Expression of Functional Interleukin-3 Receptors on Hodgkin and Reed-Sternberg Cells

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The human interleukin-3 receptor (IL-3R) is a heterodimeric complex consisting of an IL-3-specific α chain (IL-3R α) and a common β chain (β_c), this latter shared with the receptors for granulocyte-macrophage colony-stimulating factor and IL-5. Despite extensive research on cytokine circuitries regulating proliferation and survival of tumor cells in Hodgkin's disease (HD) the functional expression of IL-3Rs in this pathobiological entity has not yet been investigated. In the present study, we demonstrate that the great majority (>90%) of malignant Hodgkin and Reed-Sternberg cells of classic HD (19 of 19 analyzed cases) express IL-3R α by immunostaining of frozen sections and cell suspensions from involved lymph nodes. Accordingly, HD cell lines (L428, KMH2, HDLM2, L1236) expressed the α and β chains of IL-3R both at the mRNA and protein level, with a molecular size of IL-3R α identical (70 kd) to that expressed by human myeloid cells. Exogenous IL-3 promoted the growth of cultured Hodgkin and Reed-Sternberg cells, such effect being potentiated by IL-9 co-stimulation, and was able to partially rescue tumor cells from apoptosis induced by serum deprivation. This data suggests an involvement of IL-3/IL-3R interactions in the cellular growth of HD through paracrine mechanisms. (*Am J Pathol* 2002, 160:585–596)

Classical Hodgkin's disease (cHD) represents a unique example among human lymphomas in that the putative malignant cells, ie, the mononucleated Hodgkin (H) cells and the giant multinucleated Reed-Sternberg (RS) cells, account for the striking minority, usually 0.1 to 3%, of the total cell population in HD-involved tissues.^{1,2} Tumor cells of HD are interspersed among a heterogeneous population of nonmalignant reactive cells mainly including T cells, eosinophils, neutrophils, B lymphocytes, plasma cells, histiocytes, and stromal cells.^{1–3} Several

studies have documented that H-RS cells are highly interactive with this cellular microenvironment through direct cell contacts and elaboration of various cytokines and chemokines.^{4–8} The abnormal and unbalanced production of cytokines, chemokines, and other soluble mediators, occurring at both tissue and systemic levels, has been assumed to account for some of the peculiar histopathological features and constitutional symptoms typical of this disease.^{3–6} In addition, proliferation and survival of tumor cells in HD are affected by cytokines acting as autocrine and paracrine growth factors.^{3–6}

Primary and/or cultured H-RS cells produce a wide array of cytokines and growth factors including interleukin (IL)-1 α , IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage (GM)-CSF, tumor necrosis factor (TNF)- α and - β , transforming growth factor- β , and transforming growth factor- β -like molecules.^{3–6} Some of these cytokines such as IL-6, IL-9, M-CSF, tumor necrosis factor- α , and, more recently, IL-13, have been suggested to act as autocrine growth factors for tumor cells of HD based on the co-expression of a given cytokine and its cognate receptor and the occurrence of growth inhibition on exposure to cytokine-specific blocking antibodies.^{3,4,9–15} In addition, H-RS cells express surface receptors for cytokines and stimulatory molecules secreted or presented by surrounding reactive cells, thus gaining a paracrine/juxtacrine growth stimulation.^{3–6} In this regard, surface receptors (R) for several growth factors and cytokines such as IL-2R (α , β , and γ chains), IL-6R, IL-9R, IL-13R, M-CSFR (c-fms), SCFR (c-kit), tumor necrosis factor-R1 and -R2 and other membrane-bound ligands of the tumor necrosis factor superfamily (ie, CD30, CD40) have been detected on tissue H-RS cells and/or HD-derived cell lines.^{3–5,16–18} Signaling through such receptors results in the functional activation, enhanced proliferation, and apoptotic rescue of cultured H-RS cells.^{3–5,17,18} Unveiling the cytokine receptors repertoire of H-RS may therefore provide critical clues to fully understand

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the biological mechanisms regulating cellular growth and expansion of HD.

