a and UMC22b were kindly provided by Thomas Carey (University of Michigan, Ann Arbor, MI; Ref. 14). All seven cell lines are used by researchers nationwide for studies in SCCHN and are free of mycoplasma. SCC9, SCC15, SCC25, and SCC68 were derived from the primary tumors of separate individuals with untreated tongue cancer (Table 1). SCC66 was established from a locally advanced primary cancer of the floor of mouth (15). UMC22a and UMC22b were established from an untreated hypopharyngeal primary tumor (UMC22a) and the associated lymph node metastasis (UMC22b). The cells are routinely grown as adherent

cell cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM sodium pyruvate, and 0.1 mM nonessential amino acids with 10 μ g/ml gentamicin. For experiments, RPMI 1640 was supplemented with fetal bovine serum that had been dialyzed against PBS to eliminate proteins less than M_r 50,000 to exclude most growth factors and cytokines (dMedia). Cells were passaged by removal of growth media and exposure to trypsin and EDTA.

An EBV-transformed B-cell line established from a normal donor by standard methods in this laboratory was used as a positive control for some experiments and was grown in standard basic media as a nonadherent cell culture (16). Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation of peripheral blood obtained from normal volunteers.

Cell Proliferation Assay. Cells were harvested by using trypsin/EDTA from stock cultures. Cells were then incubated in 96-well plates in the presence of dilutions of the growth factors under study for 3 days in dMedia. The number of cells plated was adjusted for each cell line to achieve 80–90% confluence after 3 or 5 days in culture in media alone. Cells were incubated with growth factors, and cellular proliferation was measured by the incorporation of MTT dye (17, 18).

Cytokines and Abs used for these studies were obtained from a variety of sources. CD40L, IL-1R, IL-1R α , and IL-15 were provided by Immunex Corp. (Seattle, WA). The CD40L was produced as a homotrimeric protein and was capable of multimeric ligation of CD40. IL-1 β , IL-1 α , and IL-2 were provided by the Biological Response Modifiers Program (Frederick, MD). IL-11 was provided by Genetics Institute (Boston, MA). IL-4, IL-6, IL-10, and EGF were purchased (Biosource International, Camarillo, CA). TGF- α was obtained from Promega. The 528 hybridoma (anti-EGFR) was obtained from the American Type Culture Collection (Manassas, VA; Ref. 19).

Flow Cytometry. Flow cytometry was used to examine the expression of lymphoid-related cell surface markers/receptors on cell lines as described previously (17, 20). To maintain the integrity of cell surface antigens, 0.05% trypsin was used to release the cells from the flasks. The following murine mAbs were obtained for these studies: (a) anti-IL-2R α , anti-IL-2R β , anti-CD19, anti-CD80, and anti-CD86 (PharMingen, San Diego, CA); (b) IVA-12 (anti-class II), W6/32 (anti-class I), OKT3 (anti-CD3), OKT8 (anti-CD8), OKM1 (anti-CD11b), and HNK1 (anti-CD56; American Type Culture Collection); (c) SIM4 (anti-CD4; NIH AIDS Research and Reference Reagent Program, Rockville, MD); (d) B73.1 (CD16; Giorgio Trichieri; Wistar Institute, Philadelphia, PA); and (e) anti-CD40, kindly provided by Thomas Tedder (Duke University, Durham, NC) and Arnold Freedman (Dana-Farber Cancer Institute, Boston, MA). Anti-Fas (CD95) was obtained from PharMingen.

Western Blotting. Lysates were prepared from cells for Western blots as described previously. In brief, lysates of cell lines in log phase were prepared using the flow cytometry lysis buffer. Equal cell equivalents of the lysates were separated by SDS-PAGE using a discontinuous Laemmli buffer system using a 10% mini-gel (Bio-Rad, Hercules, CA). Antigens were transferred to polyvinylidene difluoride membranes (Fisher, Pittsburgh, PA) that were blocked with 1% gelatin in PBS containing 0.05% Tween 20. After 2 h, anti-CD40 Ab in mouse ascites (provided by Arnold Freedman) was added for 1 h. The mem-

Table 2 Cell surface phenotypes

	Percentage of positive cells (MFI)								
	EGF	CD40	Class I	Class II	CD80	CD86	CD95	IL-2R α	IL-2R β
SCC9	100 (81)	42 (14)	100 (200)	10 (7)	9 (6)	8 (6)	41 (13)	9 (6)	10 (6)
SCC15	100 (141)	47 (12)	100 (337)	4 (5)	3 (4)	6 (6)	97 (32)	3 (4)	2 (4)
UMC22a	100 (198)	84 (24)	100 (1218)	35 (9)	7 (4)	40 (8)	68 (10)	12 (6)	16 (7)
UMC22b	100 (205)	98 (40)	100 (538)	32 (7)	9 (6)	76 (15)	88 (22)	3 (7)	3 (8)
SCC25	100 (173)	12 (6)	100 (573)	5 (5)	5 (4)	35 (10)	91 (19)	3 (5)	5 (4)
SCC66	99 (61)	29 (11)	100 (211)	3 (9)	5 (11)	4 (10)	11 (8)	8 (9)	9 (9)
SCC68	100 (229)	100 (54)	100 (599)	5 (5)	3 (4)	8 (6)	100 (25)	5 (3)	2 (3)

brane was washed and incubated with horseradish peroxidase-conjugated goat antimouse IgG + IgM (Fisher). Reactive bands were detected by enhanced chemiluminescence following the manufacturer's suggestions (Pierce, Rockford, IL), followed by a short exposure to film.

