The Role of Immune Effector Cells in Immunotherapy of Head and Neck Cancer

Theresa L. Whiteside, Ph.D.

Immunotherapy with cytokines or cytokine-activated effector cells is a promising therapeutic approach, which has not been extensively evaluated in patients with head and neck cancer (HNC). Preclinical data obtained with human effector cells, both MHC-restricted cytolytic T lymphocytes (CTL) and MHC-nonrestricted natural killer (NK) cells, indicate that it is feasible to generate such cytokine-activated effector cells in patients with advanced HNC. Technology for separation of human effector cells and their culture on a therapeutic scale is available. Purified subsets of lymphocytes derived from autologous peripheral blood, lymph node lymphocytes or tumor-infiltrating cells can be consistently prepared by immunoselection in patients with HNC, evaluated for antitumor efficacy in vitro, using ³¹Cr-release assays, monolayer cultures or tumor spheroids and used for adoptive transfers. Locoregional transfer of such immune cells plus cytokine(s) can effectively inhibit growth of squamous cell carcinoma of the head and neck (SCCHN) xenografts established in nude mice. Human IL-2-activated NK cells (A-NK) have been shown to preferentially enter SCCHN spheroids and kill or inhibit growth of tumor cells in spheroids or in SCCHN xenografts established in nude mice. A-NK cells can be armed in vitro with chimeric monoclonal antibodies selectively reactive with squamous epithelia (e.g., cMAbs E48 or U36) to optimize their targeting to the tumor. In vivo arming with these cMAbs is likely to increase anti-SCCHN responses. Supernatants of A-NK cells cultured in the presence of IL-2 have been shown to arrest SCCHN cells in the G₀/G₁ phase of the cell cycle and to inhibit growth of SCCHN xenografts in nude mice. Efforts to transduce A-NK cells with cytokine genes are in progress. An urgent need exists in head and neck oncology for effective adjuvant therapy, and immunotherapy with immune effector cells should be considered as a feasible therapeutic modality in the adjuvant setting in HNC.

Head and neck cancer (HNC) represents about 6% of all new cases of cancer in the United States according to Cancer Statistics, 1994. Despite attempts at prevention of tobacco use and possibilities for earlier detection, the majority of patients still present with relatively advanced locoregional disease. Survival of patients with HNC has not improved in the last 30 years (50-60% five-year survival), and surgical treatment of HNC results in considerable functional and cosmetic morbidity. Standard therapy has consisted of surgery and/or radiotherapy. A better understanding of the biology of HNC is necessary in order to begin developing new, more effective approaches to therapy in order to improve both survival and quality of life of patients with this type of cancer. In spite of highly sophisticated surgical procedures in use today, the high frequency of recurrence has
been the major problem in HNC. It is likely that immunotherapy, particularly when used in the adjuvant setting, might be able to reduce this frequency and thus, have a positive impact on survival.

In the last several years, a goal of our and many other groups has been to develop an effective strategy for immunotherapy of solid tumors, including HNC. Among a variety of immunotherapies available, that with adoptively-transferred effector cells alone or in combination with cytokines has been of particular interest to us. This type of immunotherapy has not been widely evaluated in HNC, in contrast to rather extensive clinical studies of adoptive immunotherapy (AIT) in patients with metastatic melanoma or renal cell carcinoma (RCC) performed worldwide in recent years. While objective clinical benefits have been induced by the systemic administration of in vitro activated lymphoid effector cells and interleukin 2 (IL2), therapeutic efficacy has been limited, with a low frequency of complete and durable responses and appreciable toxicity associated with high doses of systemically-delivered IL2. It remains unclear why most cancer patients treated with AIT fail to respond completely, if at all. Factors such as the route of delivery, the number and state of activation of transferred effector cells or their ability to migrate in tissue and localize to the tumor are likely to be important for clinical efficacy. To begin testing these hypotheses and to clarify the role of adoptively-transferred immune cells in antitumor responses in situ, our attention has focused on locoregional AIT. Observations made in several animal models of tumor growth indicated that therapeutic effects could be achieved and systemic IL2 toxicity avoided when locally-growing tumors were treated with multiple low doses of IL2 or IL2 plus lymphocytes. These therapeutic effects were generally associated with a significant local host response involving massive accumulations of mononuclear cells and granulocytes, especially, eosinophils. In these animal studies, tumor regression appeared to be mediated through activation of the host immune reactivity, largely due to "lymphokine-activated tumor inhibition" or the LATI phenomenon, in which exogenous cytokines delivered as therapy or released by transferred and/or infiltrating host lymphocytes were responsible for tumor regression.

