The Soluble α Chain of Interleukin-15 Receptor: A Proinflammatory Molecule Associated with Tumor Progression in Head and Neck Cancer

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Abstract

Interleukin (IL)-15 is a proinflammatory cytokine, as it induces the production of inflammatory cytokines [IL-6, tumor necrosis factor α (TNFα), IL-17, etc.]. A correlation between high intratumoral IL-15 concentrations and poor clinical outcome in lung and head and neck cancer patients has been recently reported. The purpose of this study was to investigate the role of the soluble α chain of IL-15 receptor (sIL-15Rα), a natural regulator of IL-15, in head and neck cancer. Fifty-three newly diagnosed untreated head and neck cancer patients were included in this study. Quantification of sIL-15Rα was performed with a newly developed RIA. Increased serum sIL-15Rα concentrations were found in head and neck cancer patients and were closely correlated with poor clinical outcome both in terms of locoregional control and survival even on multivariate analysis. sIL-15Rα was mainly produced by tumor cells via proteolytic cleavage of IL-15Rα mediated by ADAM-17. A correlation was observed between ADAM-17 expression in tumor cells and serum sIL-15Rα concentrations. Surprisingly, sIL-15Rα did not act *in vitro* as an IL-15 antagonist but rather as an enhancer of IL-15–induced proinflammatory cytokines (IL-6, TNFα, and IL-17) that may promote tumor progression. This new tumor evasion mechanism based on amplification of the intratumoral inflammatory reaction is probably not restricted to head and neck cancer, as other tumors have been shown to release sIL-15Rα. Overall, these results support for the first time an original protumor role of sIL-15Rα in cancer. [Cancer Res 2008;68(10):3907–14]

Introduction

Interleukin (IL)-15 is considered to be a proinflammatory cytokine, as it induces the production of cytokines [IL-6, tumor necrosis factor α (TNFα), IL-17, etc.], which play a role in various inflammatory processes (1–3). IL-15 is also a critical cytokine for *in vivo* T-cell survival and function (4). It promotes the survival of $CD8^+$ T cells by protecting them from apoptosis (5, 6). IL-15 binds to the IL-15 receptor (IL-15R) α chain with high affinity, and this chain, together with the IL-2 receptor (IL-2R) β chain and the IL-2Rγ chain subunits, constitutes a trimeric receptor for IL-15. IL-15Rα can present IL-15 in *trans* to cells that express IL-2/IL-15Rβ and IL-15Rγ but not IL-15Rα (7). In preclinical models, IL-15 displays antitumor activity mainly mediated by $CD8^+$ T cells and natural killer (NK) cells (8, 9). In mice bearing leukemia, IL-15 abrogated tolerance of endogenous $CD8^+$ T cells and the rescued cells became effective in treating leukemia (10). IL-15 can also improve the *in vivo* antitumor activity of adoptively transferred $CD8^+$ T cells (11). In contrast, in other murine models, chronic stimulation of the IL-15 pathway led to the development of hematologic malignancies (12, 13). In humans, recent clinical studies reported a correlation between high intratumoral IL-15 concentrations and poor clinical outcome in patients with lung or head and neck cancers (14, 15). Head and neck cancer is heavily infiltrated by inflammatory immune cells (16). Head and neck cancer patients with increased serum concentrations of proinflammatory cytokines (IL-6, etc.), some of them regulated by IL-15, during the course of the disease have a poor survival (17). Molecules with the ability to inhibit the production of these cytokines improve the natural history of head and neck cancer (18). Because IL-15 is expressed in head and neck cancer and is associated with poor prognosis in these patients, we looked for the presence of specific regulator of IL-15 that may help to better understand its activity with the aim to modulate its function. We focused on soluble IL-15Rα (sIL-15Rα) receptor because of the recent description of a natural soluble form of this receptor in humans and mice (19–21). In some situations, sIL-15Rα behaves as an antagonist of IL-15 activity (19, 22, 23). We found increased concentrations of sIL-15Rα in the serum of head and neck cancer patients, which were highly correlated with poor clinical outcome. Interestingly, sIL-15Rα did not act as an IL-15 antagonist but rather as an enhancer of IL-15–induced proinflammatory cytokines that may promote tumor progression. Overall, these results support, for the first time, an original role of sIL-15Rα in inflammation and cancer.

Materials and Methods

Patients. Fifty-three newly diagnosed untreated patients with primary histologically proven head and neck squamous cell carcinoma (HNSCC) were included in this prospective study. Patient characteristics are

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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presented in Supplementary Table S1. Each patient's disease was staged according to the fifth edition of the International Union Against Cancer/American Joint Committee on Cancer system for head and neck cancer. Treatment modalities consisted of surgery, alone or combined with radiotherapy and chemotherapy. This study was conducted in accordance with French laws and after approval by the local ethics committee.

Cell lines and blood cells. The Fadu, SCC9, Cal27, and A253 cell lines derived from HNSCCs were obtained from the American Type Culture Collection. Cells were cultured in DMEM-F12 medium (Invitrogen) supplemented with L-glutamine. All cells were grown in 5% CO_2 at 37°C in a water-saturated atmosphere. Blood cells were collected from anonymous healthy donors at the Etablissement Français du Sang (EFS; Rungis, France) as buffy coat preparations after informed consent and following EFS guidelines. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich). $CD8^+$ T cells were purified by positive selection using CD8-conjugated MACS beads (Miltenyi Biotec). The purified cells were composed of 90% to 95% $CD8^+$ T cells.

