Abstract. Background: Most tumor tissues, especially those of non-hematopoietic origin, do not express CD80 co-stimulatory molecules, possibly as a mechanism to evade immune surveillance. The objective of this study was to determine whether abundant endogenous CD80 expression on oral squamous cell carcinoma (SCC) early during tumor progression can facilitate immune elimination and reverse immune tolerance. Materials and Methods: The growth of regressor and progressor oral SCC lines with differing endogenous CD80 expression were examined in immune-competent and -deficient mice. Immune effectors were determined by T-cell depletion experiments and immunohistochemistry. Results: Our studies show regression of early tumor growth when immunocompetent animals are inoculated with oral SCC progressor cell lines expressing abundant endogenous CD80. The CD80-induced antitumor response was due largely to induced T-cell responses. Conclusion: Our findings suggest that inadequate CD80 expression during early oral SCC formation may contribute to the escape of tumors from immune elimination. This information can be useful in the design of new approaches to generate more effective immunotherapy against this disease.

Head and neck squamous cell carcinoma (HNSCC) is a devastating disease that can destroy the structure and function of organs for deglutition, mastication, speech, taste, as well as other vital structures necessary for survival. The lack of an effective immune response contributes to the progression of HNSCC and is a prognostic marker for poor clinical response and decreased survival. These patients have shown significant immunologic dysfunctions in T- and natural killer (NK)-cell immunity that have been correlated with aggressive tumor behavior and with their poor survival (1-4). Activating or inhibitory co-stimulatory signals mediated by the interaction between CD28, CTLA-4 and other receptors on the T-cell with members of the B7 family of co-stimulatory molecules on antigen-presenting cells are crucial to determine T-cell effector function. Lack of T-cell co-stimulation may be a means to evade immune surveillance leading to T-cell anergy and inefficient/insufficient generation of anti-tumor immune responses. This process may eventually culminate in immune unresponsiveness in the host. The B7 family of co-stimulatory molecules, which are members of the immunoglobulin superfamily include CD80 (B7-1) and CD86 (B7-2) (5-7). Although others have been recently described, CD80 and CD86 molecules provide the most potent co-stimulatory signals found to date. These APC-derived ligands engage their counter-receptors CD28 (an activating surface receptor protein) and CTLA-4 (an inhibitory receptor protein) on T-cells in response to antigen, and regulate T-cell proliferation, cytokine production and generation of different functional responses. The activity of CD80 in effecting regression can be attributed to its effect on induction and propagation of Th1-mediated responses, which can mediate anti-tumor immunity.

Transfection of CD80 into murine and human cell lines has been reported to promote antigen-specific cytotoxic T lymphocytes (CTL) and NK-cell responses that result in immune recognition and destruction of tumors (8-10). In addition, studies show that genetic modifications of tumor cells to express co-stimulatory molecules serve as an effective approach for immunotherapy against cancer not only in animals, but also in limited clinical trials (11-13). However, since most established tumors not of hematopoietic origin do not express CD80, little is known...
concerning the importance of endogenous CD80 expression in tumor progression in vivo, induction of anti-tumor immunity and the nature of this immunity. In this study an experimental system has been developed in which endogenous CD80 expression by tumor cells correlates with progressor or regressor phenotypes in oral squamous cell carcinoma (SCC). Clonal murine oral SCC lines following tumor progression of transformed keratinocytes in immunodeficient SCID mice were previously derived, and a subset of these was found to be CD80 positive (14). When implanted in immune syngeneic BALB/c mice, the SCC that expressed CD80 became regressors, while those that did not express CD80 grew as progressors. These results suggested that early HNSCC that express CD80 co-stimulatory molecules might be targets for immune-mediated destruction, and that perhaps progressive tumors have escaped this surveillance partly by not expressing CD80. Lack of CD80 expression on tumor cells, thus, may contribute to mechanism of escape from immune-mediated elimination. In the present study, the role of endogenous tumor-derived CD80 in HNSCC regression was further delineated. This information can be useful to devise new approaches to generate more effective immunotherapy against HNSCC.

Materials and Methods

**Mice and reagents.** Male BALB/c mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Facility. Mice were between 4-6 weeks of age and housed in a specific pathogen-free animal facility. Animal care was provided under Animal Care and Use Committee Protocol of the University of Miami (#00-143 and 04-017) in accordance with the National Institute of Health guidelines for care and use of laboratory animals. Monoclonal antibodies against CD4 (clone H129.19); CD8a T-cells (clone 53-6.7) were obtained from BD PharMingen (San Diego, CA, USA). Monoclonal antibody against murine CD80 (clone RMMP-1) was obtained from GeneTex, Inc. (San Antonio, TX, USA) and used in immunohistochemistry. Rat IgG (BD PharMingen) was used as a negative control.