In man, locoregional AIT can only be used in a limited number of clinical circumstances. Head and neck tumors lend themselves especially well to such intervention, as they are typically confined to regional lymph nodes, contain a network of vessels readily accessible to cytokine or effector cell injections and can be easily observed, measured or biopsied to provide direct evaluation of their progression or regression. Preliminary results from a handful of clinical trials with cytokines in patients with advanced HNC indicate that locoregional cytokine therapy can produce significant responses in such patients. In addition, evidence for up-regulation of both local and systemic immune responses as a result of cytokine therapy has been obtained in these trials. For example, in a phase Ib trial recently completed at the Pittsburgh Cancer Institute, 36 patients with inoperable SCCHN were treated with peritumoral/intralymphatic IL2 given at multiple sites around the tumor daily for 2 weeks. In this dose-escalating trial, we have convincingly demonstrated significant phenotypic and functional changes in lymphocytes at the tumor site and in peripheral blood. Although these changes did not correlate with response, as only two partial responses were observed in this trial, they indicated that immune cells of patients with advanced HNC can be activated in vivo as a result of immunotherapy. Thus, up-regulation of antitumor activities by NK and LAK cells and increased proportions of CD25+ and HLA-DR+ T cells as well as CD3-CD56+ NK cells were found in fresh tumor-infiltrating lymphocytes (TIL) isolated from post-treatment tumor biopsies, as compared to TIL from pretreatment biopsies. Evidence was also obtained for the presence of numerous lymphoid cells expressing cytokine gene tran-
Table 1 Characteristics of the human A-NK cell subset

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3&lt;sup&gt;-&lt;/sup&gt;CD56&lt;sup&gt;dim&lt;/sup&gt;CD16&lt;sup&gt;dim&lt;/sup&gt; or β&lt;sub&gt;2&lt;/sub&gt; integrin&lt;sup&gt;hi&lt;/sup&gt;IL2R-β&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Rapid IL2-induced adherence to plastic or biologic substrates</td>
</tr>
<tr>
<td></td>
<td>Extensive proliferation in the presence of IL2</td>
</tr>
<tr>
<td></td>
<td>Preferential cytotoxicity against tumor cells in spheroids</td>
</tr>
<tr>
<td></td>
<td>Able to enter and accumulate in solid tissues</td>
</tr>
<tr>
<td></td>
<td>Produce and secrete a variety of cytokines</td>
</tr>
<tr>
<td></td>
<td>Efficiently mediate antitumor effects in vivo</td>
</tr>
</tbody>
</table>

scripts, as determined by in situ hybridization, in post-treatment biopsies only. In general, cytokine-mediated activation of effector cells in situ, together with direct antitumor or antiproliferative effects of cytokines on SCCHN, might be some of the mechanisms responsible for clinically beneficial results of immunotherapies. Also, loco-regional delivery of cytokines might lead to alterations of the tumor susceptibility to effector cells, as a result of changes in expression of, e.g., HLA antigens or adhesion molecules on the tumor surface or antigens on endothelial cells in the tumor vasculature.

In terms of locoregional transfer of effector cells, in addition to cytokines, it is expected that greater antitumor effects can be achieved with AIT. Not only can the transferred antitumor effector cells serve themselves as a source of cytokines, but they can also eliminate tumor cells. The expectation is based on preclinical in vivo studies. Specifically, using a nude mouse model of human SCCHN established in our laboratory, we have demonstrated complete inhibition of tumor growth by IL2-activated human NK cells, but not by IL2 alone, injected peritumorally to animals with 3-day or 7-day established tumors. These promising experimental results suggest feasibility and greater effectiveness of AIT than therapy with IL2 alone.

