Impairment of T-Cell Activation in Head and Neck Cancer In Situ and In Vitro

Strategies for an Immune Restoration

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Background: The rationale for the study was based on the hypothesis that decreased or absent expression on tumor cells of adhesion molecules, the class I or class II major histocompatibility complex (MHC) molecules, or costimulatory molecules might be responsible, in part, for the poor ability of squamous cell carcinoma of the head and neck (SCCHN) to induce generation of antitumor effector cells in vitro and in vivo.

Objective: To investigate expression of intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function associated antigen-3 (LFA-3) and distribution of the costimulatory molecules, B7.1, B7.2, and CD40, and of class I and class II MHC molecules on SCCHN cells in situ and on SCCHN cell lines.

Setting: University medical centers.

Design: Expression of ICAM-1, LFA-3, MHC molecules, B7.1, B7.2, and CD40 was evaluated in human SCCHN biopsy specimens by immunohistochemistry and on SCCHN cell lines by flow cytometry. To confirm our hypothesis that impaired T-cell activation observed in patients with SCCHN is caused by the absence of costimulatory B7 molecules, a B7-negative SCCHN cell line was transduced with the B7.1 gene, using a retroviral vector, and tested in mixed lymphocyte tumor cocultures.

Results: In contrast to abundant expression of ICAM-1, LFA-3, class I MHC molecules, and CD40, the absence of B7.1, B7.2, and class II MHC molecules on tumor cells was observed in situ and in vitro. Lymphocytes and antigen-presenting cells in inflammatory infiltrates surrounding tumor cell clusters expressed both costimulatory and adhesion molecules. The SCCHN lines negative for B7.1 and class II MHC antigens failed to induce proliferation of T cells in mixed lymphocyte tumor cocultures. However, when these cell lines were transduced with the B7.1 gene, their ability to induce T-cell proliferation in mixed lymphocyte tumor cocultures was restored.

Conclusions: The absence of B7 protein or class II MHC antigen expression on human SCCHN cells is responsible for the failure of these tumors to induce proliferation of T cells in vitro. Transduction of the B7.1 gene into SCCHN restores the ability of the tumor to induce T-cell proliferation in vitro.

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The immune response against neoplastic cells is mediated by humoral as well as cellular effector mechanisms, with T cell-mediated immunity representing an essential component of antitumor responses. Generation of effective antitumor T-cell responses involves at least 3 stages of coordinated cell-to-cell interactions. Thus, adhesion, ie, the ability of lymphocytes to bind to antigen-presenting cells (APC) or tumor targets, is the first and obligatory step in antigen presentation. This is antigen-independent adhesion, mainly mediated by intercellular adhesion molecule-1 (ICAM-1) (CD54) and lymphocyte function associated antigen-3 (LFA-3) (CD58), expressed on APC and their respective counterreceptors, LFA-1 and CD2, expressed on the surface of T lymphocytes. Next, T-cell receptor-mediated recognition of a tumor antigen by T lymphocytes in the context of MHC molecules expressed on the surface of APC takes place. Subsequent proliferation of T cells in response to this antigen requires costimulation, resulting in a signal that is delivered to the nucleus and initiates cell division. In the absence of such signaling, the antigen-MHC interactions lead to T-cell anergy or apoptosis. Among the costimulatory molecules, B7.1 (CD80) and B7.2 (CD86), which are expressed on APC and signal through the T-lymphocyte receptors CD28 and CTLA4, respectively, appear to be mandatory for T-cell activation. Recently, a new molecule,
PATIENTS AND METHODS

PATIENTS AND SAMPLES

The study group consisted of 10 randomly selected patients aged 40 to 78 years (median age, 60 years; 7 men and 3 women) with histologically confirmed diagnosis of an SCCHN. Tumor localization included oral cavity (n = 1), oropharynx (n = 3), hypopharynx (n = 2), and larynx (n = 4). Biopsy samples were obtained during surgical tumor excision, snap frozen in liquid nitrogen, and stored at −20°C for immunohistochemical staining. Sections of the corresponding paraffin-embedded tissues were stained with hematoxylin-eosin for the evaluation of histopathological tumor grading (G2, n = 6; G3, n = 4) and the pTNM classification. Seven patients had an advanced stage of disease (pT3, n = 3; pT4, n = 4) and 3 had an early stage of disease (pT1, n = 1; pT2, n = 2). Histological evaluation of neck specimens revealed the presence of cervical lymph node metastases in 6 patients (pN1, n = 1; pN2, n = 4; pN3, n = 1). Nominonvolved mucosa was used for controls.