The human IL-3R is an heterodimeric receptor complex consisting of an IL-3-specific α chain (IL-3R α) and a common β chain (β_c) that is shared with the receptors for GM-CSF and IL-5.^{19–21} Signaling through IL-3R by its cognate cytokine on normal and malignant hemopoietic cells has been shown to prevent cell death by apoptosis, promote survival, and stimulate proliferation.^{21–23} Despite the extensive investigation on cytokine circuitries underlying the development of HD, the expression and function of IL-3R on H-RS cells has not yet been analyzed in detail.

We show here that the IL-3R complex is consistently expressed on primary H-RS cells and HD-derived cell lines. Moreover, IL-3 causes a dose-dependent increase of cultured H-RS cells growth, such effect being potentiated by IL-9 co-stimulation, and is able to partially rescue tumor cells from apoptosis. This data suggests an involvement of IL-3/IL-3R in the cellular growth of HD through paracrine mechanisms.

Materials and Methods

Tissue Samples

The study included tissue samples of 19 cases of cHD (14 nodular sclerosis (NS) and 5 mixed cellularity (MC)) and 2 cases of nodular lymphocyte predominance HD. In five cHD cases, cell suspensions were also available. Tissues from six reactive lymph nodes were also included in the study. The R.E.A.L. classification was used to classify HD.²⁴ Tissues were fixed in Bouin solution or neutral-buffered formalin. In all cases a portion of unfixed tissue was snap-frozen in liquid nitrogen and stored at -80°C .

Cell Lines

The characteristics of the human HD-cell lines L428, KMH2, HDLM2, and L540 were described in detail previously.²⁵ HD-derived cell lines and the human leukemic cell lines KG1A, HL60, and NB4 were obtained through the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The HD cell line L1236²⁶ was kindly donated by Dr. A. Jox and Prof. V. Diehl (University of Köln, Köln, Germany). Cell lines were maintained in Iscove's-modified Dulbecco's medium (IMDM; Biochrom KG, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom KG), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.1% (w/v) L-glutamine (Biochrom KG).

Immunohistochemistry

In all HD cases monoclonal antibodies (mAbs) suitable for paraffin tissues—leukocyte common antigen (LCA), CD15/LeuM1, CD30/BerH2, CD40/89, epithelial membrane antigen (EMA), CD20/L26, CD21/1F8, CD74/LN2, CD79a/JCB117, CD3, CD45R0/UCHL1, CD43/L60, CD68/KP1, anti-latent membrane protein (CS1–4), and anti-cytokeratin (MN116)—were used, as previously re-

ported.^{16,27} Deparaffinized and cryostat sections were used for immunophenotyping and lineage assignment of HD cases. Source and specificity of mAbs used in this study have been reported in detail previously.^{16,27} Immunohistochemistry was performed with the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method as described.^{16,17,27} Naphthol AS-MX phosphate along with Fast Red TR salt (with the addition of 4% polyvinyl alcohol to the solution) were used for the development of alkaline phosphatase. The endogenous alkaline phosphatase was blocked by adding levamisole to the substrate solution at the final concentration of 1 mmol/L. Negative controls, which were invariably negative, consisted of omission of the primary antibody, substitution with phosphate-buffered saline (PBS), and staining with an irrelevant isotype-matched control mAb.