Recombinant proteins and chemical reagents. IL-15 and recombinant extracellular domain of human IL-15Rα lacking the domain encoded by exon 3 and linked to human Fc were purchased from R&D Systems. Recombinant sIL-15Rα comprising the complete extracellular domain of IL-15Rα was produced as follows. The plasmid corresponding to the extracellular form of IL-15Rα (pFB R15s) was generated by PCR using the sense primer 5′-CGGGATCCCAGTCCAGCGGTGTCCTGTGG (nested restriction site underlined) and the antisense primer 5′-CTAGAGCGGC-CGCTTACACAGTGGTGTCGCTGT. After amplification, the sequence was ligated between the *Bam*H1 and *Not*I sites of the pFast Bac baculovirus expression vector (Invitrogen) and controlled (Genome Express). A sequence coding for the Flag epitope and Factor Xa binding site (DYKDDDDKIEGR) was added between the endogenous signal peptide and the coding sequence. The expression cassette was recombined in baculovirus DNA using the Bac to Bac expression system (Invitrogen). The recombinant baculoviruses were used to infect SF9 cells and sIL-15Rα was expressed in the SF900 II medium and harvested 3 d after infection. For purification, the pH of the medium was increased to 8.5 with NaOH, and the medium was centrifuged at 10,000 × *g* for 15 min and filtered through a 0.45-nm filter. The medium was concentrated and changed to 50 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl in an Amicon chamber equipped with a 10,000 molecular weight cutoff membrane. The soluble receptor was purified on an anti-Flag-agarose affinity column (Sigma-Aldrich) and eluted with 0.1 mol/L glycine-HCl (pH 3.5). The purified protein was concentrated and the buffer was changed to PBS in Vivaspin (Sartorius). The purity of the protein was checked on Protein 200 Plus LabChip (Agilent Technologies) and Coomassie Blue–stained gel (Supplementary Fig. S1), and the concentration was determined by bicinchoninic acid (Pierce). Endotoxin levels determined by the limulus amebocyte lysate method were <1.0 endotoxin unit/μg recombinant protein. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich.

RIA for quantification of sIL-15Rα. Quantification of sIL-15Rα was performed as previously described (19). Briefly, a sandwich RIA was set up in which the polyclonal goat anti-human IL-15Rα antibody AF247 (R&D Systems) was used as capture antibody and radioiodinated monoclonal anti-human IL-15Rα antibody M161, kindly provided by GenMab, was used as tracer. A purified recombinant sIL-15Rα fusion protein as described above (24) was used as standard. AF247 was coated (5 μg/mL; 50 μL/well) onto high-adsorption Nunc MaxiSorp plates (breakable strips), distributed by Fisher Bioblock Scientific. Wells were saturated with 0.5% PBS-BSA for 15 min and sIL-15Rα–containing samples (50 μL/well) were incubated for 1 h at 4°C. The M161 monoclonal antibody (mAb), iodinated using the Iodogen method, was then added (1 nmol/L; 50 μL/well) for 1 h at 4°C. Supernatants of each well were collected and wells were washed twice with PBS. The radioactivity associated with the wells (bound M161 fraction) and contained in the supernatants and washings (unbound M161 fraction) was determined. This assay measures with the same sensitivity, the sIL-15Rα comprising the complete extracellular domain or deleted from the domain encoded by exon 3 of IL-15Rα. The present assay measures sIL-15Rα receptor not bound to IL-15 (19).

Induction of cytokines. Cells were incubated in 24-well sterile plates with various combinations of cytokines and/or sIL-15Rα. Supernatants were collected 24 or 72 h after stimulation. Cytokines were then measured by ELISA according to the manufacturer's recommendations. IL-6 and TNFα ELISA were obtained from Diaclone and IL-17 ELISA was purchased from Biosource.

Flow cytometry. Cells diluted in 0.1% PBS-azide were incubated for 30 min at 4°C with 5 μg/mL goat anti-IL-15Rα (R&D Systems) or isotype-matched control mAb. They were then washed twice with PBS-azide (0.1%) and incubated for 30 min at 4°C with 3 μg/mL biotinylated rabbit anti-goat antibodies (DakoCytomation). After washings, cells were incubated for 30 min at 4°C with phycoerythrin-labeled streptavidin (DakoCytomation) and then analyzed on a FACSCalibur fluorocytometer (BD Biosciences). Data were acquired and analyzed with CellQuest software.

Immunofluorescence staining. Tissue samples obtained before any treatment at initial endoscopy or surgery were immediately frozen and stored at −80°C. Frozen specimens were sectioned at 4 to 6 μm with a cryostat, placed on slides, air dried, and fixed for 10 min with 100% acetone. Before incubation with primary antibodies, the slides were treated with avidin/biotin blocker (Vector Laboratories) and Fc receptor was blocked by human serum (5%). The antibodies used for the various immunofluorescence stainings are described in Supplementary Table S2.

The various antibodies were diluted in PBS. Isotype-matched antibodies were used as negative controls. In each case, we checked that the secondary antibodies did not cross-react with the isotype or species of the other primary antibody immunoglobulin in the double immunofluorescence technique. Fluorescent images of mounted sections were analyzed with an epifluorescent microscope DMR (Leica Microsystems). Two authors (C.B. and E.T.), blinded for clinical data, independently scored the slides in at least five fields using a 40× objective.

Reverse transcription-PCR amplification. Total cellular RNAs were extracted using the Tri-reagent kit from Ambion (Applied Biosystems). Two micrograms of total cellular RNAs were reverse transcribed with oligo(dT) primers using the first-strand cDNA synthesis kit (Roche Molecular Biochemicals). PCR was performed as previously described (25). The following oligonucleotides were used: β-actin, TCGTCGACAACGGC-TCCGGCATGTGC (sense) and TTCTCCAGGGAGGAGCTGGAAGCAGC (antisense); ADAM-17, ACCTGAAGAGCTTGTTCATCGAG (sense) and CCATGAAGTGTTCCGATAGATGTC (antisense); hypoxanthine-guanine phosphoribosyltransferase (HGPRT), CCTTGGTCAGGCAGTATAATCC (sense) and TTGTATTTTGCTTTTCCAGTTTCAC (antisense); IL-15, GGA-TTTACCGTGGCTTTGAGTAATGAG (sense) and ACATTTGGACAATATG-TACAAAA (antisense); IL-6, ACGAATTCACAAACAAATTCGGTACA (sense) and CATCTAGATTCTTTGCCTTTTTCTGC (antisense); TNFα, GCGAATT-CCCTCCTGGCCAATGGCGTGG (sense) and CTAAGCTTGGGTTCC-GACCCTAAGCCCCC (antisense). Two couples of IL-15Rα primers were selected. IL-15Rα sense 1: CATGTCCGTGGAACACGCAG (hybridizing to cDNA sequence encoded by exon 2, nucleotides 189–209); IL-15Rα antisense 1: AGGAGAGACACAGCGCTCAG (hybridizing to cDNA sequence encoded by exon 6, nucleotides 733–753); IL-15Rα sense 2: GAGACCCTGCCCTGG-TTCACCA (hybridizing to cDNA sequence encoded by exon 3, nucleotides 366–387); IL-15Rα antisense 2: TCATAGGTGGTGAGAGCAGT (hybridizing to cDNA sequences encoded by exon 7, nucleotides 867–886). This last couple of primers was designed to detect alternative splicing of transmembrane exon 6, which may encode sIL-15Rα receptor as previously described for other soluble cytokine receptors.

