Regional Immunosuppression in Esophageal Squamous Cancer

Evidence from Functional Studies with Matched Lymph Nodes[1]

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Although production of immunosuppressive factor(s) by esophageal squamous cancer has been demonstrated, systemic immunosuppression occurs late. Whether local immunosuppression by tumor-derived factors occurs in vivo as a potential mechanism of escape from immune surveillance is unknown. We found that lymphocytes from nodes draining distal esophageal squamous tumors in 23 consecutive patients had depressed proliferative and cytotoxic responsiveness relative to both lymphocytes from a reference node outside the field of drainage and matched PBL from the same patient. In a subset of patients in which more than one tumor-draining node was examined, a radial or zonal immunosuppression relative to the primary tumor was evident. The findings were unrelated to surgery or anatomic location because all but 2 of 26 control patients with esophagogastric adenocarcinoma had normal or enhanced lymphocyte responsiveness in the tumor-draining node. The absence of overt or even micrometastatic nodal disease, as determined by immunostaining for cytokeratin expression, coupled with the long-term survival of several of the patients, strongly suggests that the immunosuppressive effect is due to mechanisms other than metastases, and may be a premetastatic occurrence. We conclude that regional immunesuppression does exist in patients with esophageal squamous cancer when systemic immunity is still well preserved. The local immune suppression inhibits the generation of lymphokine-activated killer (LAK) cells and may be an impediment to potential immunotherapeutic strategies. ***The Journal of Immunology,*** **1996, 157: 4717–4720.**

Escape from immunologic surveillance may account for the progression of some solid tumors in humans. In some instances, this occurs despite the presence of tumor-infiltrating lymphocytes and apparent immune reactivity to the tumor. Several mechanisms by which a tumor may confound the immune response have been described, including the elaboration of immunosuppressive cytokines and other factors (1, 2). An understanding of these escape mechanisms offers the best hope of optimizing immunotherapeutic strategies.

In human esophageal cancer, consistent alterations in the immune system have not been found. Although systemic immunesuppression may accompany esophageal cancer, it is seldom profound, and usually seen only in advanced disease. Malnutrition, advanced age, and radio- or chemotherapy are the most likely explanations. While this might suggest that there is minimal direct interplay between the host response and the tumor, the lack of evidence for tumor-specific immunologic disturbance may be because investigators have looked at the wrong immunologic compartment. The influence of tumors on the immune system might be local or zonal within the field of lymphatic drainage of the tumor. We have previously described a soluble immune suppressor factor that is produced by human esophageal squamous- but not adenocarcinoma (3). To investigate whether this factor is active in vivo and might exert a nonsystemic or regional immunosuppressive effect, we have compared the proliferative and cytotoxic responses of lymphocytes isolated from tumor-draining lymph nodes with those from a reference node outside the field of drainage and with matched PBLs. The results indicate that a profound regional immunosuppression exists in patients with esophageal squamous- but not adenocarcinoma, even though systemic lymphocyte function is well preserved. This localized immune suppression may provide the tumor with a growth and metastatic advantage, but it may also be a potential focus for exploitation and targeting by future immunomodulatory strategies.

Materials and Methods

Study population

The study was approved by the Cork University Hospitals Ethics Committee. The study population consisted of 23 patients undergoing esophageal resection for squamous carcinoma in the distal half of the esophagus (41 to 88 years of age; 11 men, 12 women). Lymph node involvement was found in 21% (5 of 23) and the number of nodes available for histologic review ranged from 1 to 17 (median, 6). The control population consisted of 26 patients undergoing esophagogastrectomy for adenocarcinoma of the esophagogastric region (48 to 78 years of age; 15 men, 11 women), of which 23% had detectable spread of cancer to lymph nodes (2 to 13 nodes were available for histologic review; median, 6). None of the patients with either squamous- or adenocarcinoma had received chemotherapy, immunotherapy, or radiotherapy before surgery.

Lymph node analysis

Lymph nodes were selected for study with careful regard to their anatomical location relative to the primary tumor. Nodes selected for comparative study included a direct tumor-draining node that was separate from and not adherent to the tumor and a distal reference node in a different lymphatic field (from the base of small bowel mesentery or mesocolon, i.e., outside the field of drainage of the tumor). Only nodes considered likely to be free of metastatic deposits on palpation at surgery were selected for immunologic studies. The histology of all nodes was reviewed a second time and in all cases in which there was no evidence of nodal involvement with tumor on conventional hematoxylin and eosin stained sections, additional sections (4 μm) were cut and stained for occult tumor cell deposits. Thus,

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Received for publication July 16, 1996. Accepted for publication August 20, 1996.

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[1] The authors are supported in part by the Health Research Board of Ireland; Forbairt, Ireland; and by the Cancer Research Appeal, Mercy Hospital (CRAM).