TUMOR CELL LINES

The human squamous cell carcinoma cell lines PCI-1 and PCI-13, established from head and neck cancer biopsy specimens, were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, 2-mmol/L L-glutamine, 50-U/mL penicillin G sodium, and 50-µg/mL streptomycin sulfate (all from Gibco, Grand Island, NY). The cell lines were routinely tested for mycoplasma at monthly intervals.

ENRICHMENT IN T LYMPHOCYTES

Peripheral blood mononuclear cells were isolated from heparinized blood of healthy donors on Ficoll-Hypaque gradients (Gibco), washed, and resuspended in complete RPMI 1640 medium. To obtain enriched preparations of peripheral blood T lymphocytes, monocytes and B lymphocytes were removed by adherence to plastic in 225-cm² flasks (Costar, Cambridge, Mass) as previously described.

CD40, which is also expressed on APC, has been identified to be involved in the process of costimulation by signaling through its counterreceptor CD40L, expressed on activated T cells. In addition, ICAM-1 and LFA-3 are now known to mediate costimulatory effects. While ICAM-1 is involved in the initiation of proliferation of antigen-responsive T cells, LFA-3 and B7 facilitate sustained proliferation of antigen-primed T cells.

The aim of the present study was to investigate expression of ICAM-1 and LFA-3 as well as the distribution of the costimulatory molecules, B7.1, B7.2, and CD40, and of class I and class II MHC molecules on squamous cell carcinoma of the head and neck (SCCHN) cells in situ and on SCCHN cell lines. The rationale for the study was based on the hypothesis that an impairment in the complex cascade of T-cell–mediated immunity exists in patients with SCCHN, it might be due to absent or inadequate expression of costimulatory or MHC molecules on tumor cells. Moreover, restoration of T-cell dysfunction would be expected upon transduction into tumor cells of genes encoding the missing protein. Our results indicate that human SCCHN cells do not express B7 proteins or class II MHC antigens and fail to induce proliferation of T cells in vitro. Transduction of the B7.1 gene into SCCHN restores the ability of the tumor to induce T-cell proliferation in vitro.

RETROVIRAL CONSTRUCTS

AND TRANSDUCTION OF TUMOR CELLS WITH THE B7.1 GENE

Expression plasmids encoding human B7.1 complementary DNA (cDNA) were cloned into a DFG-retroviral vector by the University of Pittsburgh Vector Laboratory. This DFG-B7.1-Neo construct also contained the neomycin-resistance gene. To generate appropriate controls, transfectants were prepared using Escherichia coli β-galactosidase cDNA (LaCZ).

Transduction of CD80-negative SCCHN lines with the B7.1 gene was previously described. Briefly, 1 × 10^6 tumor cells (PCI-1 or PCI-13) were plated in 10 mL of medium in 75-cm² flasks (Costar). After 24 hours, culture medium was replaced by 2 mL of the viral supernatant, including 8-µg/mL hexamethylen-bromide (Polybrene). After 4 hours of incubation, an aliquot of 8 mL of fresh medium was added, and selection was started 48 hours later by adding 700-µg/mL Geneticin 418 (Gibco) for a period of at least 2 weeks.

IMMUNOHISTOCHEMISTRY

Antibodies used for immunohistochemistry were as follows: monoclonal antibodies against ICAM-1, LFA-3 (both Boehringer Mannheim Biochemical, Mannheim, Germany); B7.1 or B7.2 (Dianova, Hamburg, Germany); CD40 (Coulter-Immunotech Diagnostics, Hamburg), class I MHC and class II MHC (both Dako, Glostrup, Denmark), and pan-cytokeratin antibody KL1 (Coulter-Immunotech). All monoclonal antibodies were titered on sections of human tonsils to determine the optimal staining dilutions. The ABC (avidin-biotin complex) method was used for staining, except for staining with B7.2 and pan-cytokeratin monoclonal antibodies, where the alkaline anti-alkaline phosphatase (APAAP) technique was used.

Frozen sections (4-µm thick) were prepared on a cryostat at −23°C and mounted onto poly-L-lysine–coated slides. Following fixation in acetone, the endogenous peroxidase activity was suppressed by treating sections in 0.3% hydrogen peroxidase in phosphate-buffered saline, followed by alkaline phosphatase (APAAP) technique was used.