**Tumor cell lines.** Oral SCC lines B6C3-4scid (regressor) and B7E3-4scid (progressor) were previously established from clonal cell lines of transformed oral keratinocytes by the carcinogen 4-NQO (4-NQO) in vitro and grown in immunodeficient SCID male BALB/c (63669) in vivo and grown in immunodeficient SCID male BALB/c mice (14). HNSCC lines were cultured at 37°C with 5% CO2 in MEM containing 10% FCS (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) and antibiotics. The B6C3-4scid and B7E3-4scid cell lines were sorted by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) and expanded into CD80-expressing (CD80+) or CD80 non-expressing (CD80−) lines for in vivo experiments.

**Plasmid construction.** To generate a CD80+ transfected line, the mouse CD80 gene (ATCC # 63669) was cloned into the Xba I and Hind III sites of the pcDNA3.1 (-) plasmid. CD80 gene insertion was confirmed by sequencing. Using lipofectamine, the pcDNA3-CD80 plasmid was transfected into the wild-type CD80-negative progressor line B7E3-4scid. The transfected line was selected in media containing G418. Further selection was achieved by FAC sorting.

**In vivo tumor growth.** The 4-NQO-induced murine oral SCC lines were previously shown to form tumors when inoculated subcutaneously in the flank (14). The wild-type progressor line B7E3-4scid form tumors that progressively grow in immunocompetent animals, while the wild-type regressor line B6C3-4scid form tumors that completely regress in immunocompetent animals by Day 30 after inoculation (14). Experiments were designed to contain 4-6 mice in each group and these experiments were repeated. For the first experiments, two groups of cell lines were grown in immunocompetent animals and compared. On day 0, a cell suspension of 1x10^7 cells of the wild-type cell line B6C3-4scid or the sorted and expanded CD80 non-expressing B6C3-4scid regressor line were inoculated into the right flanks of syngeneic BALB/c immunocompetent mice in a total volume of 100 μl. For the second experiments, three groups of experimental animals containing 5-6 animals in each group were used: a control group, which were inoculated with 1x10^7 cells of the wild-type progressor cell line B7E3-4scid, a CD80-transfected B7E3-4scid line or vector only control group into the right flanks of syngeneic BALB/c immunocompetent in a total volume of 100 μl. For the third in vivo experiments, four groups of experimental animals were compared. The first group consisted of wild-type B6C3-4scid regressor cell line grown in immunocompetent (wild-type) mice. The second group consisted of wild-type B6C3-4scid regressor cell line grown in nude BALB/c mice. The third group consisted of wild-type B7E3-4scid progressor cell line grown in immunocompetent mice and the fourth group consisted of wild-type B7E3-4scid progressor cell line grown in nude Balb/c mice. Each animal was inoculated with 1x10^7 cells in a total volume of 100 μl into the right flanks. Palpable growth was measured weekly with calipers and recorded. Tumor area was determined as the square product of tumor diameters (width x length) in centimeters. Tumor bearing mice were killed according to NCI criteria when the tumor size was greater than 2.0 cm^2 or mice developed one of the following: bleeding, ulceration of the tumor, trouble feeding or moving and greater than 10% weight loss. For animal experiments, 5-6 animals per each treatment/control group were used and experiments were duplicated for confirmation of result.

**Flow cytometry and cell sorting.** Using cells from wild-type regressor line B6C3-4scid, which has a mixed population of CD80+ and CD80 negative cells, sorted CD80 expressing (CD80+) and sorted CD80 non-expressing (CD80−) cells were obtained by flow cytometry and maintained in culture until sufficient amounts were available for in vivo inoculations into animals. The relative levels of surface CD80 expression on the cell lines were compared by immunofluorescence staining and flow cytometry as previously described (14). For quantification of CD80 expression by flow cytometry, FITC-conjugated hamster anti-mouse CD80 mAb was used with FITC- and conjugated hamster as isotype control (BD PharMingen). The stained cells were analyzed in a flow cytometer (FACScan), which had been calibrated with standard beads at an excitation wavelength of 488 nm. The log fluorescence intensity of 104 cells was plotted and analyzed.