IL-15:IL-15Rα cross-linking. sIL-15Rα was purified and concentrated from supernatants of tumor cell lines by affinity purification using the anti-IL-15Rα mAb M161, as previously described (19). Samples (recombinant IL-15Rα or purified sIL-15Rα from supernatants) were incubated with iodinated IL-15 (2 nmol/L), cross-linked with the homobifunctional cross-linker EGS (Sigma-Aldrich), and subjected to 10% SDS-PAGE and autoradiography (PhosphoImager 445 SI, Molecular Dynamics, Inc.).

Statistical analysis. Survival variables were estimated using the Kaplan-Meier method and compared by the log-rank test for categorical variables

and using the Cox model and the associated Wald χ^2 statistic for quantitative variables.

Multivariate analysis using the Cox proportional hazard model determined the influence of each variable, when adjusted to others, on locoregional control (relapse-free survival) and overall survival. The level of significance was set at $P \leq 0.05$.

Overall survival was defined as the time from initial diagnosis until death or until last follow-up (right censored data).

Locoregional control was calculated from the end of treatment and defined as the absence or either persistent or recurrent disease at the primary site or in the cervical lymph nodes. Patients with persistent disease at the end of treatment were considered to have experienced failure at time zero. Patients with no signs of relapse were censored at the time of last follow-up or death. The median follow-up for the whole population was 24 mo.

The Mann-Whitney *U* test was also used to assess whether samples of observations came from the same distribution.

Results

Increased serum sIL-15Rα levels in patients with head and neck cancers. A significant increase in the concentrations of sIL-15Rα was observed in serum samples collected from head and neck cancer patients at diagnosis before therapy compared with those of healthy donors (Fig. 1). Mean concentrations of sIL-15Rα were 5.8 pmol/L for head and neck cancer patients and 0.35 pmol/L for healthy donors. sIL-15Rα was detected in serum in 35 of 53 (66%) head and neck cancer patients versus 15% (6 of 40) in healthy subjects ($P < 0.0001$; Fig. 1). As expected, we did not detect IL-15 in the serum of these patients, as free IL-15 is rarely detectable in biological fluids.

Prognostic value of serum sIL-15Rα level on the locoregional control and overall survival rates in HNSCC patients. To assess the clinical significance of sIL-15Rα dysregulation in these cancer patients, the prognostic value of serum sL-15Rα levels was determined. Serum sIL-15Rα levels were significantly correlated with both locoregional control and survival. The locoregional control rate at 12 months was 78% for head and neck patients with no detectable serum sIL-15Rα versus only 48% for cancer patients with detectable sIL-15Rα ($P = 0.04$; Fig. 2*A*). When considered as a continuous variable, serum sIL-15Rα levels were also negatively correlated with locoregional control ($P = 0.01$; Fig. 2*B*). In addition, serum sIL-15Rα levels were positively correlated with shorter survival when considered as a continuous variable ($P = 0.003$; Fig. 2*C*). The 12-month survival rate in patients with no serum sIL-15Rα levels was 89% versus 69% for patients with detectable sIL-15Rα levels ($P = 0.04$; Fig. 2*D*). Multivariate analysis, including tumor stage, tumor-node-metastasis (TNM) grading, and serum sIL-2Rα levels considered as a powerful prognosis marker (26), showed that the only significant prognostic factor independently related to locoregional control was the serum sIL-15Rα level ($P = 0.0065$; relative risk, 1.078). On Cox multivariate analysis, only serum sIL-15Rα levels influenced overall survival probability ($P = 0.0059$; relative risk, 1.086; Supplementary Table S3).

Head and neck tumor cells secrete sIL-15Rα and express membrane IL-15Rα. The difference in sIL-15Rα concentrations observed between cancer patients and healthy subjects led us to assess whether sIL-15Rα may originate from tumor cells. We showed that, *in vitro*, tumor cell lines derived from HNSCC produced high levels of sIL-15Rα (Fig. 3). This secretion is increased by PMA-ionomycin (Supplementary Fig. S2). In contrast with other tumors (27), these cells did not secrete or express membrane-bound IL-15 (data not shown). In contrast, statistically significant lower levels of sIL-15Rα were secreted by resting PBMC from healthy donors compared with tumor cells ($P < 0.01$; Fig. 3). Because it has been shown that murine sIL-15Rα is generated by cleavage of membrane IL-15Rα (19, 20), we analyzed expression of the IL-15R by these tumor cell lines *in vitro* and directly *in situ* in biopsies from head and neck cancer patients.

In a first series of experiments, we showed that tumor cell lines expressed two transcripts of IL-15Rα mRNA (Fig. 4*A*). These PCR products were eluted and sequenced. The 564-bp PCR product corresponded to the wild-type isoform comprising the full ectodomain of IL-15Rα. The 464-bp PCR product was derived from an isoform with deletion of the exon 3 of IL-15Rα mRNA (Fig. 4*A*). The same isoforms of IL-15Rα were present in PBMC (Fig. 4*A*). Characterization of the sIL-15Rα protein showed that the wild-type isoform not deleted from domain encoded by exon 3 was the predominant form detected in the supernatant of these tumor cells (Supplementary Fig. S3*A*).