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RESULTS

IMMUNOHISTOCHEMICAL

AND FLOW CYTOMETRY ANALYSES

Expression of ICAM-1

As shown in Figure 1, A, lymphocytes and APC in the stromal infiltrate of tumor specimens were strongly positive for ICAM-1, as detected by immunohistochemistry.
incubation with primary antibodies. After several washings, the sections were treated with a biotinylated rabbit anti-mouse IgG secondary antibody and the avidin-biotin peroxidase complex (Vectastain, Burlingame, Calif). The respective antigens were visualized by means of the peroxidase reaction with 0.01% 3-amino-9-ethylcarbazole as chromogen (Sigma Chemical Co, St Louis, Mo). After counterstaining with Mayer hematoxylin, slides were coverslipped with Kaiser glycerol gelatine (Merck, Darmstadt, Germany). In addition, control sections were stained using rabbit nonimmune serum instead of the specific antibodies (negative controls). As positive controls, sections of human tonsils were stained in parallel with tumor sections.

For the APAAP method, the following modification was used. After pretreatment with normal rabbit serum (Dako) and incubation with the primary antibodies for 30 minutes, sections were washed with TRIS-buffered saline and further incubated with rabbit anti-mouse IgG bridging antibody (Dako). After an additional treatment with mouse APAAP complex (Dako), staining was developed with new-fuchsine, while counterstaining was performed with Mayer hematoxylin. Controls were prepared as outlined above.

For double-staining experiments to simultaneously detect malignant cells (as stained by KL1) and B7-positive cells (lymphocytes and APC), the ABC technique was applied for B7.2, as described above. As chromogenic substrate, 3,3-diaminobenzidine was used. The APAAP method was performed for staining with KL1. Fast red stain (Sigma Chemical Co) was used as a chromogenic substrate.

FLOW CYTOMETRY

Flow cytometry on SCCHN cell lines, PCI-1 and PCI-13, was performed after staining with the following antibodies: anti-B7.1 (CD80), anti-B7.2 (CD86), anti–ICAM-1, anti–LFA-3, anti–class I MHC, anti–class II MHC (HLA-DR and HLA-DQ), and CD40. The antibodies were purchased from Dianova.

For staining of PCI-1 and PCI-13 cell lines, which grow as adherent monolayers, cells were dissociated using 0.05% trypsin-EDTA solution (Sigma Chemical Co) and washed. Tumor cells in suspension were incubated for 30 minutes on ice with the respective antibodies at appropriate dilutions. After 2 washings with phosphate-buffered saline containing 0.1% (wt/vol) sodium azide (Sigma Chemical Co) and 0.1% (vol/vol) fetal calf serum (Gibco), anti-mouse IgG-fluorescein–labeled antibody (Boehringer Mannheim Biochemical) was added, followed by an incubation for 30 minutes on ice in the dark. Cells were washed again and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, Calif) after a final fixation in 0.2% (wt/vol) paraformaldehyde (Sigma Chemical Co). Appropriate controls were performed using isotype-matched monoclonal antibodies (IgG1 and IgG2a; Becton Dickinson).

MIXED LYMPHOCYTE TUMOR COCULTURES

Peripheral blood T lymphocytes (2 × 10^8 per well) were cultured in 96-well flat-bottom microtiter plates (Costar) with 5 × 10^5 irradiated (100 Gy) tumor cells (PCI-13 parental, LacZ transduced, or B7.1 transduced) in complete RPMI 1640 medium (Gibco) supplemented with 1-µg/mL phytohemagglutinin-P (Gibco). After a 5-day incubation, 3.7 × 10^10 μBq-aliquot of tritiated thymidine (New England Nuclear, Boston, Mass; 147.9 GBq/mmol) was added to each well, and the incorporated radioactivity was measured by liquid scintillation counting (Wallac, Gaithersburg, Md) 16 hours later. Effector (peripheral blood T lymphocytes) or stimulator (PCI-13) cell preparations alone served as controls.

STATISTICAL ANALYSIS

The significance between experimental and control groups was analyzed using the Student t test. Differences were considered to be significant at P<.05.

Furthermore, 9 of 10 carcinoma specimens showed a positive membrane staining for ICAM-1, varying from moderate to strong within various tumor areas, especially those localized next to inflammatory cells. There was no ICAM-1 expression detectable in the patients’ mucosa except for interspersed Langerhans cells, as demonstrated by positive staining with the anti-CD1a monoclonal antibody (data not shown).

By flow cytometry, LFA-3 (CD58) was found to be constitutively expressed on the surface of PCI-1 (Figure 2, B) and PCI-13 (data not shown) cells. Staining for LFA-3 was stronger than that for ICAM-1 (Figure 2, A).