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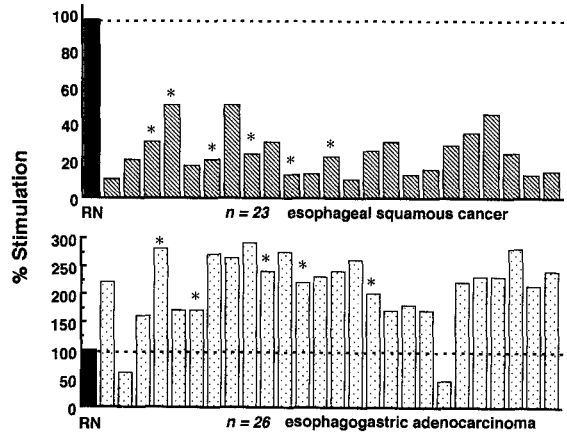

FIGURE 1. Regional suppression of lymphocyte proliferation in patients with esophageal squamous carcinoma. The proliferative response of lymphocytes in the tumor-draining node was compared with that of the reference node (RN) outside the field of drainage of the tumor in each of consecutive patients with esophageal squamous cancer and esophagogastric adenocarcinoma. The proliferative response of the RN was standardized to 100%, and the response of lymphocytes in the tumor-draining node was expressed as a percentage of the matched RN response for each individual patient. Asterisks denote patients known to have survived 5 yr after tumor excision.

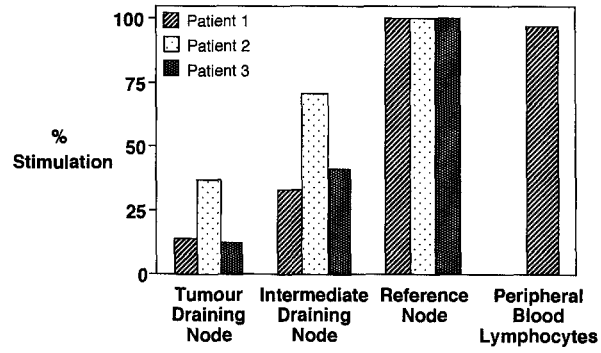

FIGURE 2. Zonal suppression of lymphocyte proliferation in tumor-draining and more distal (intermediate) draining node within field of drainage relative to the reference lymph node that is outside the field of drainage of the tumor.

detection of micrometastases was performed using the pancytokeratin Ab MCF 116 (DAKO Ltd., High Wycombe, Bucks, England) to identify aberrant epithelial cells within lymph node mesenchymal tissue (4, 5). Ab staining was performed at 1/200 dilution for 15 min, and developed using the universal strep-avidin biotin kit (DAKO) and the sections were counterstained with Mayer's hemalum. Samples with cytokeratin-positive staining that were clearly cell-associated and had dysplastic cellular/nuclear morphology were considered to be micrometastases. In all surveys of micrometastases, the pathologist was kept blind to the patient identity and diagnosis.

Cell isolation

Single-cell suspensions were made by gentle extrusion through a 60 μ mesh wire screen and were washed and resuspended in DMEM containing 10% FCS (complete medium). Lymphocytes were isolated by Ficoll-Hypaque density centrifugation (6), and adherent cells were depleted by incubation in plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C in humidified atmosphere containing 5% $C0_2$ in air for 2 h. Viability of lymphocytes determined by trypan blue exclusion was consistently ≥98%. Peripheral blood was collected during anesthesia at time of lymph node harvest and lymphocytes were isolated as for nodal lymphocytes.

Lymphocyte proliferation and cytotoxicity assays

Proliferative responsiveness was assessed by seeding lymphocytes in triplicate conditions in U-shaped 96-well tissue culture plates (Costar) at 2×10^5 per well with 200 μl complete medium alone or medium containing 3 μg/ml PHA (Sigma Chemical Co., St. Louis, MO). The plates were incubated in a 5% CO_2 humidified incubator at 37°C. After 2 days, 1 μCi [^{3}H]thymidine was added to each well and incubation was continued for a further 18 h. Cells were then harvested onto glass fiber filters and macromolecules precipitated with cold 5% TCA. Precipitates were filtered in a minifold microsample filtration unit (Schleicher & Schuell, Dassel, Germany). The dried filters were transferred into scintillation vials containing 10 ml scintillation fluid (Ready Safe, Beckman, Fullerton, CA) and radioactivity was measured in a liquid scintillation counter.