Fluorescence-activated cell sorting analysis confirmed the membrane expression of IL-15Rα by these tumor cells

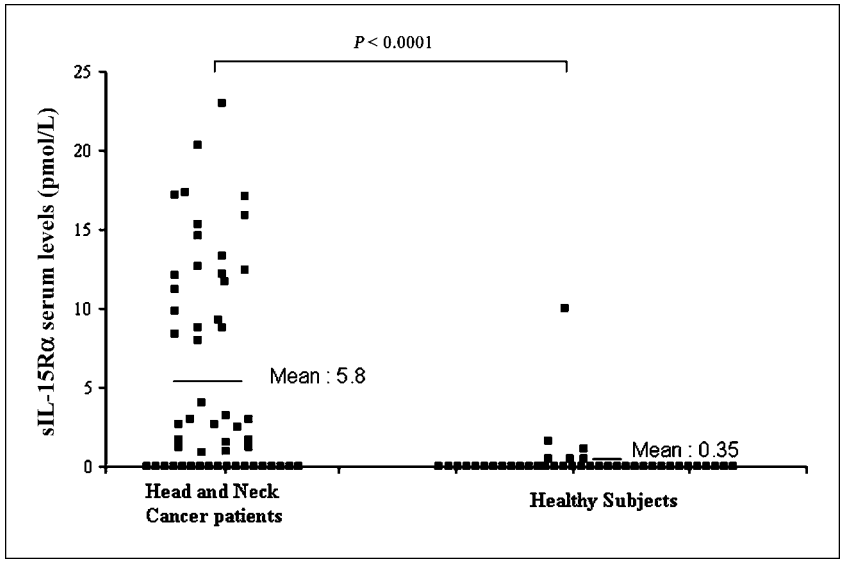

Figure 1. Increased serum levels of sIL-15Rα in patients with head and neck cancers. sIL-15Rα concentrations were determined using a RIA assay in serum samples from 53 head and neck cancer patients collected at diagnosis before the start of therapy. Forty serum samples from healthy donors were included as controls.

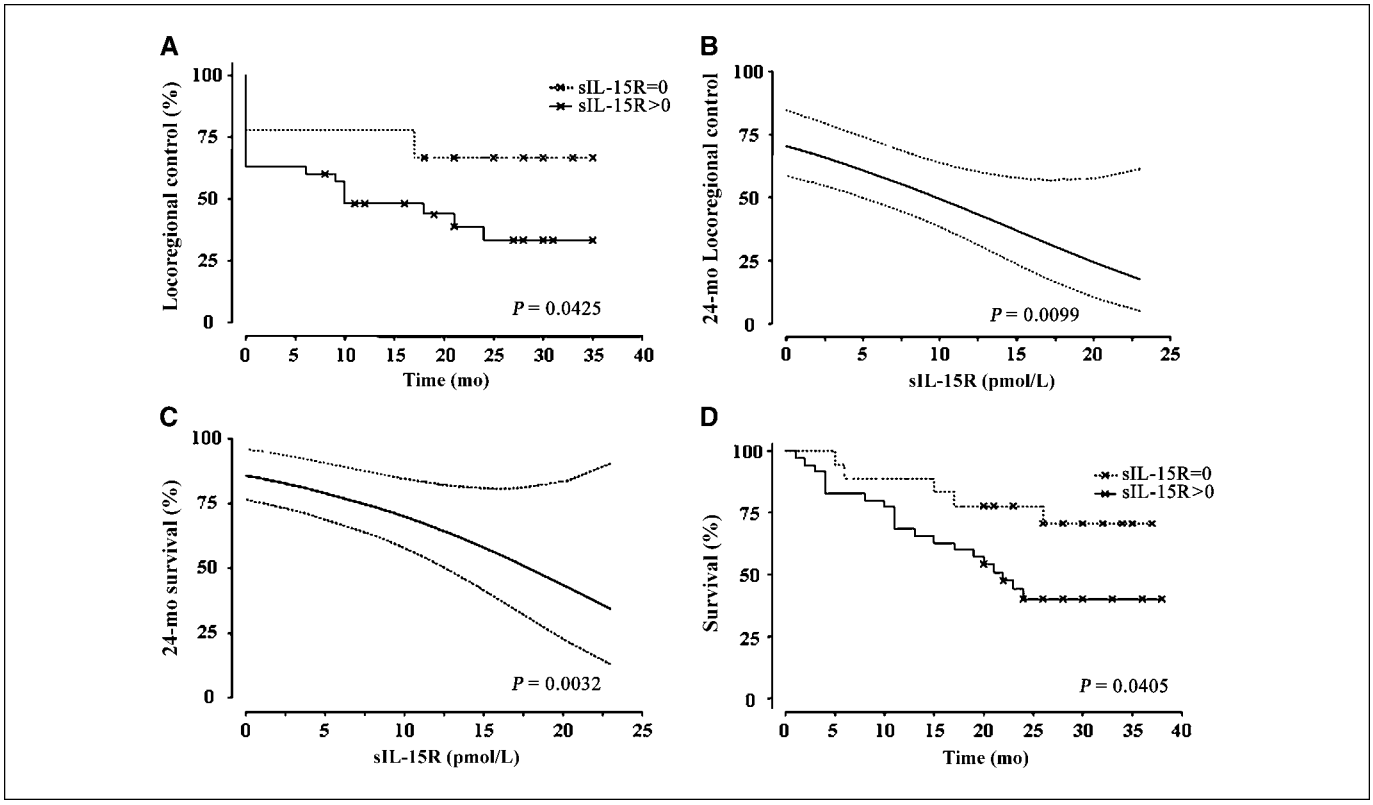

Figure 2. Locoregional control and survival at 24 mo for patients with HNSCC in relation to their serum sIL-15Rα levels. *A* and *B,* relationships between locoregional control and serum sIL-15Rα levels. *C* and *D,* correlation between survival and serum sIL-15Rα levels. *B* and *C, dashed lines,* upper and lower 95% confidence interval.