Expression of B7.1 and B7.2

All carcinoma cells were found to be negative for B7.1 and B7.2 by immunohistochemistry (Figure 1, C and D). This finding was confirmed by double-staining experiments, using the pancytokeratin antibody KL1 as a selective marker for epithelial cells, in conjunction with anti-B7.2 monoclonal antibody. KL1-positive carcinoma cells exhibited no reactivity for B7.2. This was in contrast to inflammatory infiltrates where a high level of expression for B7.2 on lymphocytes and APC was observed (Figure 1, E). Furthermore, inflammatory cells were positive for B7.1. The uninvolved mucosa was negative for B7, except for interspersed Langerhans cells, as demonstrated by positive staining with the anti-CD1a monoclonal antibody (data not shown).

The absence of B7.1 and B7.2 on carcinoma cells in situ was in accordance with the flow cytometry results,
Figure 1. Immunostaining of squamous cell carcinoma of the head and neck biopsy specimens. A, Carcinoma cells show high levels of intercellular adhesion molecule-1 (ICAM-1) expression (anti–ICAM-1 avidin-biotin peroxidase complex [ABC-PO], original magnification ×400). B, Similar staining pattern for lymphocyte function associated antigen-3 (LFA-3), with intensive staining of both the inflammatory cells and carcinoma cells (arrow) (anti–LFA-3 ABC-PO, original magnification ×200). C and D, Absence of B7.1 (CD80; part C) and B7.2 (CD86; part D) on carcinoma cells (arrow). In contrast, lymphocytes and antigen-presenting cells (APC) exhibit strong positivity for both antigens (anti–B7.1 ABC-PO and anti–B7.2 alkaline anti-alkaline phosphatase [APAAP], original magnification ×400). E, Double staining allows discrimination between B7.2-negative carcinoma cells as detected by pancytokeratin antibody KL1 (red) and CD86 (B7.2)-positive lymphocytes and APC (brown; arrow) (anti–B7.2 ABC-PO and anti–pancytokeratin AAPAP, original magnification ×400). F, Expression of CD40 on APC and on carcinoma cells. The endothelium of blood capillaries, used as an “internal standard” also exhibited a well-delineated and strong positivity (arrow) (anti–CD40 ABC-PO, original magnification ×200). G and H, Expression of class I major histocompatibility complex (MHC) on the surface of carcinoma cells (arrow) and inflammatory cells (part G); class II MHC molecules are only detectable on APC but not on tumor clusters (arrow) (part H) (anti–class I MHC and class II MHC ABC-PO, original magnification ×200).
which showed no B7.1 (CD80) or B7.2 (CD86) reactivity on SCCHN cell lines (Figure 2, C and D).

**Expression of CD40**

Immunohistochemistry showed CD40 expression (Figure 1, F) on the surface of inflammatory cells and on neoplastic cells of all tumor specimens. Similar to ICAM-1 and LFA-3, vascular endothelium also demonstrated strong positivity. In the uninvolved mucosa, only the basal cell layer revealed a weak staining for CD40.

PCI-1 and PCI-13 cell lines were positive for CD40 expression (Figure 2, E).

**Expression of Class I and Class II MHC**

Immunohistochemical analysis of class I MHC on carcinoma cells showed surface expression on 7 of 10 tumors (Figure 1, G). The inflammatory cells also stained positively for this antigen. In contrast, tumor cells were found to be negative for class II MHC, while the inflammatory cells showed strong positivity (Figure 1, H). These results were confirmed by flow cytometry on SCCHN cell lines, which were class I MHC positive and class II MHC negative (Figure 2, F and G).

**FUNCTIONAL STUDIES**

When allogeneic peripheral blood T lymphocytes were coincubated with SCCHN tumor cells (PCI-13), no proliferation of lymphocytes was observed in 5-day cultures (Figure 3). However, peripheral blood T lymphocytes coincubated with B7.1-transduced SCCHN cells showed a significantly increased tritiated thymidine incorporation ($P < .001$) compared with effector cells coincubated with parental or LacZ-transduced tumor cell lines. Thus, in the presence of B7.1, the proliferative capacity of peripheral blood T lymphocytes was completely restored, indicating that expression of B7.1 on tumor cells is necessary for SCCHN-induced proliferation of T cells in vitro.