Tumor Samples and Immunohistochemistry. Tissue was obtained from patients undergoing surgery at the Beth Israel-Deaconess Medical Center and Brigham and Women's Hospital. All material was subject to immediate review by the pathology department before acquisition and was obtained under an institutional review board-approved protocol. Tissues specimens were obtained as fresh as possible from the operating room and processed immediately for storage. Samples were embedded in OCT and snap-frozen in liquid nitrogen. OCT-embedded tissue was cut and stained according to the method of Peguet-Navarro *et al.* (10) mAb 89 (Immunotech). Staining of normal tonsillar tissue and basal keratinocytes in nonkeratinizing oral epithelium served as positive controls. Thirteen samples from 12 patients with SCCHN, excluding nasopharyngeal carcinoma and salivary gland cancers, were arbitrarily selected on the basis of sample availability for this study. No attempt was made to otherwise preselect a specific cohort or prognostic group.

RESULTS

Phenotypic Analysis of SCCHN Lines. Flow cytometry was used to examine the expression of lymphoid-related cell surface markers and receptors on SCCHN cell lines. The data are summarized in Table 2. Three cell lines, UMC22a, UMC22b, and SCC25, were studied extensively for expression of a major lymphoid subset defining surface antigens, such as CD3, CD4, and CD8 for T cells and CD19 and CD20 for B cells, and found to be negative (data not shown). However, all seven cell lines uniformly expressed high levels of MHC class I antigen. Differential expression of a number of lymphoid-related antigens among all seven cell lines was noted. IL-2R α was expressed only minimally by UMC22a (12%) and was not expressed by the other six cell lines. CD95 (Fas) is expressed on the cell surface in varying degrees in all seven cell lines. This is an important regulator of apoptosis in lymphoid cells and is notably minimally expressed on SCC66. CD86, another important B lymphoid regulatory molecule, is present on three of the seven cell lines tested. CD80, a related B-cell signaling molecule, is not expressed any of the cell lines tested. All seven cell lines were tested for expression of the nonlymphoid antigen EGFR, which was used as a positive control, and all were highly

positive (data not shown). MFI values for EGFR varied between 61 and 229. As shown in Fig. 1 and Table 2, all seven cell lines also expressed CD40; however, there were significant differences in the intensity of staining as determined by MFI. For example, the following MFI values for CD40 expression were obtained for the cells depicted in Fig. 1: 24 for UMC22a; 40 for UMC22b; 12 for SCC15; 6 for SCC25; 11 for SCC66; 14 for SCC9; and 54 for SCC68. With the exception of SCC25, CD40 expression did not vary. Expression of CD40 varied dramatically for SCC25, ranging from a MFI of 6, with 12% of the cells positive (as shown in Fig. 1) to 90% of cells expressing CD40 with a MFI of 78 in another experiment (data not shown). Thus, CD40 was expressed on all human SCCHN cell lines tested to date.

Because the Ab to CD40 may be binding to a related molecular variant of CD40 in SCCHN tumor cell lines, the molecular weight of CD40 expressed on SCCHN cells was confirmed by Western blot. As shown in Fig. 2, anti-CD40 reacted with a band corresponding to M_r 48,000 in lysates from seven SCCHN cell lines as well as peripheral blood mononuclear cells and EBV (data not shown). This band was not present in control blots consisting of irrelevant mouse immunoglobulin (data not shown). The identical molecular weights strongly support the notion that CD40 is identical in all tissues tested. Staining was proportional to cell surface expression based on MFI, and the least staining was observed with SCC25 (Fig. 2, Lane E), and the most staining was observed with SCC68 (Fig. 2, Lane G).

Effect of Cytokines on SCCHN Growth. A number of cytokines, growth factors, and Abs were studied for their effects on SCCHN cell growth. EGF was selected as a positive control for its known effects on some cell lines. Cytokines were chosen for testing if they might be predicted to be present or have been shown by others to be in the immediate tumor microenvironment or the local lymph node. It has been shown, for example, that the cytokines IL-1, IL-4, IL-6, IL-8, and granulocyte macrophage colony-stimulating factor are present in the microenvironment of SCCHN tumors (21–23). When the dose-dependent effects of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-11, and IL-15 were studied ($n = 4–6$), none altered the cell growth of SCC25, UMC22a, or UMC22b in a consistent manner (data not shown). The results with IL-2 are in contrast to those described previously whereby IL-2 inhibited SCCHN tumor growth (24, 25). However, the SCCHN cell lines studied thus far in our laboratory failed to express more than a single chain of the heterotrimeric IL-2R. This lack of expression of IL-2R is in