(Fig. 4*B*). To assess the *in vivo* relevance of these results, tissues from head and neck cancer patients or normal mucosae were stained with anti-IL-15Rα antibodies. Tumor cells were clearly labeled with anti-IL-15Rα antibodies (Fig. 4*C, left*). This expression of IL-15Rα was found in 17 of 48 tumors tested. In contrast, normal epithelium did not significantly express IL-15Rα (Fig. 4*C, right*). It should be noted that isolated cells with a macrophage morphology in both tumor stroma and normal mucosa also seemed to express IL-15Rα (Fig. 4*C*).

As it has been reported that ADAM-17 was responsible for the cleavage of IL-15Rα (20), we wondered whether tumor cells expressed this metalloproteinase. Reverse transcription-PCR analysis showed mRNA expression of ADAM-17 in all three head and neck tumor cell lines tested (Fig. 5*A*). Double immunofluorescence analysis on fresh biopsies of head and neck cancers extended this result to cancer tissues. Tumor cells were clearly costained with anti-AE1/AE3 antibodies (Fig. 5*B, middle* and *right*), a pancytokeratin marker used to identify tumor cells, and anti-ADAM-17 (Fig. 5*B, left* and *right*).

Using primers overlapping the transmembrane exon to track putative alternative splicing of this exon to generate mRNA encoding sIL-15Rα, no specific mRNA for sIL-15Rα was detected (Supplementary Fig. S2*B*). In addition, as previously shown in murine models (19, 20), ADAM-17 small interfering RNA (siRNA) partially inhibited the release of sIL-15Rα in supernatants of head and neck tumor cell lines (Supplementary Fig. S4). Overall, these results support previous data obtained in murine model that sIL-15Rα released by tumor cells is produced by cleavage of IL-15Rα mediated by ADAM-17 (20).

In addition, a significant correlation was found between ADAM-17 expression in tumors and serum sIL-15Rα levels, as the mean serum sIL-15Rα concentration was 1.35 pmol/L when ADAM-17 was expressed by <50% of tumor cells, whereas this concentration increased to 8.65 pmol/L in patients with high tumor expression of ADAM-17 (Supplementary Table S4). No correlation was found between serum sIL-15Rα concentration and IL-15Rα expression by

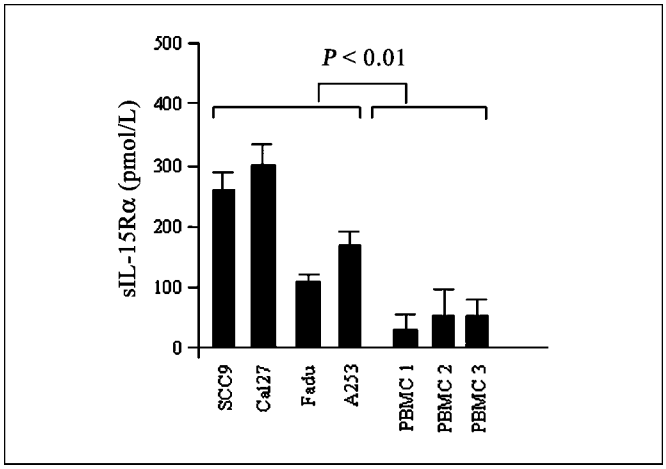

Figure 3. Head and neck cancer cell lines produced high levels of sIL-15Rα. Head and neck cancer cells (SCC9, Cal27, Fadu, and A253) and three PBMCs derived from healthy donors were cultured for 24 h. Supernatants were then collected and sIL-15Rα was measured by a RIA assay. All measurements were performed in duplicate and each experiment was performed twice.