Staining with Anti-IL-3R α Antibody

Anti-IL-3R α mAb S-12 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied to frozen tissues from all 21 HD cases included in the study. Air-dried frozen sections were kept under vacuum for 3 hours and then fixed in a 1:1 solution of acetone and chloroform for 10 minutes; sections were then hydrated with PBS, incubated with normal rabbit serum (1:50 for 20 minutes at room temperature) and with anti-IL-3R α mAbs for 1 hour at room temperature, and immunostained by the APAAP method. Cytospin smears of HD-derived cell lines were fixed in acetone-chloroform at room temperature for 10 minutes and immunostained with anti-IL-3R α mAbs by the APAAP method.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA (1 μg) extracted by the guanidium thiocyanate method²⁸ was reverse-transcribed using avian myeloblastosis virus RT (Promega Co., Madison, WI) in a 20- μl reaction mixture containing hexadeoxyribonucleotide random primers (0.5 μg), as previously described.²⁹ Two μl of the same cDNA preparation were amplified in a 50- μl volume of final reaction mix in a PTC150 thermal cycler (MJ Research, Watertown, MA) with 25 pmol/L of primer pairs specific for IL-3R α (sense, 5'-TCT CCA GCG GTT CTC AAA GTT CCC ACA TCC-3', region 664 to 693; antisense, 5'-CCC AGA CCA CCA GCT TGT CGT TTT GGA AGC-3', region 1218 to 1189), β_c (sense, 5'-AGA TGC AGG GGA GGA AGA GTG-3', region 874 to 894; antisense, 5'-CGC AGC CTG TAC CCG TAG ATG-3', region 1440 to 1420), IL-3 (sense, 5'-ATG AGC CGC CTG CCC GTC CTG-3', region 10 to 30; antisense, 5'-GCG AGG CTC AAA GTC GTC TGT TG-3', region 458 to 436) and β -actin (sense, 5'-AGC ACA GAG CCT CGC CTT TG-3', region 27 to 46; antisense, 5'-CGT GGT GGT GAA GCT GTA GCC-3', region 693 to 673). Conditions for hot-start PCR were 3 minutes at 94°C followed by 35 cycles (28 cycles for β -actin) of 45 seconds at 94°C , 45 seconds at 60°C , 1.0 minute and 45 seconds at 72°C , and a final elongation of 5.0 minutes at 72°C . A total of 10

μ l of amplified cDNAs, were run in a 1.5% agarose gels and stained with ethidium bromide. RNA extracted from the human leukemic cell lines KG1A and NB4 was used as positive control for IL-3R α and β_c , respectively, whereas activated peripheral T cells²⁹ were used as a positive control for IL-3 transcripts. Normal human thyroid follicular cells (courtesy of Dr. R. De Filippi, University of Udine, Italy) or the omission of cDNA were used as RT-PCR-negative control.

Immunofluorescence and Flow Cytometry

Expression of the IL-3R complex on HD cell lines was analyzed by direct and indirect immunofluorescence as previously described.^{17,29} For IL-3R α chain detection, cells were incubated with the phycoerythrin (PE)-conjugated anti-IL-3R α mAb 7G3 (BD PharMingen, San Diego, CA) for 30 minutes at 4°C. For β_c detection, cells were sequentially incubated with the biotin-conjugated anti- β_c mAb 3D7 (BD PharMingen) and with PE-conjugated streptavidin (Becton Dickinson Immunocytometry System, San Jose, CA). Nonspecific binding of antibodies was assessed by labeling cells with isotype-matched PE-conjugated and biotin-conjugated irrelevant mouse IgG (BD PharMingen). Viable, antibody-labeled cells were identified according to their forward- and right-angle scattering, electronically gated, and analyzed on a FACS-calibur flow cytometer (Becton Dickinson) by means of the CellQuest software (Becton Dickinson).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Cells were washed twice in ice-cold PBS and immediately lysed in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 10 mmol/L ethylenediaminetetraacetic acid, and 1.0 mmol/L phenylmethyl sulfonyl fluoride at 4°C for 30 minutes. After centrifugation for 10 minutes at 10,000 rpm (4°C), samples (50 μ g) were mixed with 7 μ l of 3 $\times$ sodium dodecyl sulfate sample buffer containing 2-mercaptoethanol, heated at 100°C for 5 minutes, and subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Proteins were blotted onto a polyvinylidene difluoride membrane (Bio Rad Laboratories, Hercules, CA) and residual binding sites were blocked by incubation for 2 hours in 0.5% casein dissolved in PBS-0.1% Tween 20. Membranes were then washed with PBS-0.1% Tween 20 and incubated for 2 hours with 1.5 μ g/ml of the anti-IL-3R α mAb (S12), (Santa Cruz Biotechnology, Inc.) in PBS containing 0.5% casein, followed by a 2-hour incubation with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Life Science, Amersham Place, UK) in PBS-0.5% casein. Filters were shown by the enhanced chemiluminescence system (ECL-Plus, Amersham) following the manufacturer's instructions.