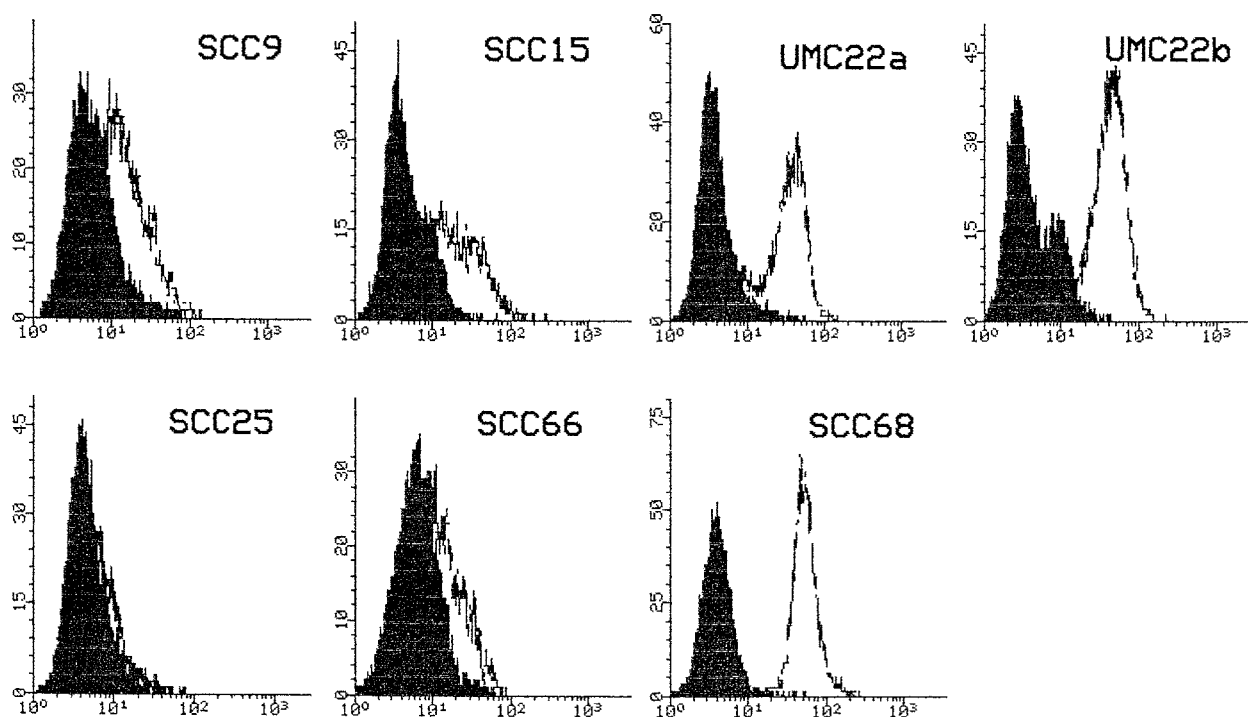


Fig. 1. Flow cytometry demonstrating the expression of CD40 on the surface of tumor cell lines. A negative control isotype-matched Ab is shown for each cell line. The value shown in Table 2 is the percentage of positive cells. The value given in parentheses is the MFI.

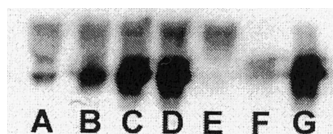


Fig. 2. Western blot demonstrating anti-CD40 Ab binding to a M_r 48,000 band present in cell lysates from seven squamous cell lines. Each lane is a single cell line. Lane A, SCC9; Lane B, SCC15; Lane C, UMC22A; Lane D, UMC22B; Lane E, SCC25; Lane F, SCC66; and Lane G, SCC68.

contrast to the expression of multiple chains of the IL-2R on a number of cell lines used in the studies by Yasumura *et al.* (24) and Weidman *et al.* (25). We also failed to see growth enhancement with IL-4 as reported by Myers *et al.* (26). Because these growth effects occur in only a fraction of cell lines in each of these studies, they may not have been evident in the limited number of cell lines used in this study.

EGF, our control factor, consistently had an effect on SCCHN cell growth in some cell lines, as shown in Fig. 3. Each curve represents the average of three experiments. At the highest concentration tested, 10 ng/ml, EGF reduced cell growth by 30% for UMC22a and 40% for UMC22b. In contrast, EGF stimulated the growth of SCC25 by over 40% but had no effect on the growth of SCC9, SCC15, SCC68, or SCC66. In our experiments, the effects on individual cell lines were purely dose dependent, regardless of inhibitory or stimulatory direction. In addition to EGF, TGF- α , which is a known ligand of the EGFR, also affected the proliferation of SCCHN cell lines.