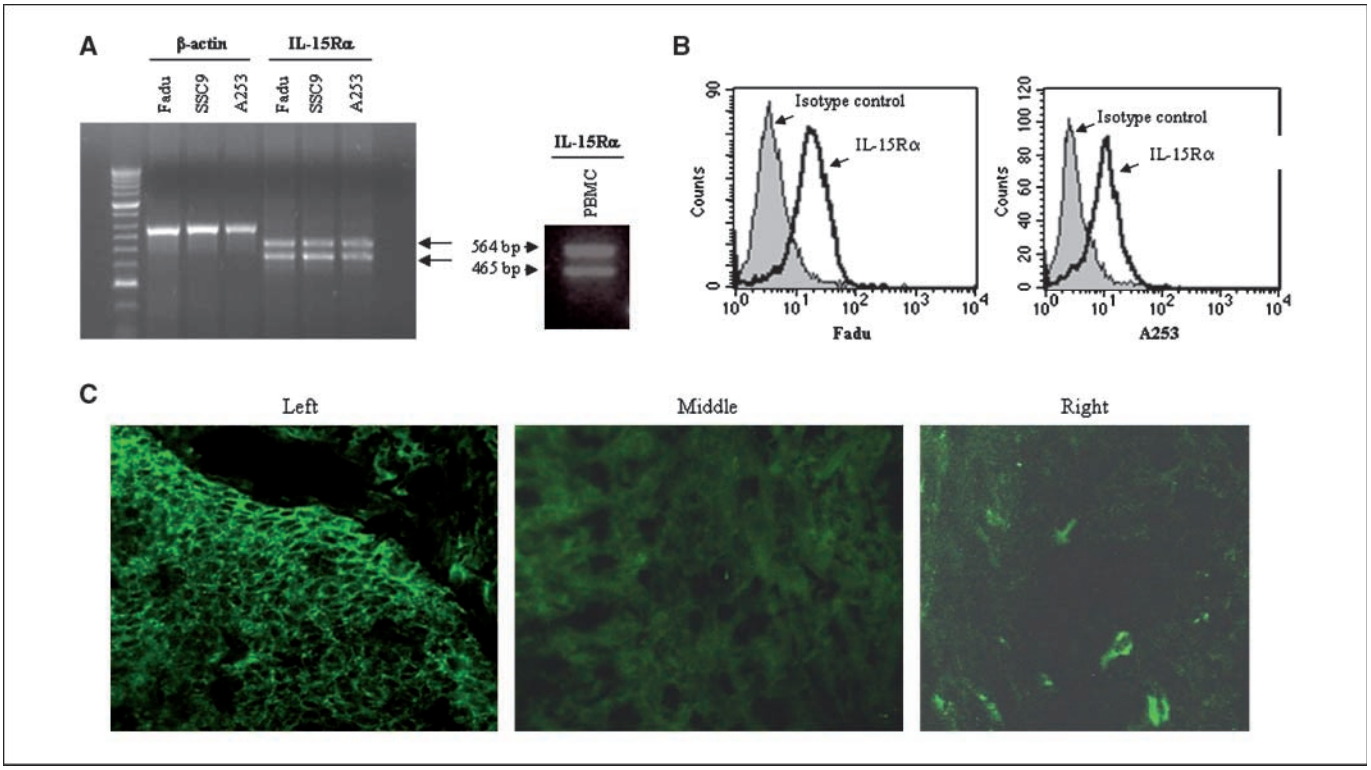

Figure 4. Tumor cells from head and neck patients express IL-15Rα. *A,* three cDNA derived from mRNA extracted from three head and neck cell lines (Fadu, SCC9, and A253) or PBMC were amplified by PCR using oligonucleotide primers specific for β-actin and IL-15Rα. Amplified PCR products were loaded onto a 2% agarose gel and stained with ethidium bromide for UV visualization. *B,* two head and neck cancer cell lines (Fadu and A253) were stained with goat anti-IL-15Rα polyclonal antibody. Polyclonal isotype control antibodies were included in each experiment. The labeling was analyzed by fluorescence-activated cell sorter. *C,* frozen tissues derived from head and neck cancer biopsies (*left* and *middle*) or normal head and neck mucosa (*right*) were stained with primary goat antibodies against IL-15Rα (*left* and *right*) or normal isotype control (*middle*). The reaction was revealed with biotinylated rabbit anti-goat IgG and then CyTM2-labeled streptavidin.

tumor cells (data not shown). These results therefore suggest that ADAM-17 is a critical factor for the release of sIL-15Rα *in vivo*.

sIL-15Rα increased IL-15 activity on the production of proinflammatory cytokines. As two sIL-15Rα isoforms were expressed by tumor cells, although one of them was predominant at the protein level (Fig. 4; Supplementary Fig. S3*A*), the role of these two isoforms on the modulation of IL-15 activity was tested *in vitro* in various models. IL-15 is known to play a major role on $CD8^+$ T-cell survival and homeostasis, which may enhance their antitumor activity (28). Unexpectedly, both isoforms were found to increase IL-15–induced proliferation of purified $CD8^+$ T cells (Fig. 6*A*). This potentiation of the activity of IL-15 was seen at various doses of IL-15 ranging from 10 to 100 ng/mL (Fig. 6*A*; data not shown). No effect of sIL-15Rα alone on $CD8^+$ T-cell proliferation was recorded (data not shown). To check for the specificity of the action of sIL-15Rα, these soluble IL-15Rs were shown not to have any activity on IL-2–dependent $CD8^+$ T-cell proliferation (Fig. 6*A*).

IL-15 has been reported to increase the production of several proinflammatory cytokines, such as IL-6 and TNFα (1, 2). Low doses of IL-15 up to 10 ng/mL were not efficient to induce significant levels of IL-6 and TNFα by human PBMC and high doses of IL-15 (100 ng/mL) were required to elicit large amounts of IL-6

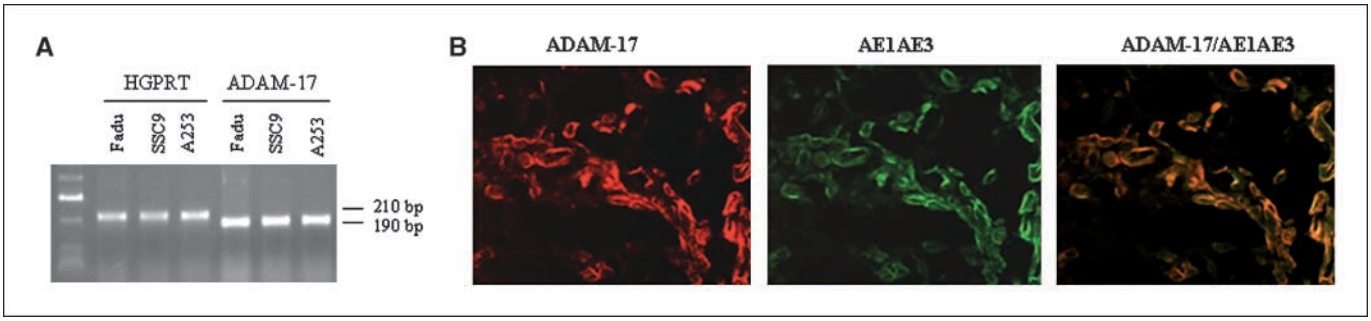

Figure 5. Expression of ADAM-17 by head and neck cancer cells. *A,* cDNA derived from mRNA extracted from three head and neck cancer cell lines were amplified by primers specific for ADAM-17 and HGPRT. Amplified PCR products were loaded onto a 2% agarose gel and stained with ethidium bromide for UV visualization. *B,* tissues derived from head and neck cancer specimens were stained with antibodies to human ADAM-17 and pancytokeratin AE1AE3. *Left* and *middle,* results of simple immunofluorescence acquisition with each antibody; *right,* double immunofluorescence staining. *Yellow,* colocalization between the two markers recognized by the specific antibodies. Isotype control antibodies gave no staining.