Proliferation Assays

Clonogenic growth of HD cell lines was assayed as previously described.¹⁷ Briefly, 1.0×10^3 cells were suspended in 1.0 ml of IMDM containing 15% FCS and 0.8% methylcellulose in the absence and presence of different concentrations (5.0, 10, 25, and 50 ng/ml) of human recombinant IL-3 (R&D Systems, Minneapolis, MN) and cultured in 100- μ l aliquots (eight replicates) in 96-well flat-bottomed microplates. Control clonogenic experiments were also performed in the presence of 10 μ g/ml of mouse anti-human IL-3 mAb (R&D Systems). The effects of the simultaneous stimulation with IL-3 (5.0 ng/ml) and IL-9 (5.0 ng/ml) on the clonogenic growth of HD cell lines were also analyzed. After 14 days of incubation, plates were observed under phase-contrast microscopy and aggregates with ≥ 40 cells were scored as colonies.

For proliferation assays in liquid culture, HD-derived cell lines (2.0×10^4 cells/ml) were seeded in IMDM medium supplemented with 2% FCS on 24-well flat-bottomed tissue culture plates in the presence of IL-3 (50 ng/ml). After 72 hours cells were pulsed with 1 μ Ci/well ³H-thymidine for the final 12 hours of culture, harvested onto glass fiber membranes, and counted in a liquid scintillation β -counter (Tri-carb 1600TR; Camberra-Packard, Meriden, CT). Results are expressed as mean counts per minute (cpm) $\pm$ SEM of triplicate cultures. When indicated, the L1236 HD-derived cell line (2.0×10^4 cells/ml) was cultured as above in the presence of different concentrations (1 to 50 ng/ml) of IL-3.

Detection of Apoptosis

Apoptosis was induced in HD-cell lines by partial serum deprivation or CD95/Fas ligation. Exponentially growing HD cell lines from serum-containing (10% FCS) cultures were washed twice in PBS, resuspended in IMDM containing 2% FCS, and seeded (2.0×10^4 cells/ml) into 6-well flat-bottomed microplates in the presence or absence of IL-3 (50 ng/ml). CD95-mediated apoptosis was induced by incubating HDLM2 cells (5.0×10^4 /ml) in IMDM containing 5.0% FCS in the presence of 100 ng/ml of the agonistic anti-CD95 mAb CH-11 (Medical & Biological Laboratories, Nagoya, Japan) or an isotype-matched control antibody in the absence and presence of IL-3 (50 ng/ml). At different time intervals, cells were harvested and assayed for viability by the trypan blue exclusion test and apoptosis/necrosis by flow cytometry. Apoptosis was measured by staining cells with PE-conjugated APO2.7 mAb³⁰ (Coulter-Immunotech, Marseille, France) and with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Pharmingen), according to the manufacturer's instructions. Briefly, cells were fixed with 1.0% paraformaldehyde and permeabilized for 20 minutes on ice with digitonin (100 μ g/ml; Sigma-Aldrich, Milan, Italy), washed once in cold PBS containing 2.5% FCS and 0.01% NaN₃ (PBSA), and incubated for 15 minutes at room temperature in the dark with 10 μ l of PE-conjugated APO2.7 mAb. Cells were then washed twice in PBSA and analyzed by flow cytometry. In some

Figure 1. Expression of IL-3R α in reactive lymph node tissues. Expression of IL-3R α is detected on the endothelium of a high endothelial venule and on surrounding mononuclear cells. Frozen section of a reactive lymph node stained with anti-IL-3R α mAb. APAAP immunostaining, hematoxylin counterstain. Original magnification, $\times 250$.

experiments cells were double-labeled with the FITC-conjugated anti-CD30 mAb BerH2 (DAKO, Milan, Italy) and PE-conjugated APO2.7. For detection of Annexin V binding, cells were resuspended in binding buffer (10

mmol/L Hepes/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂) and incubated for 15 minutes in the dark with 10 μ l of Annexin V-FITC. Cells were then washed and resuspended in binding buffer. Before flow cytometry 10 μ l of propidium iodide solution (10 μ g/ml in binding buffer) were added to each sample.

Results

Reactive Lymph Nodes

By applying a mAb directed against IL-3R α on frozen sections of human lymph nodes, an intense immunoreactivity was detected on mononuclear cells with histiocytic/monocytic morphology closely associated with high endothelial venules, high endothelial venule endothelium and scattered B cells, whereas most T cells were IL-3R α -negative (Figure 1).