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In vivo T-cell subset depletion. In vivo T-cell depletion was performed using monoclonal antibodies of anti-CD4+ (GK 1.5) or anti-CD8+ T-cell (53-6.72) hybridomas (American Type Tissue Collection, Manassas, VA, USA). Balb/c mice were depleted with 0.1 ml of anti-CD4+ or anti-CD8+ mAb suspended in PBS intraperitoneal on days-3, -2 and -1 and tumor cell inoculations of 1x10^7 cells were started on Day 0. Treatment with anti-CD4+ or anti-CD8+ T-cell mAb was continued during the entire period of tumor growth on days 5, 10, 15, 20 and 25. Three days after inoculation of anti-CD4+ or anti-CD8+ mAb, a mouse was sacrificed and spleen cells harvested to confirm appropriate elimination of CD4+ and CD8+ cells using flow cytometry. The control group of mice was inoculated with 0.1 ml of purified normal rat IgG (Sigma, St. Louis, MO, USA).

Immunohistochemical staining for CD4+ and CD8+ T-cells. Tumor specimens formed from wild-type B6C3-4scid regressor cells and wild-type progressor B7E3-4scid were harvested and fragments processed for frozen sections (American HistoLab, Gaithersburg, MD, USA). The presence of CD4+, CD8+ T-cells was compared between regressor and progressor tumor specimens by immunohistochemical staining on glass slides. Briefly, sections were fixed in cold acetone and incubated with 10% rabbit serum for 20 minutes at room temperature. Monoclonal antibodies against CD4+, CD8+ T-cells or CD80 were added to the slides at a concentration of 1:250 in a humidity chamber for 1 hour. After several PBS washes, an immunoperoxidase staining system containing biotinylated antibody was used, according to instructions provided by the manufacturer (Vectastain Elite ABC Kit; Vector, Burlingame, CA, USA). The substrate for the peroxidase reaction was obtained from a commercially available kit (DAB, Vector). Slides were counterstained with hematoxylin, dehydrated with ethanol and permanently mounted using Permount (Fisher, McLean, VA, USA).

Rechallenge experiments. BALB/c mice that showed complete regression of the CD80+ transfected progressor of the B7E3-4scid were re-inoculated with 1x10^7 wild-type B7E3-4scid progressor cells on the contralateral flank after complete tumor regression was confirmed by 60 or more days of observation. Tumor growth was measured weekly with calipers and recorded. Tumor area was determined as the square product of tumor diameters (width x length) in centimeters. Animals were killed according to NCI criteria.

Results

Endogenous CD80 expression by tumor cells is sufficient for tumor regression of early HNSCC. We took advantage of our murine model in which the wild-type regressor line, B6C3-4scid, which contains 89-95% of cells expressing CD80 (inset, Figure 1) to sort and expand these cells into CD80 non-expressing populations by using FACS. The CD80 non-expressing cell population of B6C3-4scid contained 5% of cells expressing CD80 (inset, Figure 2). To study the role of CD80 in mediating tumor regression, we compared in vivo growth between CD80 non-expressing sorted cells of the regressor line B6C3-4scid and the wild-type cells of the same cell line. The CD80 non-expressing sorted cells showed MHC class I staining similar to that of wild-type counterparts (data not shown). The results of a representative experiment are shown in Figures 1 and 2. For these experiments, the experimental groups contained 5-6 animals each and were duplicated. Figure 1 shows complete regression of tumor growth by day 20 after tumor inoculation in the wild-type line in which a majority of cells express CD80. In the experiment represented by Figure 2, 3 out of 5 (60%) animals showed progression of tumor growth when CD80 is not expressed in a majority of tumor cells. These findings suggest that, during early tumor development, abundant CD80 expression on oral SCC facilitates tumor regression. Although CD80 expression may not be the sole factor determining regression, our findings suggest that tumors not expressing sufficient density of endogenous CD80 co-stimulatory molecules may likely escape immune surveillance and grow progressively in the host.