Searching for antitumor effector cells that are highly effective in AIT, we have focused attention on two subsets of human lymphocytes: A-NK cells and SCCHN-specific cytolytic T lymphocytes (CTL). The former are a small subset of peripheral blood NK cells (5-30%), which have the ability to rapidly adhere to solid surfaces in response to IL2 (22 nM) and which have been named by us Activated/Adherent NK or A-NK cells. After selection by adherence, A-NK cells proliferate rapidly in the presence of IL2, produce high levels of various cytokines and mediate antitumor effects in vitro against SCCHN and other tumor cell lines and in vivo in xenograft models of tumor growth or metastasis. Table 1 lists various characteristics of A-NK cells, emphasizing those that might be especially useful for preparation and use of this effector cell population for AIT. Based on A-NK cell attributes and their presence in various solid tissues, including tumors, we hypothesize that they represent a solid-tissue seeking subset of NK cells, which is responsible for antitumor activities against SCCHN. To test this hypothesis, we determined the potential of normal human peripheral blood A-NK cells to infiltrate SCCHN spheroids and to kill tumor cells as well as to inhibit tumor growth in vivo in a xenograft model of SCCHN established in nude mice. A-NK cells were separated from nonadherent NK (NA-NK) cells by adherence after 5 h incubation in the presence of IL2 of NK cells purified from peripheral blood by negative immunoselection on magnetic beads (≥95% CD3<sup>-</sup>CD56<sup>-</sup>CD16<sup>-</sup>). Both subsets of NK cells were cultured for 14d in 22 nM of IL2 and then compared for antitumor effects. Using multicellular tumor spheroids of two SCCHN cell lines (PCI-1 and PCI-50), we demonstrated by confocal microscopy that fluorescently-labeled A-NK cells infiltrated the spheroids significantly better than did...
Table 2 Entry of radiolabeled A-NK cells, NA-NK cells and PCI-50-specific CTL into PCI-50 SCCHN spheroids

<table>
<thead>
<tr>
<th>Time of co-incubation</th>
<th>Effectors</th>
<th>% of effector cells in spheroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Individual spheroids</td>
</tr>
<tr>
<td>2 h</td>
<td>A-NK</td>
<td>39, 29, 28, 23</td>
</tr>
<tr>
<td></td>
<td>NA-NK</td>
<td>13, 9, 5, 4</td>
</tr>
<tr>
<td></td>
<td>CTL</td>
<td>1.7, 1.5, 1.4</td>
</tr>
<tr>
<td>5 h</td>
<td>A-NK</td>
<td>95, 43, 14, 11</td>
</tr>
<tr>
<td></td>
<td>NA-NK</td>
<td>25, 18, 14, 14</td>
</tr>
<tr>
<td></td>
<td>CTL</td>
<td>14, 10, 10, 6</td>
</tr>
</tbody>
</table>

a) $^{51}$Cr-labeled effector cells were co-incubated with PCI-50 spheroids, and the radioactivity associated with the spheroids was determined. Two representative experiments of 5 performed. PCI are SCCHN cell lines established at the Pittsburgh Cancer Institute. A-NK and NA-NK cells are subsets of human IL2-activated NK cells. CTL is a T cell line specific for PCI-50.

NA-NK cells. Also, the $^{51}$Cr-labeled A-NK cells accumulated inside the spheroids better than NA-NK cells or CTL (Table 2). These findings suggest that A-NK cells preferentially have the ability to enter into multicellular tissue-like formations. We have also shown that A-NK cells are better able to lyse SCCHN spheroids than NA-NK cells (Fig. 1).

To explain the excellent ability of the A-NK cell subset to enter solid tissues, we postulated that their enzymatic activities may play an essential role in this function. Indeed, we have preliminary evidence that A-NK cells can produce collagenase-like activity, urokinase and tissue-plasminogen activator as well as degrade gelatin. Also, bestatin, an inhibitor of ectoaminopeptidases, was able to block entry of A-NK cells into PCI-50 spheroids (data not shown). These observations support our hypothesis that A-NK...
Table 3  Human A-NK cells kill SCCHN targets by direct cell-to-cell mediated necrosis, direct cell-to-cell induced apoptosis or through soluble factor-induced apoptosisa).