Expression of IL-3R α in HD

Expression of IL-3R α was investigated, in conjunction with that of the HD typical marker CD30, by immunostaining frozen sections from 21 cases of HD. Because IL-3R α and CD30 antigens are co-localized at the cell membranes and cytoplasm of H-RS cells, the distribution of the relative expression in HD-involved lymph nodes was studied by using a serial frozen sections strategy. H-RS cells from 19 cases of cHD (Table 1, Figure 2) and lymphocytic and/or histiocytic (L&H) cells from two cases of nodular lymphocyte predominance HD (Table 1) showed a consistent reactivity with anti-IL-3R α mAbs that was observed in most tumor cells, albeit at a variable staining intensity. In cHD cases membrane staining of H-RS cells was commonly associated with a cytoplasmic

Table 1. IL-3R α Expression and Immunophenotype in Hodgkin's Disease

Case no.	Histologic diagnosis	IL-3R α	CD15	CD30	CD40	Immunophenotype*
1	NLP HD	+	—	—	+	B
2	NLP HD	+ (w)	—	—	+	B
3	MC HD	+ (w)	+	+	+	B
4	MC HD	+ (w)	+	+	+	B
5	MC HD	+	+	+	+	B
6	MC HD	+	+	+	+	Undetermined
7	MC HD	+	+	+	+	Undetermined
8	NS HD	+	+	+	+	B
9	NS HD	+ (w)	+	+	+	Undetermined
10	NS HD	+	+	+	+	Undetermined
11	NS HD	+	+	+	+	Undetermined
12	NS HD	+ (w)	+	+	+	Undetermined
13	NS HD	+	+	+	+	Undetermined
14	NS HD	+ (w)	+	+	+	Undetermined
15	NS HD	+ (w)	+	+	+	Undetermined
16	NS HD	+	—	+	+	Undetermined
17	NS HD	+	+	+	+	Undetermined
18	NS HD	+	+	+	+	Undetermined
19	NS HD	+	+	+	+	Undetermined
20	NS HD	+	+	+	+	Undetermined
21	NS HD	+ (w)	+	+	+	T

*Immunophenotype was determined by the expression of B- and T-associated antigens on H-RS cells.

Abbreviations: NLP, nodular lymphocyte predominance; HD, Hodgkin's disease; MC, mixed cellularity; NS, nodular sclerosis; w, weak; +, positive (most tumor cells labeled); —, negative (no tumor cells labeled).

Figure 2. Expression of IL-3R α on H-RS cells of cHD. Serial frozen sections of a lymph node involved by cHD (**A** and **B**) are presented to show that H-RS cells, detected by their typical marker CD30 (**A**), express IL-3R α (**B**). In a frozen section from another lymph node involved by cHD (**C**) a H-RS cell expressing IL-3R α displays a cytoplasmic labeling of weak intensity. In the **inset**, another H-RS cell expressing IL-3R α with a cytoplasmic labeling of a higher intensity is reported. APAAP immunostaining, hematoxylin counterstain. **Arrows** indicate morphologically recognizable IL-3R α -expressing H-RS cells. Original magnifications: $\times 250$ (**A**, **B**); $\times 630$ (**C**).

labeling (not shown). Both of these patterns of staining were also found on H-RS cells from cell suspensions obtained from lymph nodes involved by cHD (Figure 3, A and B). In these cases, stained H-RS cells could be clearly detected amid a population of reactive IL-3R α -negative lymphoid cells (Figure 3, A and B). Differences

in the staining patterns between H-RS cells of cHD and L & H cells of nodular lymphocyte predominance HD were not perceptible. IL-3R α expression did not disclose any apparent correlation with the histological subtypes of HD or the supposed lineage derivation of tumor cells (T cells, B cells, undetermined) (Table 1).

Figure 3. Expression of IL-3R α on H-RS cells. Cytospin preparations from lymph nodes involved by cHD. **A:** H-RS cells expressing IL-3R α display a membrane staining associated with cytoplasmic labeling. **B:** In this case, staining of H-RS cells is mainly membranous. APAAP immunostaining, hematoxylin counterstain. Original magnification, $\times 630$.