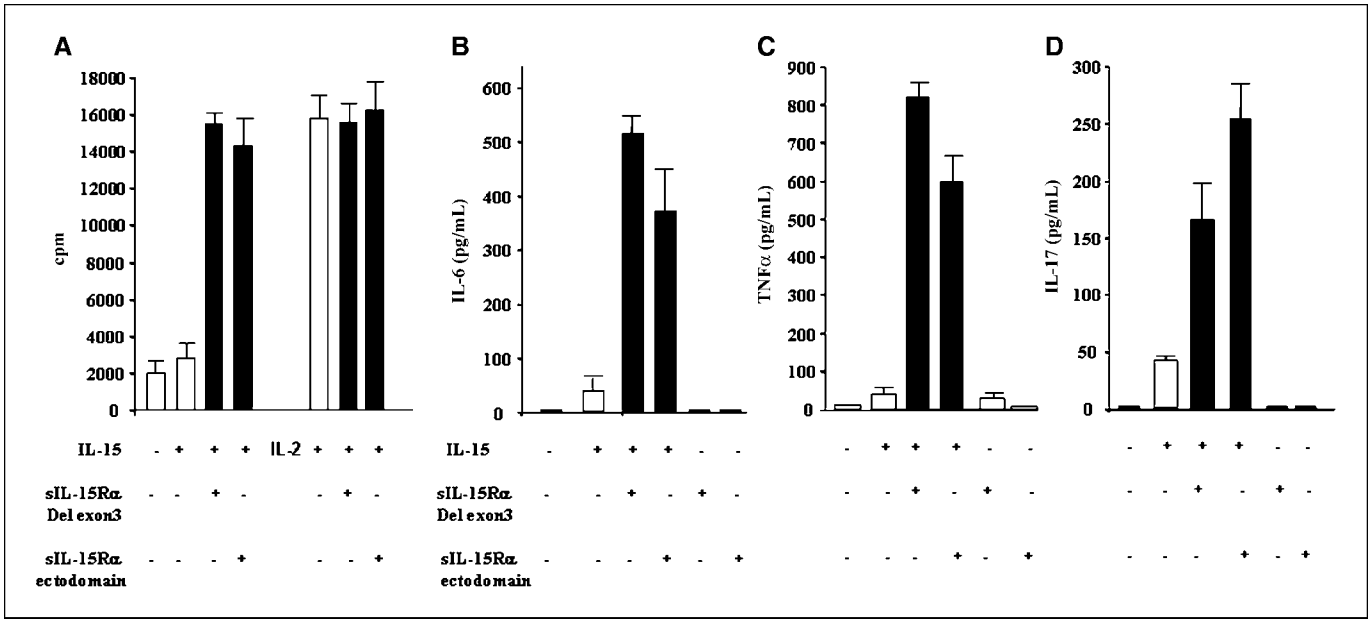

Figure 6. sIL-15Rα increased IL-15 activity on CD8$^+$ T-cell proliferation and production of inflammatory cytokines. *A,* purified CD8$^+$ T cells (10^5) were seeded on 96-well plates and cultured in medium or IL-15 (10 ng/mL) or IL-2 (1,000 IU/mL) alone (□) or in combination with sIL-15Rα (350 ng/mL) comprising either the full ectodomain of sIL-15Rα or deleted from the domain encoded by exon 3 (■). *B* to *D,* PBMCs (2×10^6/mL) were stimulated or not with IL-15 [10 ng/mL (*B* and *C*) or 100 ng/mL (*D*)] in combination with the two kinds of sIL-15Rα described in *A*. Seventy-two hours later, supernatants were collected and concentrations of IL-6 (*B*), TNFα (*C*), and IL-17 (*D*) were measured by ELISA. These results are representative from three experiments performed with three different PBMCs.

and TNFα (Fig. 6*B* and *C*; data not shown). In contrast, in combination with both sIL-15Rα isoforms, low doses of IL-15 (10 ng/mL) were shown to markedly increase the production of IL-6 and TNFα by PBMC (Fig. 5*B* and *C*). The secretion of these cytokines was only observed in the presence of the IL-15/sIL-15Rα complex, as addition of sIL-15Rα alone had no effect (Fig. 6*B* and *C*). All recombinant molecules used were tested for the absence of significant lipopolysaccharide contamination (<1.0 endotoxin unit/μg recombinant protein). The possible role of sIL-15Rα on the induction of IL-17, a proinflammatory cytokine with protumor activity (29, 30) produced by a subpopulation of CD4$^+$ T cells (TH 17 cells) and previously shown to be regulated by IL-15, was also assessed. As shown in Fig. 6*D*, high doses of IL-15 (100 ng/mL) were required to elicit weak production of IL-17, supporting previous studies reporting that CD4$^+$ T cells are less sensitive to IL-15 than other T and NK cells. sIL-15Rα synergized with IL-15 to greatly enhance the secretion of IL-17. Both isoforms of sIL-15Rα therefore synergized with IL-15 to enhance the release of proinflammatory cytokines. These data seem to be relevant *in vivo* as we showed that mRNA coding for IL-15, sIL-15Rα, IL-6, and TNFα could be expressed in the same biopsy of head and neck cancer patients (Supplementary Fig. S5).

Discussion

This study reports increased serum sIL-15Rα levels in head and neck cancer patients compared with healthy subjects. Serum sIL-15Rα levels were strongly associated with poor clinical outcome both in terms of locoregional control and survival. On multivariate analysis, this biomarker was more powerful than the conventional clinical variables (TNM) used for the initial staging of head and neck cancer patients. Surprisingly, sIL-15Rα did not seem to act via inhibition of IL-15 but rather as an amplifier of IL-15 activity. We showed that sIL-15Rα synergized with IL-15 to enhance the production of proinflammatory cytokines. sIL-15Rα was first described as an antagonist of IL-15 (22, 23), but recent data have shown that administration of soluble IL-15/IL-15Rα complexes markedly enhanced IL-15 half-life and bioavailability *in vivo* (31–33), which supports our results. sIL-15Rα does not seem to exert any synergy with IL-15 on cells expressing the high-affinity IL-15R (19). However, it promotes IL-15 action on cells expressing intermediate- or low-affinity IL-15R (34). Thus, in addition to the ability of membrane-anchored IL-15Rα to present IL-15 in *trans* to neighboring cells during cell-to-cell contact (7), IL-15-sIL-15Rα heterocomplexes may also perform a similar function of IL-15 transpresentation in a soluble form. Similar observations have been reported with soluble IL-6 receptors, as shedding of sIL-6Rgp80 from colon adenocarcinoma cells allowed the formation of IL-6/sIL-6R complexes, which could lead to tumor growth of colon carcinoma cells expressing gp130 but not IL-6Rgp80 (35). It should be noted that, in the present study, IL-15 alone or combined with sIL-15Rα did not exert any effect on the growth of head and neck tumor cells (data not shown).