<table>
<thead>
<tr>
<th>Targets</th>
<th>Effectors</th>
<th>51Cr</th>
<th>3H-TdR</th>
<th>MTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCI-1</td>
<td>Untreated</td>
<td>LU</td>
<td>6583</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Fixed</td>
<td>LU</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
<td>%</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>PCI-50</td>
<td>Untreated</td>
<td>LU</td>
<td>6612</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Fixed</td>
<td>LU</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
<td>%</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

*a) Cytotoxicity of A-NK cells, which were either untreated or fixed with 1% paraformaldehyde or supernatants of A-NK cells was tested in various assays. Data for effector cells are LU20/10^7 effectors based on % of specific lysis in 4 different E:T ratios each in 6 replicates. Results for supernatant (1/10 dilution) are mean % specific lysis in 6 replicates. 51Cr = 4 h 51Cr-release assay39); 3H-TdR = 1 h thymidine-release assay38); MTT = 24 h MTT assay.40)

Cells abundantly produce certain proteolytic enzymes, which are likely to facilitate their entry and function in solid tumor tissues. The reasons for this superior antitumor activity mediated by A-NK cells against CA spheroids, and the mechanisms utilized by these cells in elimination of tumor cell targets, have been under intense scrutiny in our laboratory. We find that A-NK cells might be able to utilize multiple cytolytic mechanisms, including perforin-mediated lysis, apoptosis dependent on cell-to-cell contact and apoptosis mediated by soluble products (cytokines) released from effector cells. These observations were made by measuring cytotoxic activity of untreated and fixed A-NK cells as well as their culture SN against SCCHN targets in three different cytotoxicity assays: 4 h 51Cr-release assays (presumably measuring perforin-mediated lysis or necrosis); 1 h [3H]-Tdr-release assays (measuring DNA fragmentation or apoptosis); and 24 h MTT assays (measuring cytostasis as well as cell death). Our results showed that fixed A-NK cells mediated activity only in [3H]-Tdr-release and MTT assays, while untreated A-NK cells had antitumor cytotoxicity in all three assays (Table 3). In addition, while paraformaldehyde (PF)-fixed A-NK cells or their SN had greater cytotoxic activity against various CA targets in [3H]-Tdr and MTT assays than NA-NK cells, the latter were more effective than A-NK cells in lysing the same target cells in 51Cr-release assays (data not shown). Together, these findings indicate that in addition to perforin-mediated lysis, A-NK cells utilize at least two other killing pathways, one direct, presumably mediated by cell membrane-bound cytokines, and the other indirect, presumably mediated by secreted cytotoxic molecules. Both of these newly defined cytotoxic mechanisms seem to be apoptotic and might involve the TNF family of surface-associated molecules.20)

The second population of effector cells we have targeted for further studies is the CTL line reactive with HLA-compatible SCCHN cells.21,22) This T-lymphocyte line (CD3+ CD8+, MHC class I-restricted) was established from peripheral blood of a patient with SCC of the tongue.21) This T-cell line was specific for antigens expressed by AuTu and several allogeneic SCCHN lines and contained at least two different clones of cytolytic T cells by the T-cell receptor Vβ analysis (Vβ6+ and Vβ2+). In order to be able to investigate...
the nature of shared antigens recognized by these T cells on SCCHN targets or use this CTL in AIT experiments, an attempt was made to immortalize the T-cell line by infection with Herpesvirus saimiri, a lymphoma-inducing virus of non-human primates. The immortalization studies were conducted by Drs. Muller-Fleckenstein and B. Fleckenstein at the Institute of Molecular Virology, Erlangen, Germany. Following infection, the T cells have been cultured in the presence of 300 IU/ml of interleukin 2 (IL2) and 300 U/ml of IL4 for over 12 months without restimulation with AuTu. In contrast, the parent cells required frequent AuTu restimulation and stopped growing by 20 weeks. In the parental T cell line, containing 90% of CD8+ and 10% CD4+ cells at week 10-12 of culture, the ratio of CD4+/CD8+ cells shifted to 1 by week 20. The immortalized T cells were CD3+CD4+ (100%) and the expression of this phenotype remained stable in culture. These immortalized T cells mediated AuTu cytotoxicity and retained the same specificity of lysis as the parental line. They did not kill K562 or Daudi targets. However, AuTu cytotoxicity mediated by the immortalized CD4+ cells was not inhibited by mAbs specific for CD3, CD4 or MHC-class II molecules. This cytotoxicity tended to reach a plateau at the 10:1 effector-to-target cell ratio in 4 h 51Cr-release assays, as shown in Figure 2A. By comparing activities of CD4+