Lymphokine-activated killer (LAK)[3] activity was assessed by incubating lymphocytes in complete medium (1×10^6 per ml) with rIL-2 (1000 U/ml, Cetus Corp., Emeryville, CA) for 5 days at 37°C in humidified atmosphere of 5% CO_2 in air. They were then washed and resuspended in complete medium. The esophageal squamous carcinoma cell line (OC2), generated and described by us elsewhere (7), was used as a target cell in cytotoxicity assays. Targets were seeded in monolayers in flat-bottom 96-well tissue culture plates at 37°C for 24 h, and LAK cells were added in triplicate at varying E:T ratios in 200 μl of medium. After contact for 18 h, medium was aspirated from the wells and the target cells were washed gently with PBS to remove nonadherent cells and lysed cell debris. Effector cells remaining were detached by brief (30 s) treatment with 0.02% EDTA followed by two washes with saline. Surviving target cells were quantitated using protein assay determination. Cells were lysed by adding 50 μl of H_2O followed by two cycles of freeze-thaw. The amount of protein present, which is dependent on the number of surviving cells, was determined by adding 200 μl of 1/5 diluted Bio-Rad protein assay reagent (Bio-Rad laboratories GmbH, Munich, Germany). The absorbence at 600 nm was determined using a Bio-Rad model 2550 enzyme immunoassay plate reader. Percent cytotoxicity determined by this colorimetric assay is calculated as follows:

$$\% \text{ cytoxicity} = 1 - [(\text{OD experimental})/(\text{OD control})] \times 100$$

The OD experimental is defined as the mean absorbence of target wells with added LAK cells and OD control is defined as the mean absorbence of target wells without LAK cells.

Results

The results indicate that lymphocytes from the tumor-draining nodes of each of 23 consecutive patients with esophageal carcinoma had suppressed proliferative responsiveness to PHA when compared with lymphocytes from a reference node from the same individual (Fig. 1). Peripheral lymphocyte responsiveness did not differ from that of the reference node (±10%) in 12 of 12 patients tested. In contrast, in all but 2 of 26 patients with esophagogastric adenocarcinoma, there was normal or enhanced proliferative responsiveness in the tumor-draining node when compared with the matched reference node (Fig. 1).

In three patients with esophageal squamous cancer, a second, more distal lymph node (intermediate draining node) was obtained from within the tumor-draining area, and in each case lymphocyte proliferation was suppressed relative to the reference node but not to the same extent seen in the more proximal tumor draining lymph node (Fig. 2). This suggests a radial zone of immune suppression in relation to the tumor.

Consistent with suppressed lymphocytic proliferative activity of regional nodes in esophageal squamous cancer, there was failure to generate LAK cell activity in the tumor-draining lymph nodes of these patients relative to those from the matched reference node or peripheral lymphocytes from the same patients (Fig. 3). In contrast, the lymphocytes in the tumor-draining nodes of patients with gastroesophageal adenocarcinoma exhibited normal LAK function (63 ± 5%; % cytotoxicity ± SD at E:T ratio of 40:1; $n = 5$) relative to the reference node (56 ± 9%) and the peripheral blood (61 ± 2.5%).

[3] Abbreviations used in this paper: LAK, lymphokine-actived killer; RN, reference node.

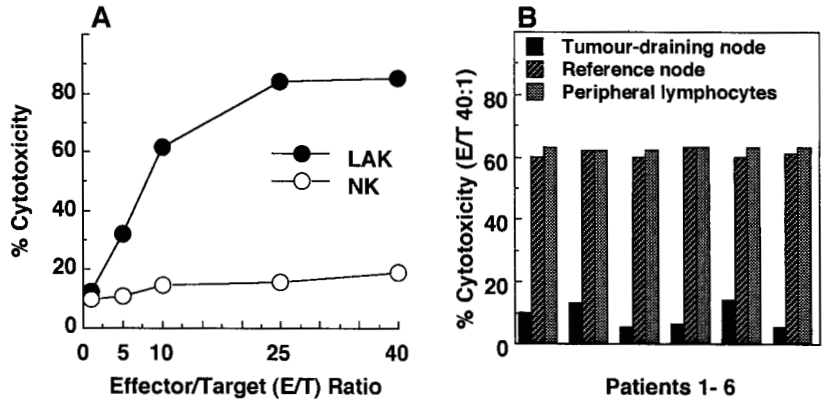

FIGURE 3. Regional suppression of LAK cell activity in patients with esophageal squamous cancer. *A*, Resistance of the esophageal squamous cell line (OC2) to natural killer (NK) and sensitivity to LAK activity were confirmed using normal PBLs that were either untreated (NK) or were preincubated for 5 days in 1000 U/ml IL-2 (LAK), before exposure to the OC2 target cells. *B*, Within each patient, LAK activities of lymphocytes from the tumor-draining node, the reference node, and peripheral blood were compared.