Expression of the IL-3R Complex and IL-3 in HD-Derived Cell Lines

Expression of the IL-3R α and β_c on a panel of HD-derived cell lines was first assessed at the mRNA level by RT-PCR. Amplified products specific for IL-3R α and β_c were detectable in all HD cell lines analyzed (L428, L540, KMH2, HDLM2, and L1236), although the levels of IL-3R α -specific transcripts were significantly higher in the L428 and HDLM2 cell lines (Figure 4). Conversely, low levels of the cognate cytokine IL-3 were seen in the HD cell line L540 (Figure 4).

The presence of surface IL-3R α and β_c on cultured H-RS cells was then assessed by flow cytometry. In agreement with RT-PCR data, L428 and HDLM2 cells expressed IL-3R α at a highest surface density (Figure 5). The same cell lines were also found to consistently express surface β_c , albeit at lower fluorescence intensity (Figure 5). Moreover, a lower expression level of both components of IL-3R was detected by L1236 and KMH2 cells, and an almost negative fluorescence signal was obtained on staining of L540 cells with both anti-IL-3R α and β_c mAbs (Figure 5). On cytospin preparations, L428 and HDLM2 cells were strongly immunoreactive with anti-IL-3R α mAbs displaying an intense membrane staining in some cases associated with a strong cytoplasmic reactivity (Figure 6). Such double pattern of staining was

Figure 4. Expression of mRNAs encoding for α and β chains of the IL-3 receptor and IL-3 in HD-derived cell lines. Total RNA was isolated from L428, L540, KMH2, HDLM2, and L1236 cells, reverse-transcribed, and amplified with primers pairs specific for the α (IL-3R α) and the common β chain of the human receptor for IL-3, and the human IL-3 as detailed in Materials and Methods. Sources of negative (–) and positive (+) control RNAs, are specified in Materials and Methods. Amplified products were resolved on 1.5% agarose gel and visualized by ethidium bromide and ultraviolet light. In all cases, the same cDNA bulks were also amplified with primers specific for the housekeeping gene β -actin. MW, 100-bp molecular weight marker; the double-intensity band corresponds to 600 bp.

closely reminiscent of results obtained in primary H-RS cells from cHD-involved lymph nodes (Figures 2 and 3). Despite the detection of low amounts of specific mRNA and protein (Figures 4 and 5), L540 cells were not stained by anti-IL-3R α in immunocytochemistry (not shown).

Finally, immunoreactive IL-3R α was demonstrated as a single component of 70 kd identical to that found in human cells of myeloid derivation^{31,32} in all of the HD cell lines analyzed, the highest levels being found in L428 and HDLM2 cells (Figure 7). Notably, a weak but specific band, consistent with RT-PCR and flow cytometry data, was detected also in cell lysate from L540 cells.

Effects of IL-3 on Growth of Cultured H-RS Cells

Exposure of HD cell lines to increasing concentrations of recombinant human IL-3 resulted in a dose-dependent

Figure 5. Expression of surface IL-3R α and β chains on HD-derived cell lines. Flow cytometry profiles were generated by incubating cells with PE-conjugated anti-IL-3R α mAb 7G3 (**closed histograms**) and with the biotin-conjugated anti- β chain mAb 3D7, followed by PE-conjugated streptavidin (**shaded histograms**). **Dotted- and continuous-line histograms** represent staining profiles obtained with irrelevant isotype-matched PE-conjugated and biotin-conjugated control Igs, respectively. The x and y axes indicate the logarithm of relative red fluorescence intensity and the relative cell counts, respectively.

enhancement of their clonogenic growth (Figure 8A). At the highest concentration of 50 ng/ml, IL-3 was able to induce a 2.1- to 3.2-fold increase in the total number of colonies generated by L428, KMH2, and HDLM2 cells. Such effect was strikingly more evident on L1236 cells, in which IL-3 (50 ng/ml) induced a more than 11-fold increase of the clonogenic capacity over control cultures (Figure 8A). The enhancement of colony formation was abolished by the presence of a neutralizing anti-human IL-3 mAb (10 μ g/ml) (Figure 8A). Colony cells developed under IL-3 stimulation displayed an increased cell survival (up to 20 to 22 days) as opposed to control cultures that underwent degeneration starting from the 16th day of culture (not shown). In a subsequent series of experiments (Figure 8B), we evaluated the cooperative effects of IL-3 with IL-9, a cytokine involved in the growth of H-RS cells,^{11,12} in our panel of HD-derived cell lines, all expressing IL-9R (Degan M, unpublished observation, 1999).¹² As for IL-3, IL-9 (5.0 ng/ml) was able to produce

