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,
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 if an impairment in the complex cascade of T-cell–mediated immunity exists in patients with SCCHN, it might be due to absence 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.

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 (AAPAP) technique was used. Following fixation in acetone, the endogenous peroxidase activity was suppressed by treating sections in 0.3% hydrogen peroxidase in phosphate-buffered saline, followed by}

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Expression of LFA-3

Strong surface expression of LFA-3 was detected on mononuclear cells in the cellular infiltrate in situ (Figure 1, B). The vascular endothelium serving as an internal standard and the mucosal epithelium also were intensively stained, as were carcinoma cells, showing LFA-3 positivity in 9 of 10 cases. Similar to ICAM-1 expression, that of LFA-3 varied within the individual tumor and among the different tumor specimens.

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) formaldehyde (Sigma Chemical Co). Appropriate controls were performed using isotype-matched monoclonal antibodies (IgG1 and IgG2a; Becton Dickinson).

MIXED LYMPHOCTYE TUMOR COCULTURES

Peripheral blood T lymphocytes (2 × 10⁵ per well) were cultured in 96-well flat-bottom microtiter plates (Costar) with 5 × 10⁵ 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 × 10¹⁰ Bq–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.

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 panicytokeratin 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,

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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 $\times 400$). 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 $\times 200$). 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 $\times 400$). 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 APAAP, original magnification $\times 400$). 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 $\times 200$). 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 $\times 200$).
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-
lular immune cascade and is mainly mediated by the adhesion molecules ICAM-1 and LFA-3. Recently, it was demonstrated that these molecules not only strengthen cell-to-cell interactions but also provide costimulation for T-cell activation. Moreover, Webb et al reported that cytokine-induced enhancement of ICAM-1 expression resulted in increased susceptibility of melanoma and colon carcinoma cell lines to lysis by monocytes. In the present study, we demonstrated that the majority of SCCHN cells showed strong ICAM-1 expression in situ, especially those cells localized next to inflammatory infiltrates. Squamous cell carcinomas of the head and neck are usually characterized by the presence of abundant inflammatory infiltrates surrounding tumor cell clusters. Proinflammatory cytokines such as interleukin 1, tumor necrosis factor, and interferon γ are able to up-regulate the surface expression of ICAM-1 both on fibroblasts and on SCCHN cell lines. Thus, strong staining for ICAM-1 on tumor cells located next to inflammatory cells might be attributable to such proinflammatory cytokines, especially since its expression on SCCHN tumor cell lines was only moderate, as detected by flow cytometry. The staining pattern for ICAM-1 and LFA-3 in our study was similar to that described by Kornfehl et al, who also found expression of these molecules on endothelial cells, lymphocytes, APC, and carcinoma cells, varying within individual tumors as well as among patients. Compared with ICAM-1, we observed a 3 times higher level of LFA-3 expression on cultured lines in vitro, which contrasted with no significant difference in expression of these molecules in situ. A possible explanation for this observation might be a differential regulation of adhesion molecules in vivo and in vitro due to microenvironmental factors, including cytokines.

Optimal activation of T cells is known to depend on 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 cytolytic 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. Mattijssen 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.23,24 These observations are in accordance with results reported by Pardoll25 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 suppressed lymphocyte proliferation in mixed lymphocyte-tumor cocultures, thus bypassing the absence of class II MHC, CD80, or CD86 (Figure 4).

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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REFERENCES