TGF- α also inhibited UMC22a and UMC22b and, in contrast to EGF, slightly inhibited SCC25. TGF- α had no effect on SCC9, SCC15, SCC66, and SCC68 (data not shown). Distinct growth effects were observed when the cells were incubated with the EGFR-specific murine mAb 528 (19). As shown in Fig. 3, similar to the results seen with EGF, growth of UMC22a was inhibited by the 528 Ab. However, 528 Ab stimulated the growth of UMC22b and inhibited the growth of SCC25. Also, whereas EGF had no effect on the growth of SCC68 cells, 528 Ab significantly inhibited the growth of this cell line in a dose-dependent manner. The 528 Ab to the EGFR functions much as the 225 Ab does in blocking EGF engagement and may also directly affect cell proliferation, as has been reported in A431 cells with Ab 225. This functional effect of Ab ligation is qualitatively different than EGF ligation and acts on cell growth in A431 cells through a different mechanism of action (27–29). The functional activity of 528 Ab was compared against an isotype-matched control mAb reactive with human light chain to control for FcR interactions (data not shown). It should also be noted that EGF, as opposed to the Abs, has dual effects on A431 cells, depending on the dose (19).

In an analysis of cell surface receptors by flow cytometry (described above), we identified CD40 as being expressed on the cell surface of the SCCHN. Given the expression of CD40 on SCCHN cell lines, we evaluated the effect of CD40 ligation on cell growth. The results of CD40 ligation are shown in Fig. 4. It is noteworthy that aside from EGFR-associated reagents, no other cytokine consistently affected the growth of the SCC cell lines. Moreover, the magnitude of the changes are consistent with other known active cytokines. The growth of UMC22a,

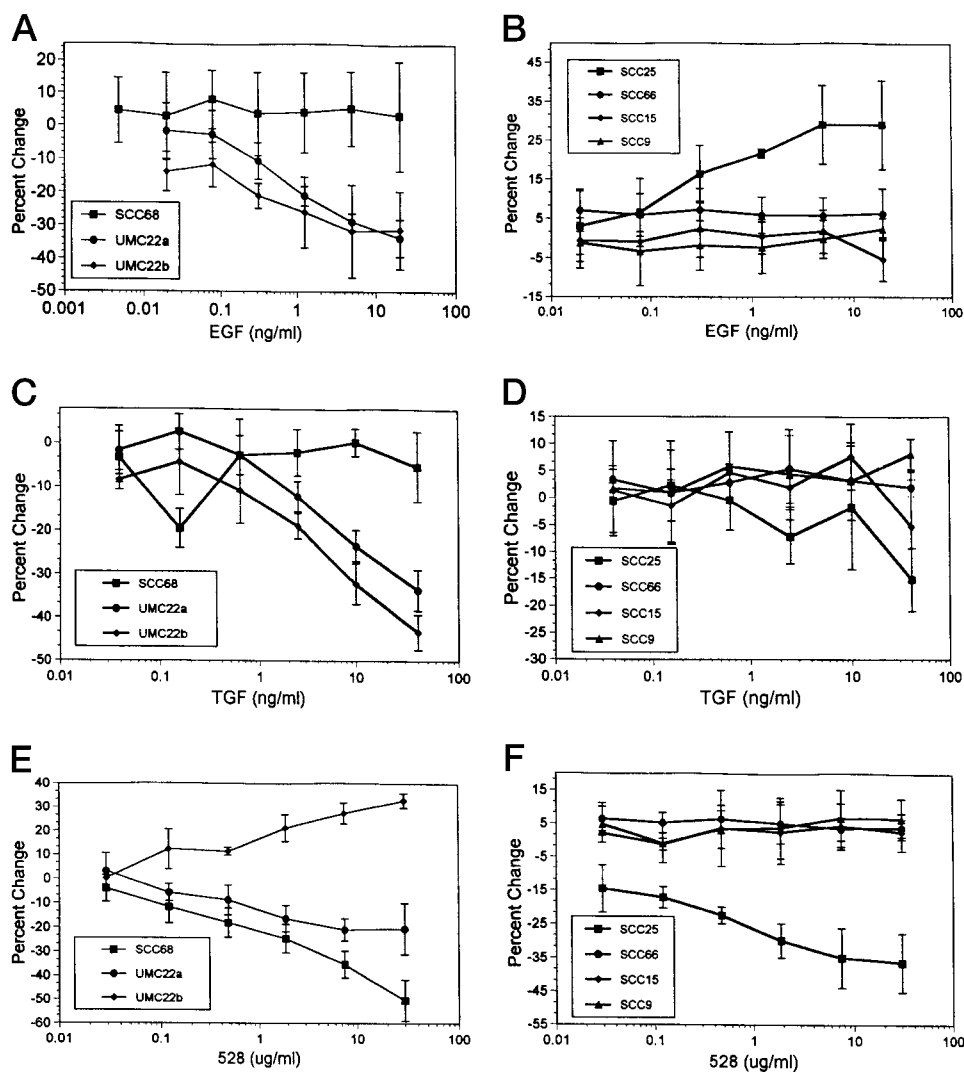


Fig. 3. A–E, effect of EGF, TGF- α , and 528 Ab on the growth of SCCHN cell lines as measured by the MTT/MTS cell growth assay. Each set of curves depicts stimulation (the positive percentage change from control) or inhibition (the negative percentage change from control) induced by the specific ligand on the identified cell lines. A and B, EGF; C and D, TGF- α ; E and F, 528 Ab.