In our study, tumor cells only secreted sIL-15Rα but the formation of sIL-15Rα-IL-15 *in situ* is expected, as high levels of IL-15 have been reported in biopsies derived from head and neck cancers (15). Because tumor cells did not seem to produce IL-15, monocytes or dendritic cells present in tumor stroma may secrete this cytokine. We could not also exclude that membrane IL-15 expressed in monocytes is the target of sIL-15Rα. From preclinical studies, IL-15 was considered to be an antitumor cytokine mainly due to its role in the activation of antitumor CD8$^+$ T cells promoting the expansion of CTL with high avidity (9, 10, 36). This activity of IL-15 may be not predominant in head and neck cancer patients as antitumor function of T lymphocytes is often compromised (37, 38) and CD8$^+$ T lymphocytes seem to be particularly sensitive to apoptosis in these patients (39). However, sIL-15Rα amplified the IL-15–induced production of various proinflammatory cytokines,

such as IL-6, TNFα, and IL-17, which may promote tumor progression. TNFα and IL-6 are known to act as growth factors for many tumor cells. They are required for the development of tumors induced by chemical carcinogens. IL-6 activates signal transducers and activators of transcription 3, which blocks apoptosis of cancer cells and contributes to the tolerant state of dendritic cells in the tumor microenvironment (40). IL-17 also promotes tumor progression partly via its proangiogenesis effect (29, 30). These *in vitro* results on the role of IL-15 amplified by sIL-15Rα in the secretion of inflammatory cytokines seem relevant with respect to the *in vivo* inflammatory environment of these head and neck cancer patients, as mean concentrations of proinflammatory cytokines, such as IL-6, are increased in these patients (41). We detected serum IL-6 levels suggesting an ongoing inflammatory reaction in some patients included in this study (data not shown). The detrimental role of these inflammatory cytokines for the host is documented by preclinical and clinical data. In mice implanted with murine head and neck tumors, orally delivered lactoferrin, an iron-binding secretory protein, inhibited tumor growth. This effect seemed to be mediated by the ability of lactoferrin to inhibit the cellular release of proinflammatory and prometastatic cytokines (18). Increased serum concentrations of proinflammatory cytokines have been correlated with poor outcome in head and neck cancer patients (17). Other activities of IL-15 that may contribute to the protumor activity of this cytokine include its role in tumor protection from apoptosis, its action on vascular endothelial cells to induce angiogenesis, and its ability to enhance cell motility and invasiveness of cancer cells (42). This protumor role of IL-15, possibly amplified by sIL-15Rα, has been supported by recent clinical prognostic results. Using microarray analysis, expression of IL-15 in biopsies of oral cancer was associated with the development of metastasis in these patients (15).

More generally, murine models and epidemiologic studies have established that chronic inflammation caused by infectious microorganisms or chemical irritants orchestrates a tumor-supporting microenvironment that is indispensable in the neoplastic process (43–45). Recent reports suggest that inflammatory cytokines are more likely to contribute to tumor growth and progression than to mount an effective host antitumor immune response (46, 47), as also suggested by this study, where IL-15-sIL-15Rα complex seemed to be an important player coordinating the induction of the proinflammatory cascade.

As previously reported for other cytokines (47), IL-15 could be considered to be ambivalent cytokine in the modulation of tumor growth with a fine balance between antitumor activities mainly mediated by $CD8^+$ T and NK cells and protumor activities mediated by amplification of inflammatory reactions.

The increased concentrations of sIL-15Rα in head and neck cancer patients could be explained by the release of sIL-15Rα by tumor cells. Tumor cells express IL-15Rα *in vitro* and *in vivo*. Expression of this receptor was not found in normal corresponding mucosa, suggesting dysregulation of the expression of this receptor with malignant transformation. An increased expression of IL-15Rα by epithelial cells in benign prostatic hyperplasia compared with normal prostate has also been previously observed (48).

With respect to the mechanisms of sIL-15Rα production, its release seems to be dependent on proteolytic cleavage, as no mRNA was detected with alternative splicing of transmembrane exon encoding sIL-15Rα. Recently, sIL-15Rα only comprising the Sushi domains of the receptor was shown to be generated by alternative splicing mechanism within intron 2 of IL-15Rα in mice (21). The predicted molecular mass of this isoform was 12 kDa. We could not completely exclude the presence of a similar soluble Sushi isoform in human, although this study and others found that the human sIL-15Rα had a molecular mass of about 42 to 45 kDa (Supplementary Fig. S3; ref. 19).

In addition, as previously described (20), various arguments converge to support the role of ADAM-17 in the shedding of sIL-15Rα: (*a*) head and neck tumor cell lines and tumor cells *in situ* expressed ADAM-17, (*b*) ADAM-17 inhibitors decreased the *in vitro* release of sIL-15Rα by head and neck tumor cell lines, and (*c*) a correlation was shown between ADAM-17 expression by tumor cells *in situ* and serum sIL-15Rα levels. ADAM-17 has been postulated as a tumor promoter. Targeting this protease with small molecular inhibitors or siRNAs reverted the malignant phenotype in a breast cancer cell line by preventing mobilization of two crucial growth factors, transforming growth factor-α and amphiregulin, which bind to erbB (49). Oral administration of a small-molecule inhibitor of ADAM-17 to tumor-bearing mice resulted in a significant decrease of tumor proliferation (50). Inhibition of cleavage of sIL-15Rα by these ADAM-17 inhibitors may counteract this new tumor evasion mechanism and improve the prognosis of these cancer patients. This new tumor evasion mechanism, possibly dependent on amplification of the intratumoral inflammatory reaction, reported in this study for head and neck cancers could also be potentially extended to leukemia and other solid tumors, which have been shown to express and release sIL-15R.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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