**COMMENT**

The ability of immune effector cells to recognize and bind to tumor cells is the obligatory first step in the cel-

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**Figure 2.** Expression of adhesion, costimulatory, or major histocompatibility complex (MHC) molecules on human squamous carcinoma cell line PCI-1, as detected by flow cytometry. The cell line PCI-1 was stained either with the fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies of interest (black line) or with the FITC-labeled isotype-matched control monoclonal antibody (gray line). A total of 5000 cells were analyzed for each sample. A, intercellular adhesion molecule-1 (CD54); B, lymphocyte function associated antigen-3 (CD58); C and D, absence of B7.1 (CD80; C) or B7.2 (CD86; D); E, CD40; F, class I MHC; and G, absence of class II MHC (HLA-DR).
et al reported that cytokine-induced enhancement of costimulation for T-cell activation. Moreover, Webb demonstrated that these molecules not only strengthen cell-to-cell interactions but also provide costimulatory signals. The interaction between B7.1, B7.2, and the counterreceptor CD28 has been suggested to play a critical role in preventing the induction of clonal anergy. We therefore investigated expression of B7.1 and B7.2 on tumor cells and in the inflammatory infiltrate. We could not find expression of these costimulatory molecules in situ, as demonstrated by double-staining experiments, or in vitro on SCCHN cell lines. This is in accordance with previous observations demonstrating expression of CD80 and CD86 on APC-like macrophages, dendritic cells, monocytes, and activated T lymphocytes, but not on neoplastic cells from human solid tumors. The importance of B7 molecules for generation of an efficient antitumor immune response was demonstrated recently. Transduction of the B7.1 gene into CD80-negative tumor cell lines converted them into strong stimulators of allogeneic as well as autologous lymphocytes in vitro and in vivo, thus preventing clonal T-cell anergy. To confirm our hypothesis that impaired T-cell activation observed in patients with SCCHN is caused by the absence of costimulatory B7 molecules, we transduced the CD80-negative SCCHN cell line PCI-13 with the B7.1 gene. The B7.1-positive SCCHN cell line was able to mediate costimulatory activity with respect to lymphocyte proliferation. Thus, the failure of SCCHN cell line to elicit an adequate lymphocyte proliferation response could be overcome, at least in vitro, by B7.1-mediated costimulation.

It is noteworthy that many of the costimulatory molecules act cooperatively. Azuma et al demonstrated by transduction of the B7.1 gene that interactions between B7/CD28 and between ICAM-1/LFA-1 are necessary for the optimal generation of cytokytic T lymphocytes. Damle et al reported that the ICAM-1/LFA-1 pathway is required for the optimal CD28-dependent CD4+ T-cell proliferation. Cooperation also exists between ICAM-1/LFA-1 and CD40/CD40L in the costimulation of T-cell proliferation, as demonstrated by Cayabyab et al. Furthermore, the CD40/CD40L pathway is also involved in up-regulating the expression of B7 molecules on APC. The triggering of CD40 on APC cells, binding to CD40L on T cells, permits activation of B cells and cytokine production, thus further activating T cells and allowing their proliferation. Our observations of preserved CD40 expression on carcinoma cells in vitro and in vivo and on APC in situ are in accordance with previous results and, to our knowledge, are reported for the first time for SCCHN.

As adhesion and costimulation provide neither antigen-specific nor MHC-restricted signals, another step in the successful generation of a tumor-specific immune response is mandatory. This involves recognition by T-cell receptor of the tumor antigen presented by MHC molecules. Our results showed a preserved class I MHC expression on the majority of carcinoma specimens in situ as well as on SCCHN cell lines in vitro, and an absence of class II MHC in both experimental settings. In contrast, the inflammatory infiltrate exhibited a strong positivity for both MHC molecules in situ. No correlation was found between the pTNM classification or grading and expression of MHC or adhesion and costimulatory molecules. Mattijsen et al, who reported class I MHC expression in a high percentage of SCCHN tumors (49 of 66 lesions investigated), and class II MHC expression in a minority of SCCHN specimens, did not
find staged-related differences in class I and class II MHC expression. Furthermore, for patients with SCCHN, it has been demonstrated that 75% to 100% of tumor specimens from primary lesions express class I MHC. These observations are in accordance with results reported by Pardoll24 that most epithelial tumors express class I MHC and lack class II MHC.

In conclusion, our data provide an explanation for an impairment in the generation of antitumor immunity in patients with SCCHN. The preserved expression of the adhesion molecules ICAM-1 and LFA-3 allows adhesion of T lymphocytes to tumor cells. However, the absence of class II MHC expression might impair class II MHC–restricted T-lymphocyte responses. Furthermore, the absence of the costimulatory molecules, B7.1 and B7.2, despite normal expression of CD40, additionally limits the ability of SCCHN cells to deliver costimulatory signals to lymphocytes. Transduction of retroviral vectors or expression plasmids encoding human B7.1 cDNA into SCCHN cells completely restores the proliferative response of antigen-specific, tumor-reactive, and antigen-primed CD4+ T lymphocytes.

An improved understanding of interactions between molecules involved in the cascade of T-cell activation and of signaling impairments present in SCCHN might lead to the development of new immunotherapy or gene therapy strategies for head and neck cancer.

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