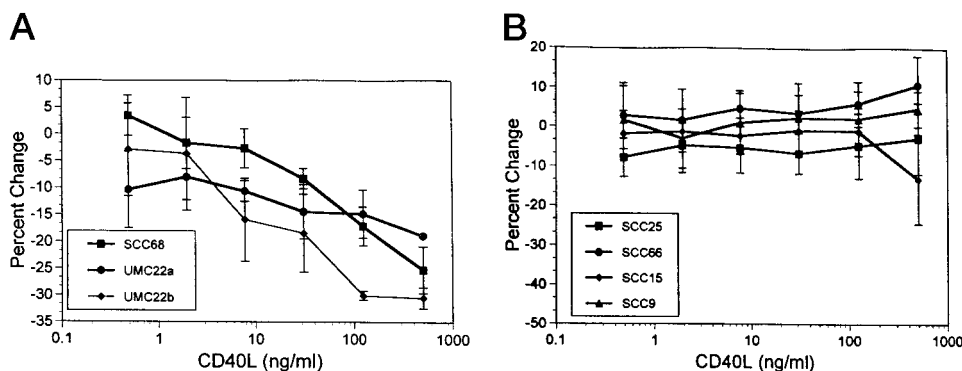


Fig. 4. A and B, effect of CD40L on the growth of SCCHN cell lines as measured by the MTT/MTS cell growth assay. Each set of curves depicts stimulation (the positive percentage change from control) or inhibition (the negative percentage change from control) induced by CD40L.

UMC22b, and SCC68 was inhibited by 20%, 30%, and 25% (the mean of four separate experiments), respectively, by the highest concentrations of CD40L tested, whereas the growth of SCC9, SCC15, SCC25, and SCC66 was unaffected. As can be seen in

Fig. 4, growth inhibition is dose dependent. Despite the expression of CD40 by the seven cell lines, there are distinct differences in the functional activity of this receptor upon ligation of CD40L.

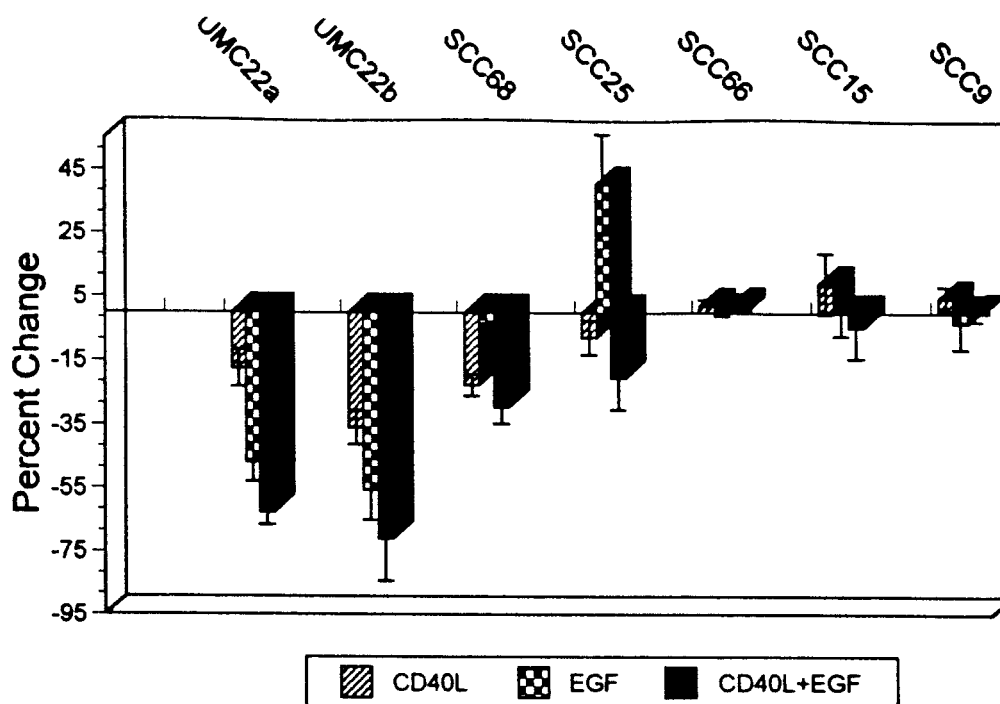


Fig. 5. The effect of EGF (10 ng/ml), CD40L (250 ng/ml), and the combination of EGF and CD40L on the growth of all seven cell lines. Each set of bar graphs depicts stimulation (the positive percentage change from control) or inhibition (the negative percentage change from control) induced by the specific ligand on the identified cell lines.