Abundant expression of CD80 in established oral HNSCC promote tumor regression. In the experiments above, it was demonstrated that lack of endogenous CD80 expression on the surface of tumor cells promote tumor growth of oral SCC (Figure 2). We studied the wild-type progressor line B7E3-4scid, which grows progressively in immunocompetent animals and transfected the CD80 gene in order to determine whether transfected CD80 can effect regression in progressor tumors, which may have a genetic predisposition to progression, as it occurs in regressing tumors. The wild-type progressor line B7E3-4scid has minimal constitutive expression of CD80 co-stimulatory molecule (2-5%) and grows progressively in immune competent animals (14). The growth of tumors derived from the CD80+ transfected progressor line was examined and compared to growth of tumors derived from wild-type progressor in immune competent mice. Prior to in vivo experiments, CD80 expression in the transfected line was analyzed by flow cytometry, which showed 82% transfected cells expressing CD80 compared to wild-type (4.83%) and 4.67% for the vector only control (Figure 3, inset). Our results showed decreased tumor growth in immune competent animals bearing tumors formed from the CD80+ B7E3-4scid transfected line compared to growth from vector only or wild-type progressor line (Figure 3). Regression of the transfected line was seen by day 30 similar to the regression pattern observed in vivo with wild-type regressor lines expressing endogenous CD80 (Figure 1). In addition, one cell line named “CD80– re-isolate” outgrew from one mice bearing CD80+ transfected cells after complete tumor regression was achieved for 30 days. Significantly, after re-isolation of tumor and passage in vitro, this cell line expressed low CD80 (12%) by flow cytometry (Figure 4). Thus, it was demonstrated that the substantial expression of CD80 on progressor cells can...
facilitate regression of these tumors as well, and our findings suggested that in established tumors that have escaped immune elimination, small proportions of cells expressing CD80 may not be sufficient to effect complete and persistent tumor regression.

Host CD8+ T-cell immunity is required for CD80-mediated regression. To study immune effector involved in CD80-mediated tumor regression, the growth of CD80 expressing regressor wild-type line B6C3-4scid was compared with CD80 non-expressing progressor wild-type line B7E3-4scid in immune competent and nude mice (Figure 5). In these experiments, no tumors outgrew in wild-type animals challenged with B6C3-4scid regressor line, as compared to nude animals in which these tumor grew progressively. Animals challenged with the progressor CD80 non-expressing line B7E3-4scid showed a significant increase in growth in nude mice lacking T-cell responses as compared to growth in wild-type animals, suggesting that an immune response is generated against these CD80 negative cells, but are ineffective in achieving regression. Moreover, T-cell depletion studies (Figure 6) confirmed that CD8+ T-cells may be the subset of T-cells that has an effector role in inducing tumor regression in this model of oral SCC.

In addition, regressing tumors derived from CD80 expressing B6C3-4scid regressor and tumors derived from CD80 non-expressing B7E3-4scid progressor cells were harvested and the presence of CD4+ and CD8+ T-cells was examined by immunohistochemistry. Figure 7 shows significantly higher numbers of CD8+ than CD4+ T-cells in B6C3-4scid regressing tumors; while these T-cell numbers were not significantly different in CD80 non-expressing B7E3-4scid progressor tumors. These findings suggest that HNSCC expressing endogenous CD80 generate effective T-cell cytotoxic (CD8+) immune responses that may play a critical role in tumor regression.

Animals bearing endogenous or transfected CD80 are resistant to rechallenge. To determine whether CD80 induced anti-tumor responses stimulated resistance to rechallenge with
Figure 2. Growth of CD80 non-expressing B6C3-4scid sorted regressor cells from the same clonal lineage as in Figure 1. This figure shows representative tumor growth curves of 5 animals bearing CD80 non-expressing regressor cells. Out of 5 mice inoculated, 3 (60%) showed progressive growth of tumor and 2 (40%, 2 curves at 0 cm growth are superimposed on each other) showed no growth of tumor for up to 80 days after inoculation.

Figure 3. Growth of transfected CD80+, vector and wild-type progressor line B7E3-4scid. Immunocompetent Balb/c mice were challenged with 1x10^7 cells/mouse of either CD80+ transfected cells, vector only transfected cells or wild-type progressor B7E3-4scid cells. This figure shows representative growth curve. Tumor area was determined as the square product of tumor diameters (width x length) in millimeters and expressed as mean ± SEM.
wild-type cells, animals that underwent regression after inoculation with the CD80+ transfected progressor line B7E3-4scid or after inoculation with the vector only transfected line were re-challenged with wild-type B7E3-4scid progressor line. The results showed that 100% of mice had regression of tumor growth by day 20 after inoculation (Figure 8). The regression after being rechallenged with the wild-type progressor line in all the recipients is consistent with development of immunity.