Fig. 2 Cytotoxicity of immortalized CD4+ T cells or parental CTL against AuTu. Cytotoxicity was measured in 4 h 51Cr-release assays using PCI-50 monolayers labeled in situ with 51Cr (A). In (B), DNA fragmentation and lysis of PCI-50 cells induced by immortalized T cells. [3H]-thymidine or 51Cr-labeled PCI-50 cells were co-cultured with immortalized T cells at the E: T ratio of 40:1 for different times before harvest. For DNA fragmentation assay, 3H] PCI-50 cells were frozen and thawed three times and harvested with automated cell harvester. Activity of 3H incorporated in unfragmented DNA or of 51Cr released in culture medium was measured in a beta-counter. DNA fragmentation was calculated as:

\[
\text{% DNA fragmentation} = \left(1 - \frac{\text{mean experimental cpm}}{\text{mean control cpm}}\right) \times 100
\]

51Cr-release assays were performed as described by us earlier.39
Table 4  Spontaneous and AuTu-induced cytokine production by PCI-50 (SCCHN)-specific immortalized CD4+ T lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>GM-CSF</th>
<th>IL2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>1</td>
<td>&lt;20</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>Immortalized CD4+</td>
<td>118</td>
<td>&lt;20</td>
<td>&gt;400</td>
<td>&lt;6</td>
</tr>
<tr>
<td><strong>AuTu induced:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AuTu alone</td>
<td>&lt;6</td>
<td>ND</td>
<td>ND</td>
<td>&lt;6</td>
</tr>
<tr>
<td>Immortalized CD4+</td>
<td>316</td>
<td>ND</td>
<td>ND</td>
<td>11,000</td>
</tr>
</tbody>
</table>

*a)Parent or immortalized CD4 T cells (1×10⁶) were incubated in AIM-V medium alone for 2d or in the presence of irradiated AuTu (PCI-50) monolayers. Supernatants were harvested and assayed for cytokines by ELISA. The data are expressed in pg/ml.

b)These assays are in progress.

t in [3H]-TdR assays for DNA fragmentation and ⁴⁰Cr-release assays with PCI-50 monolayers over a 5 h period, we obtained evidence indicating that these effector cells were able to induce apoptosis in AuTu targets, as evidenced by rapid DNA fragmentation observed to occur within 1 h of co-incubation (Fig. 2B). In addition, CD4+ T cells spontaneously produced considerably higher levels of interferon-γ (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) than the parental line. Upon stimulation with AuTu, these CD4+ T cells produced significantly more IFN-γ and IL2 than before stimulation (Table 4). These data suggest that the immortalized CD4+ T cells have the TH1 phenotype. Co-incubation with irradiated AuTu monolayers promoted proliferation of the parental as well as immortalized CD4+ T cells (data not shown). The parent CD8+ T cells proliferated significantly better (up to 200% better by [3H]-TdR incorporation) when cocultured with the immortalized CD4+ cells. The requirements of these cells for co-stimulatory signals is under investigation, and their ability to recognize the same panel of SCCHN targets as the parent should facilitate studies of T cell responses specific for SCCHN-associated antigens. Very recently, an immortalized CD8+ T cell line was derived from the original CTL by Drs. Fleckenstein and is being characterized in our laboratory. The availability of both CD4+ and CD8+ SCCHN-specific immortal T-cell lines opens an exciting possibility of future studies aimed at dissecting the role of AuTu-specific CD4+ and CD8+ T cells in anti-SCCHN responses.

In a series of in vivo experiments, we evaluated effects of peritumoral AIT with effector cells on growth of SCCHN in the nude mouse xenograft model. Human A-NK cells, which are currently available in large numbers for AIT were utilized as effectors in these experiments, one of which is summarized in Table 5. Antitumor effectiveness of A-NK vs. NA-NK cells was compared in vivo. Immunosuppressed nude mice with subcutaneous 7d established tumors were treated as follows: The first group of mice received daily peritumoral injections of 0.1 ml PBS for 3d (controls). The second group of mice received daily peritumoral injections of 6,000 IU IL2 for 3d. The third group of mice was injected peritumorally once with 5×10⁶ NA-NK cells, followed by daily peritumoral injections of IL2 alone for 3d. The fourth group received one peritumoral injection of 5×10⁶ A-NK cells, followed by daily injections of IL2 for 3d. The tumor size was measured 2 times per week for the next 4 weeks, using calipers. The results shown in Table 5 are
products of two diameters (in mm) of tumors measured on day 20 after the start of treatment. Tumor sizes in animals treated with PBS, IL2 or NA-NK cells and IL2 were not significantly different. In contrast, tumor sizes in mice injected with A-NK cells plus IL2 were significantly smaller than those injected with PBS ($p<0.001$), IL2 ($p<0.001$) or NA-NK cells plus IL2 ($p<0.004$). These in vivo experiments show that A-NK cells and IL2 are more effective than either IL2 alone or NA-NK in inhibiting growth of SCCHN in vivo.