EGF (10 ng/ml) was combined with CD40L (250 ng/ml) at concentrations determined from prior experiments to yield significant but not maximal stimulation or inhibition. At this dose, additive effects for inhibition of UMC22a, UMC22b, and SCC68 were observed (Fig. 5, mean of three experiments). Growth of UMC22a was inhibited 17% by CD40L alone, 47% by EGF alone, and 63% with a combination of EGF and CD40L. Similarly, UMC22b growth was inhibited 36% by CD40L alone, 56% by EGF alone, and 71% by the combination of EGF and CD40L. For SCC68, CD40L inhibited growth 23%, EGF had no effect, and the combination of EGF and CD40L inhibited growth 30%, which was of borderline significance. The combination of CD40L and EGF had unexpected effects on the growth of SCC25. EGF stimulated SCC25 growth by 41%, whereas CD40L had no effect (7% inhibition; not significant); however, when combined, CD40L significantly reversed the EGF stimulation and further decreased SCC25 cell growth by 20%. To determine whether coligation with other B-cell cytokines might result in alterations in CD40 function, we performed coligation experiments with IL-4, IL-10, and IL-15. None of these cytokines affected the cells independently or perturbed the effects of CD40 ligation (data not shown).

Immunohistochemical Studies. To determine whether CD40 is expressed on tumor cells *in vivo*, surgical specimens from patients with previously untreated SCCHN undergoing initial surgical management of local disease were studied for expression of CD40. Patient and tumor characteristics span a broadly representative population of those who present with previously untreated SCCHN and are summarized in Table 3.

Positive control material consisted of nonkeratinizing mucosa from normal and tumor-bearing patients and tonsillar specimens from patients undergoing tonsillectomy. CD40 staining was specific for the expected basal layer and the germinal center cells, respectively (data not shown). CD40 was expressed within the primary tumors (12) and in the lymph node metastases (1) of all of the patients. In Fig. 6, H&E staining (A and D) of representative samples of well-differentiated (A–C) and poorly differentiated (D–F) tumors are shown in conjunction with negative control Ab staining (B and E) and CD40 staining (C and F). Within the primary tumors, there was differential staining of different areas of the tumor. Reduced CD40 staining was seen primarily in areas of differentiating cells and keratin pearls in well-differentiated tumors (A–C), suggesting that reduced CD40 expression can occur in areas of cellular differentiation, consistent with loss of CD40 expression during terminal differentiation of normal cells. Differential staining was seen in the moderately differentiated tumors, with more consistent staining seen in the invading tumor fronts (data not shown). In the poorly differentiated tumors (D–F), tumor staining was scattered throughout the tumor. Membrane and cytoplasmic staining of the tumor cells with Ab is readily evident in all tumor specimens.

DISCUSSION

Based on clinical data, we have hypothesized that the limitation of SCCHN growth to the local site and regional lymph nodes is a tissue-specific effect and that regional tumor

Table 3 Patient characteristics^a

Patient	Age (yr)	Sex/race	Stage	PS/L	Smoking	D	Site
JP	43	M/C	T ₂ N ₀	PS	+	W	FOM/OC
GB-1	74	M/C	T ₂ N ₂	PS/L	+	P	Larynx
JR	55	M/A	T ₄ N ₂	PS	+	MP	H
LR	72	F/C	T ₁ N ₀	PS	-	W	Tongue/OC
PA	79	F/C	T ₄ N ₀	PS	+	M	FOM/OC
BR	62	M/C	T ₃ N ₀	PS	+	MP	Maxilla/PNS
BS	71	M/C	T ₃ N ₀	PS	-	M	Tongue/OC
SO	49	F/C	T ₃ N ₀	PS	+	M	OP
NR	63	F/C	T ₁ N ₂	PS	+	P	H
MM	87	F/C	T ₂ N ₂	PS	-	P	H
MS	44	M/C	T ₁ N ₁	PS	-	MP	Tongue/OP
GY	58	M/A	T ₃ N ₃	PS	-	M	H
SG	58	M/C	T ₃ N ₀	PS	+	M	Tongue/OP

^a OP, oropharynx; H, hypopharynx; OC, oral cavity; FOM, floor of mouth; PNS, paranasal sinus; PS, primary site; L, lymph node; +, yes; -, no; D, differentiation; W, well differentiated; M, moderately differentiated; P, poorly differentiated; C, caucasian; A, African.