Discussion

The expression and function of tumor-derived endogenous CD80 in inhibiting neoplastic growth of non-hematopoietic tissue during early tumor development has not been defined. Using expanded CD80 expressing and non-expressing cells from recently characterized murine oral SCC lines derived from clonal populations of transformed keratinocytes, we examined the effects of tumor-derived CD80 co-stimulatory molecule expression during tumor development of HNSCC in vivo to determine whether CD80 expression can facilitate immune elimination. Our present studies show that during early tumor formation, substantial CD80 expression on tumor cells induces tumor regression of oral SCC, as evidenced when CD80 non-expressing expanded cells of the wild-type regressor line B6C3-4scid show progressive tumor growth in immunocompetent animals. Cells of the wild-type line B6C3-4scid, which mostly express CD80, have previously and uniformly shown tumor regression when inoculated into immunocompetent mice (14). Additionally, our study shows that tumors formed from the wild-type progressor B7E3-4scid cells transfected with CD80 completely regressed in immunocompetent mice, while the wild-type cell line, with approximately 5% expression of CD80, form tumors that grow progressively in immunocompetent mice. Our results not only provide evidence for the acquisition of growth advantage of oral SCC tumors in the host environment with low or absent CD80 surface expression, but also suggest that endogenous or transfected CD80 when expressed at high density in a tumor population may facilitate regression of early tumor growth.

While our studies show that tumor regression in murine oral SCC was dependent on a large proportion of cells expressing endogenous CD80, these results contrast with other published studies. Wendtner et al. (1997) showed that only a small number of transduced CD80– or CD86 positive human multiple myeloma cells by recombinant adeno-associated virus (as few as 300 CD80 or CD86-transduced cells) are sufficient to induce T- and NK-cell proliferative response (15). Whiteway et al. (2003) in their study of the effect on clinical remission of CD80 and CD86 co-stimulatory molecules on acute myeloid leukemia (AML) blasts showed that AML blasts samples, which had less than 10% of cells expressing CD86 were still able to deliver co-stimulatory signal capable of inducing allogeneic T-cell proliferation and Interleukin-2 production of similar levels to those samples were greater than 60% of AML cells expressed CD86 (16). The above studies used cancer cells derived from hematopoietic cells, which may have an enhanced expression of other co-stimulatory molecules and may exhibit high immunostimulatory ability compared to epithelial cells. Thus, the discrepancy seen between our results and those from the studies above may be related to the histological type of tumor. The outgrowth from the CD80 transfected progressor line B7E3-4scid (re-isolate) and the relatively low expression of CD80 in that line, argues that these cells have escaped immune elimination. Furthermore, these results are in agreement with our recent studies on CD80 expression on tumor specimens from patients with HNSCC. Preliminary results of these studies show only minimal and focal CD80 expression pattern in human specimens of patients with advanced and aggressive HNSCC (data not shown).

The effects of CD80 on tumor growth may be directly tied to its role in the regulation of antigen-specific immune responses (18, 19). Although, co-stimulatory molecules on tumor cells do not influence CTL priming, they enhance primed CTL responses in peripheral solid tumors (20, 21). In our studies, mice that rejected the CD80+ expressing regressor line B6C3-4scid or CD80+ transfected progressor line B7E3-4scid were resistant to rechallenge with the sorted CD80 non-expressing cells of the regressor.
line or with wild-type B7E3-4scid progressor line, and the response to CD80 expression was associated with an increase in CD8+ T lymphocytes within tumor specimens, consistent with the development of an immune response. In addition, in vivo CD8+ T-cell depletion allowed growth of regressor tumors. These findings suggest that sufficient endogenous tumor-derived CD80 may generate an anti-tumor CD8+ T-cell response against murine oral SCC.

Taken together, the data presented here directly implicate a supportive role for CD80 expression in tumor progression of oral SCC and supports the hypothesis that lack of CD80...
co-stimulatory molecule expression facilitates HNSCC escape from immune elimination. Since immunotherapeutic clinical trials using CD80 tumor transfection have shown mixed benefits to local control in patients with carcinoma (22-24), our findings, indeed, may be an indication of the complexity of the system of tumor immune surveillance. Moreover, the stimulatory and inhibitory roles in anti-tumor immunity of more recently described co-stimulatory molecules, such as PD-L1 (25, 26), PD-L2 (27), B7-H3 (28, 29) and B7-H4 (30, 31) is presently being investigated. Certainly, co-stimulatory molecule expression may not be the sole determinant of tumorigenicity and tumor progression in immunocompetent animals, and mechanisms of tumor escape from immune recognition and destruction are likely multiple in HNSCC. Alterations in expression of signal transduction factors, alterations in cell death receptor signaling, loss of tumor antigens and loss or down-regulation of HLA I may be additional mechanisms of escape from immune elimination. However, our study may have potential applications in the immunotherapy of HNSCC using co-stimulatory molecules, as low expression of these molecules on HNSCC may result in inefficient delivery of co-stimulatory signals. Therefore, mechanisms that achieve high transduction efficiency of CD80 co-stimulatory molecule on tumor cells may provide a means for more effective immunotherapy. This study provides additional data to support the adjuvant use of CD80 transfection in the immunotherapy of HNSCC.

References


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