The experiments described above confirmed that locoregional delivery of A-NK cells to established tumors results in substantial therapeutic benefits. Similar in vivo experiments with the SCCHN-specific immortalized T cells are now in progress. It remains unproven, however, whether the delivery of effector cells, as described above, is adequate for meeting the goals of AIT with lymphoid effector cells and IL2. In order to achieve the goals of increasing the number and maintain the state of activation of these cells in tumor tissue, of eliminating most, if not all, tumor cells and thereby improving survival of the host, it might be necessary to optimize AIT. The recent availability of chemically- or genetically-modified antibodies introduces the possibility that a combination of mAbs and effector cells, such as A-NK cells, might be therapeutically more beneficial than either of these biological agents alone. Chimeric human/murine mAbs specific for antigens on human carcinomas have been successfully used for arming of LAK cells, which were then transferred to SCID mice bearing established liver metastases of human colon carcinoma.\(^\text{24}\) In our hands, anti-carcinoma chimeric mAbs were able to arm human NK or A-NK cells for antibody-dependent cellular cytotoxicity against NK-resistant SCCHN targets.\(^\text{25}\) In SCID mice, tumor regression could be induced by bispecific mAbs that were able to target T cells (anti-CD3 specificity) to the tumor expressing an antigen recognized by the bispecific mAb (anti-tumor specificity).\(^\text{26}\) Considering this type of strategy for future AIT, we have evaluated in vitro bispecific mAbs recognizing the CD16 antigen on A-NK cells and CD33 antigen on HL60 leukemia cells.\(^\text{27}\) The presence of the bispecific mAb significantly up-regulated cytotoxicity of these effector cells against usually quite resistant leukemia cells. Similar approaches are being developed for SCCHN targets. We expect that therapeutic use in xenograft tumor models, and subsequently in patients with cancer, of effector cells armed with mAbs or pre-treated with a bispecific mAb might lead to more effective inhibition of tumor growth than that mediated by A-NK cells and IL2.

Another strategy aimed at improving AIT that is being implemented in our laboratory is transduction of the IL2 gene into SCCHN. The rationale for this goal has been provided by our earlier observations that: (a) A-NK cells as well as SCCHN-specific CTL

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### Table 5 Effects of peritumoral therapy with human A-NK or NA-NK cells and IL2 on growth of subcutaneous human SCCHN established in immunosuppressed nude mice\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Individual tumors</th>
<th>Mean±SEM</th>
<th>% of control</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>48, 45, 35, 35</td>
<td>41±3</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>IL2</td>
<td>48, 35, 35, 33</td>
<td>36±3</td>
<td>88</td>
<td>-</td>
</tr>
<tr>
<td>NA-NK + IL2</td>
<td>56, 48, 45, 25, 16</td>
<td>38±2</td>
<td>93</td>
<td>-</td>
</tr>
<tr>
<td>A-NK + IL2</td>
<td>15, 6, 4, 0, 0</td>
<td>5±3</td>
<td>12</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)See text for experimental details.
are strictly IL2-dependent, and their antitumor activities are compromised in the absence of IL2; and (b) in view of the presence of IL2R on SCCHN, it seems likely that an autocrine pathway of tumor growth regulation may be induced in such transfectants, resulting in a stronger growth inhibitory signal in the presence of tumor-derived IL2. Production of IL2 by SCCHN transfectants might up-regulate expression of IL2R and thus, increase tumor cell sensitivity to growth inhibitory effect of IL2.