Immunohistochemical analysis for micrometastases (cytokeratin-positive tumor cells) within the resected nodes showed that micrometastases were present in ≥1 node from 6 of the 18 patients with esophageal squamous cancer for whom conventional histology showed no tumor involvement. Micrometastases were absent in 10 of these 18 patients and were indeterminate in 2 because of the presence of coexisting anthracosis. Analysis of nodes from patients with gastroesophageal adenocarcinoma showed that of the 20 patients who had no nodal involvement by conventional histology, 4 had detectable micrometastases and 16 had no evidence of micrometastases.

At present, six of the patients with squamous carcinoma have survived in excess of 5 yr post-excisional surgery. These patients had node-negative disease by conventional histologic criteria and only one was found to have micrometastases on immunohistology for cytokeratin. In each of these patients, the tumor-draining nodes were suppressed relative to the reference node (Fig. 1) and results were similar to the nodes of the remaining patients with squamous cancer. In the patients with adenocarcinoma, there are to date five long term survivors (≥5 yr), one of whom had nodal disease (one positive node with metastases by conventional histology). The remaining four patients were negative for nodal disease by conventional and immunohistologic criteria. The results of this subset of patients were similar to those of the remaining study patients with adenocarcinoma (Fig. 1).

Discussion

The study demonstrates that regional suppression of lymphocyte proliferation in vivo is a consistent finding in patients with distal squamous- but not adenocarcinoma of the esophagus. This cannot be attributed to normal anatomic variation because of our use of adenocarcinoma of the gastroesophageal junction as a disease and anatomic control, nor can it be accounted for by local ischemia or other surgically-induced effect, because surgical manipulation for either histologic type is similar. Furthermore, matching lymph nodes for each patient excludes generalized immunologic variations from systemic influences introduced nonspecifically by malnutrition, senescence, or therapeutic agents. It also reveals that regional immunosuppression exists at a relatively early stage (in patients with operable tumors) when systemic immune function is still well preserved.

In this study, there were six patients with esophageal squamous cancer with node-negative disease confirmed by immunohistochemical analysis for occult deposits (in five cases), with post-excisional survival in excess of 5 yr. Each of these patients had regional immunosuppression in the matched nodes and their results were similar to those of the rest of the squamous cancer population. This strongly suggests that the regional immune suppression is due to mechanisms other than metastases and may be a premetastatic phenomenon.

The concept of a regional immunosuppression in vivo within the zone of influence of human tumors has been raised by others in relation to malignant melanoma and breast cancer (8, 9). The mechanism(s) is uncertain; although production of immunoregulatory products in vitro by human tumor cells, including gastrointestinal malignancies, is well established (1–3, 10, 11), their potential significance in vivo is unclear. We previously reported the production of a soluble factor in vitro by squamous carcinoma of the esophagus that is distinct from known immunosuppressive cytokines (3). The activity of this factor in vitro differs from that described for other tumor-derived immunomodulatory agents in that it is preferential for activated lymphocytes and is irreversible. This irreversibility may account for our findings here, and supports the interpretation that these findings reflect local immunosuppression in vivo. Indeed, the finding of an intermediate level of suppression in the more distal tumor-draining nodes of three patients is further evidence for a local humoral influence on target tissue that would be diluted in the systemic circulation.

In adenocarcinoma of esophagogastric region, lymphocytes from the tumor-draining nodes tended to be hyperresponsive relative to the reference node. This cannot be attributed to metastatic disease and may be a feature of adenocarcinomas of the gastrointestinal tract, because we have observed similar results in a cohort of patients with colonic adenocarcinoma (unpublished observations). The mechanism may be a reflection of reactive hyperplasia that is frequently seen in tumor-draining lymph nodes. However, it is unlikely to confer a prognostic advantage because patients with adenocarcinomas of the esophagogastric region generally have a poor outcome and usually a less favorable prognosis than those with squamous carcinoma of the esophagus (12–14).

Failure to generate significant LAK responses in draining nodal lymphocytes of patients with squamous esophageal cancer is consistent with the inhibited proliferative responsiveness. However, this finding suggests that lymphocytes in the range of influence of esophageal squamous tumors are incapable of generating an activated or lytic ability even in response to systemic therapy with IL-2. This could account, in part, for the failure of LAK/IL-2 therapy in some clinical and experimental protocols (15). Future immunomodulatory strategies including lymphokine therapy will have to take into account the local immunomodulatory properties of certain tumors if they are to achieve optimal success.

In conclusion, esophageal squamous cancer consistently induces regional immune suppression in the absence of metastases. This is evident in patients with relatively early and operable disease. It

appears to be a local and zonal effect occurring before systemic immune suppression. The local immune suppression inhibits the generation of LAK cells and may be an impediment to potential immunotherapeutic strategies.

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