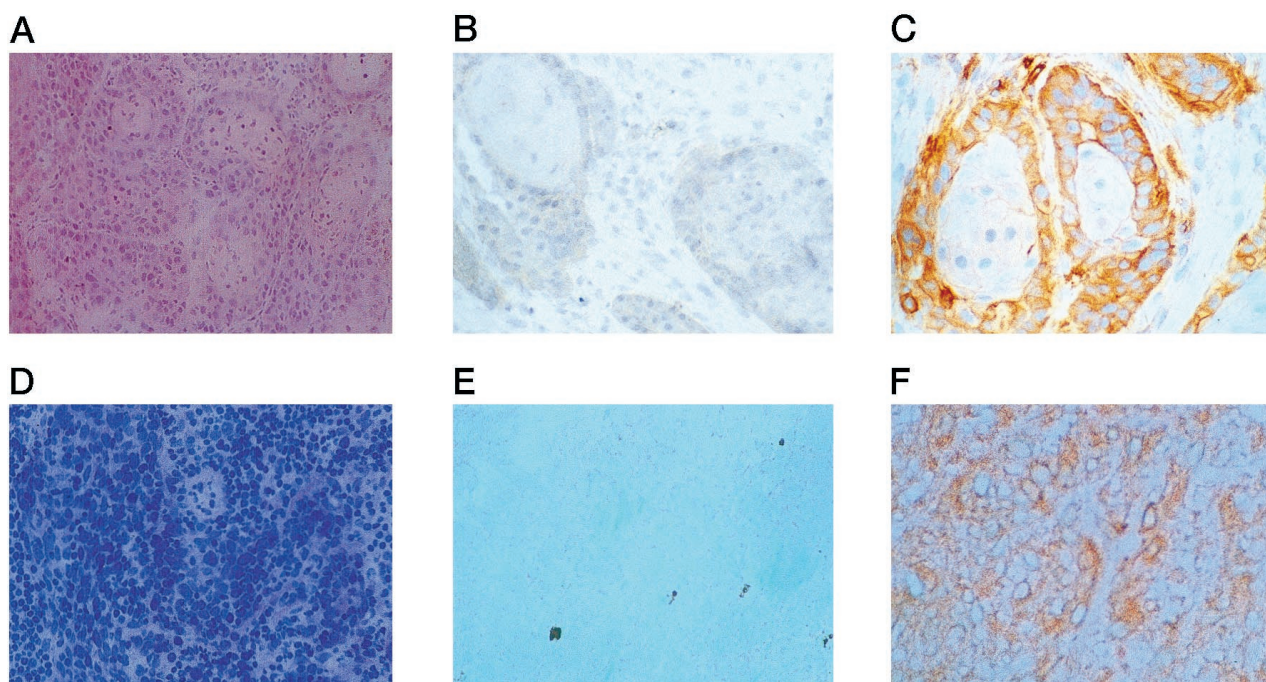


Fig. 6. CD40 expression in frozen sections of tumors obtained during surgery from patients with well-differentiated (A–C) or poorly differentiated (D–F) SCCHN. Each tumor type is stained with H&E (A and D), a negative control isotype-matched antibody (B and E), and anti-CD40 Ab (mAb 89); C and F.

cell growth is mediated by cell surface receptors. We have predicted that SCCHN tumor cell surface receptors would be responsive to lymphoid cytokines or growth factors found in the regional lymph node environment and would be identical or related to lymphoid cell surface receptors. In the present study, we have identified CD40 as a molecule expressed on cell lines derived from SCCHN and SCCHN tumor cells *in vivo*. Ligation of CD40 resulted in growth inhibition in a subset of cell lines. Of importance, CD40 ligation and coligation of EGFR resulted in significant, integrated effects on cell growth in affected cell lines. Furthermore, SCCHN cell lines also expressed CD95 (Fas) and the important B-cell regulatory molecule CD86 but

did not express other, selected B-cell, T-cell, or monocyte-associated regulatory molecules. Finally, CD40 has been demonstrated to be expressed *in vivo* on tumor cells from a selection of previously untreated tumor tissues from a broadly representative group of patients with SCCHN.

Previous investigators have evaluated the effects of lymphoid cytokines on the growth of SCCHN (21, 22, 24–26). IL-1 and IL-4 have been suggested as possible growth-promoting cytokines on the basis of growth studies of dispersed tumor samples from patients (23). Tumor cells grown *in vitro* and patient tumors from *in vivo* biopsies have been shown to produce IL-1 and IL-6, but these cytokines were not shown to

influence cell growth (30, 31). Recent studies with cell lines derived from a variety of patients have demonstrated that the γ -chain of the IL-2R is expressed on a fraction of cell lines, and that an IL-2R composed of the γ - and β -chains of the IL-2R is capable of inhibiting tumor cell growth by 10–30% in these cell lines. Only approximately 20% of cell lines express the minimal, dimeric IL-2R (24). IL-4 has also been shown to enhance growth by 10–50% in approximately 15% of cell lines tested. In the present study, a number of cytokines were tested, and none were found to have an effect on the growth of the SCCHN cell lines used in these studies either alone or coligated with CD40.