Using an episomal expression vector pREP4 (Invitrogen Inc., San Diego, CA), we have recently transduced two SCCHN cell lines and the HR cell line with the human IL2 gene (a 528 bp DNA fragment obtained from the plasmid PBCII/RSV/T by digestion with restriction enzymes BamHI and HindIII), selected transduced tumor cells for hygromycin resistance and shown by immunostaining that they contain intracytoplasmic IL2 and by RT-PCR that mRNA for IL2 is present in the cells (Fig.3). These transduced tumor cell lines secreted minimal levels of IL2, however, as determined by IL2-specific ELISA. Transductions with the DFG-IL2 retroviral supernatant are in progress, and IL2 production by transiently induced PCI-1 or PCI-50 cells (tested 48 h after transduction) was in the range of 1-2 ng/ml/10^6 cells/48 h, as compared to none for non-transduced cultures. We expect that these tumor cell transfectants will be able to form tumors in nude mice and to produce IL2 at the desired concentrations in vivo. This strategy has been successfully used by others in animal models of tumor growth.

In considering the reasons for in vivo effectiveness of human A-NK cells in AIT of SCCHN in our xenograft model, it might be important to remember that interactions between tumors and effector cells, whether endogenous, that is, present in the tumor, or adoptively-transferred into the tumor, are complex. Convincing evidence exists for the ability of SCCHN to suppress immune cells through the release of soluble factors such as PGE_2 or p15E. Also, immune effector cells in the peripheral blood of patients with HNC have been reported to be functionally suppressed. On the other hand, we have reported that SCCHN can induce activation of human resting NK cells or up-regulate activation of A-NK cells in vitro and in vivo in the xenograft model, as evidenced by expression of surface activation markers and of mRNAs for several cytokines. In vivo, SCCHN might be able to regulate not only the state of activation but also proliferation of certain subsets of effector cells by means of soluble factors, one of which has been recently purified and, in part, characterized in our laboratory. This SCCHN-derived factor has been shown to
Table 6  Future approaches to AIT in HNC

1. Adjuvant locoregional therapy
2. Combination with other therapies to treat residual disease
3. Combination with SCCHN-specific mAbs to arm effector cells
4. Genetically-modified effector cells to change tumor microenvironment
5. Generation of immunologic memory (vaccination) to induce tumor-specific endogenous effector cells

have growth-promoting effects on several different human hematopoietic cell lines of myeloid, T, B or NK cell origin. Since adoptively-transferred A-NK cells might thus encounter activating or growth-promoting signals upon encounter with the tumor in situ, it is quite likely that they will be induced to secrete soluble factors with potent growth-inhibitory effects on the tumor. Supernatants (SN) of A-NK cells have been shown to inhibit proliferation of SCCHN in vitro or in vivo in nude mice. In addition to IL2, these SN were shown to contain IFN-γ, TNF-α, and other cytokines, and the mixture proved to be able to arrest SCCHN targets in the G0/G1 phase of the cell cycle, alter expression of MHC-class I and adhesion molecules on SCCHN cells and increase sensitivity of SCCHN cells to lysis mediated by CTL but not by NK cells. Interestingly, all of the above effects on SCCHN targets were also mediated by exogenous IL2 alone, but to a lesser degree than by SN of A-NK cells. Of course, IL2 is one of the most prominent components of the mixture of factors present in this SN. In addition, however, we have demonstrated earlier that receptors for IL2 (IL2R) are present on SCCHN and other human carcinomas and that binding of IL2 to its receptor on SCCHN cells induces a proliferation-inhibitory signal(s) and results in the other target-cell alterations listed above. In aggregate, our findings suggest that cytokines and soluble factors produced by activated effector cells bind to SCCHN cells by specific receptors and are able to regulate tumor cell growth.

In the SCCHN xenograft model established in our laboratory, and another xenograft model of gastric CA metastatic to liver, we have had an opportunity to deliver antitumor effector cells and IL2 to the tumor milieu, observe their movement in the tumor, determine functional properties of these cells and of the tumor cells in situ and, most important, relate these observations to tumor regression. This preclinical model has proven to be useful in obtaining a better understanding of the SCCHN biology and the type of effector-tumor cell interactions that might determine therapeutic efficacy of AIT. In the future, lessons learned from this model and other basic studies are likely to contribute to a more rational design of clinical trials in patients with HNC. Table 6 is a list of options that are now open or will shortly become opened to clinicians treating patients with HNC. The knowledge acquired through preclinical, basic studies can now begin to be applied to clinical studies in order to improve therapeutic efficacy for patients with HNC.

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