CD40 is expressed in varying degrees on all the cell lines tested; CD40 expression is qualitatively similar to that of the EGFR in this respect, and CD40 ligation affected growth in four of seven cell lines tested, either as a single agent or in combination with EGFR. Although it is possible that functional effects of CD40 ligation in SCCHN cell lines are a function of receptor density on the cell surface, the relationship is most likely complex and not simply explained as shown by the results with SCC25 and SCC66 (Table 2; Fig. 5). Thus, the relative effects of CD40 on cell growth in the different cell lines are most likely due to mechanistic differences in receptor signaling rather than receptor density. As described above, CD40 is a M_r 45,000–50,000 glycoprotein member of the TNF receptor family expressed on the surface of a variety of cells (7, 9). Although CD40 was originally thought to be B-cell specific, it has been identified on many lymphoid cells in which it functions as a cognate receptor (5–8). Multimeric CD40 ligation by the CD40L, a M_r 35,000 glycoprotein expressed on the surface of T cells, causes a series of events that, in concert with other signals, results in the growth or death of B cells (7, 9, 32–34). Multimeric ligation of CD40 is a cognate receptor interaction requiring cell-cell contact. In the presence of costimulation via additional cell surface receptors, including CD80 and CD86, B cells will proliferate. This appears to be a major consequence of CD40 ligation. Under some circumstances, in the absence of a costimulatory signal, CD40 may also induce cell death by an apoptotic mechanism different from apoptosis induced by other B-cell molecules or inhibit the growth of malignant B cells (7, 32, 33, 35–38). Among the important receptors known to act with CD40 as costimulators are the B cell receptor, cytokine receptors for IL-2 and IL-4, and B7.1 (CD80) and B7.2 (CD86) in B cells. It is important to emphasize that CD40 ligation can directly mediate growth inhibition, apoptosis, and cell proliferation and that the ultimate fate of the cell after ligation is a function of the cytokine and cognate environment of that cell. It is important to note that regarding the predicted role of CD40 in SCCHN, it is the subsequent interplay of signaling in conjunction with CD40 ligation that determines cell response to CD40 ligation.

Furthermore, and relevant to our hypotheses regarding CD40 as a regulator of cell growth in SCCHN, in addition to affecting B cell growth or survival, CD40 ligation also up-regulates the expression of cell adhesion molecules, Fas, and the regulatory molecules CD80 and CD86 in lymphoid cells (33, 39, 40). More recently, CD40 regulation has been associated with the production of IL-12, IL-8, matrix metalloproteinases, and TNF by dendritic cells and macrophages (41–46). Like CD40, the receptors CD80 and CD86 are cognate receptors and func-

tion through cell-cell contact and ligation. These receptors, in concert with T-cell receptor ligation by HLA-DR on B cells, provide costimulatory support for the growth of T cells (39, 44, 45, 47, 48). The ligation of CD40L on T cells by CD40 up-regulates the secretion of cytokines and the expression of regulatory molecules in T cells, including CD40L. Thus, CD40-CD40L interactions have profound effects on both cell partners in the cognate interaction, and many of the features of this interaction affect the growth of the CD40-bearing cell. These data support the notion that CD40 may have a specific role in lymph node metastases in SCCHN.

Recently reported studies have demonstrated the presence of CD40 on basal keratinocytes and have demonstrated that ligation of CD40 expressed on cultures of normal skin keratinocytes up-regulates the expression of differentiation antigens and inhibits proliferation. CD40 ligation in keratinocytes increases secretion of cytokines including IFNs (10, 49). This suggests that CD40 is a relevant molecule in the regulatory control of normal keratinocyte function. CD40 has also been shown to be expressed on normal pulmonary fibroblasts (50). In this setting, CD40 ligation is associated with cytokine production. Of interest, CD40 has been shown to be expressed on endothelial cells, supporting the growth of renal cell cancer (11). Furthermore, CD40 has now been reported to be expressed by a fraction of lung cancer, melanoma, and ovarian cell lines and tumors, and ligation of CD40 on the surface of these tumor cell lines also results in growth inhibition (12, 13, 51).

The mechanism of CD40 signaling in SCCHN and the differences in each cell line in the integration of CD40 signaling are likely to be relevant to regional metastatic behavior. CD40 lacks an enzymatic cytoplasmic tail and primarily mediates signaling through associated molecules (9, 52). These molecules are the TNF receptor-associated factors that associate in various combinations with TNF family receptors. The TNF receptor-associated factors have no known enzymatic activity but appear to be linking molecules to additional effectors downstream of the membrane CD40. Downstream intracellular signaling via CD40 in B cells is associated with the activation of the extracellular signal-regulated kinase and stress-activated protein kinase pathways (53–55). Others have shown that CD40 ligation activates the RAS pathway in human lymphoma cells and that *src* kinases may mediate CD40 signals (56). Recent studies have shown a direct effect of CD40 ligation on p38 mitogen-activated protein kinase in B cells (57). These studies demonstrate that the stage and state of the lymphoid or lymphoma cells used for studies strongly influence CD40 signaling.

The mechanisms by which CD40 receptor ligation affects cell growth in SCCHN were not investigated in these studies, which serve to identify the potential importance of CD40 in epithelial cell growth. It is apparent that CD40 is a central cell surface molecule involved in lymphocyte growth regulation, and the mechanisms by which it acts in lymphocytes are now under intense investigation. The role of CD40 in the regulation of epithelial cell growth and epithelial tumor growth and the mechanisms by which it mediates these effects remain to be determined. This study supports the hypothesis that CD40 is an important regulatory molecule in the growth of SCCHN. As we have described, CD40 has functional effects on SCCHN cell lines. Our data in SCCHN tumor cells and data from others

studying CD40 in keratinocytes and tumors suggest an *in vivo* role for CD40 in SCCHN. The important role of CD40-CD40L interactions in the regulation of immune cells in the lymph node and the unique high-level expression of CD40L by these immune cells lend support to the hypothesis that this ligand/receptor pair is an important mediator of cell growth in SCCHN.

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