Figure 6. Immunostaining patterns of HD-derived cell lines with anti-IL-3R α antibody. **A:** L428 cell line. **B:** HDLM2 cell line. Cultured H-RS cells show an intense membrane staining associated with a strong cytoplasmic reactivity or a selective labeling of cell membranes. Cytospin preparations. APAAP immunostaining, hematoxylin counterstain. Original magnifications, $\times 250$.

a 2.5- and 1.9-fold increase in colony formation of L428 and KMH2 cells, respectively, whereas showing a more striking effect on L1236 cells, as witnessed by a more than sixfold increase in the total number of colonies. The exposure to a combination of IL-3 (5.0 ng/ml) and IL-9 (5.0 ng/ml) resulted in a further enhancement in the clonogenic growth of L428 and KMH2 cells (5.5-fold and 3.2-fold increase, respectively), up to an 18-fold increase over control in colony formation in L1236 cells (Figure 8B). In particular, the combined action of IL-3 and IL-9 yielded a 2.8- to 4.0-fold and 1.7- to 5.6-fold increase of colony formation by HD cell lines with respect to IL-3 or IL-9 alone, respectively (Figure 8B). To provide further evidence that IL-3 serves as growth factor for HD-derived cell lines, additional proliferation assays were performed (Figure 8, C and D). In particular, with the only exception of L540 cells, exposure of HD-derived cell lines to IL-3 (50 ng/ml) in liquid culture for 72 hours resulted in a significant increase of proliferation (fold increase ranging from 1.38 to 2.5), as assessed by 3 H-thymidine uptake (Figure 8C). Consistently, IL-3 was able to induce a dose-dependent increase of radiolabeled nucleotide incorporation in L1236 cells reaching a maximum at 50 ng/ml IL-3 (Figure 8D).

Figure 7. Detection of IL-3R α in HD-derived cell lines by Western blotting. Cell lysates (50 μ g/lane) from the different HD-derived cell lines, KG1A cells (positive control), and HL60 cells (negative control) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The blots were then incubated with 1.5 μ g/ml of anti-IL-3R α mAb and shown by chemiluminescence. The position of molecular weight markers is indicated.

Figure 8. Growth of HD-derived cell lines by IL-3. **A:** Cells were cultured in semisolid medium (0.8% methylcellulose) in the presence of increasing concentrations of IL-3. After 14 days of incubation, plates were observed under phase contrast microscopy and aggregates with ≥ 40 cells were scored as colonies. Results represent the mean $\pm$ SEM of eight replicate wells from three different experiments. The data (filled squares) is presented as percent growth relative to spontaneous colony formation (medium only). Filled circles represent clonogenic growth obtained at the maximal IL-3 concentration (50 ng/ml) in the presence of 10 μ g/ml of a mouse anti-human IL-3 mAb. **B:** Cooperative effect of IL-3 and IL-9 on the clonogenic growth of cultured H-RS cells. Clonogenic assays were performed in the presence of medium alone (open bars), IL-3 (5.0 ng/ml; filled bars), IL-9 (5.0 ng/ml; shaded bars), and their combination (striped bars). Results represent the mean $\pm$ SEM of eight replicate wells from three different experiments. The data are presented as percent growth relative to spontaneous colony formation (medium only). **C:** Cells from HD-derived cell lines (2.0×10^4 /ml) were cultured in 96-well flat-bottomed microplates in the presence of IL-3 (50 ng/ml). After 72 hours cells were pulsed with 1 μ Ci/well 3 H-thymidine for the final 12 hours of culture, harvested onto glass fiber membranes, and counted in a liquid scintillator β -counter. Data are presented as percent $\pm$ SEM of 3 H-thymidine uptake in the presence of IL-3 relative to control (medium alone). **D:** L1236 cells (2.0×10^4 /ml) were cultured as in **C** in the presence of increasing concentrations (0 to 50 ng/ml) of IL-3. Results are expressed as cpm $\pm$ SEM. Data in **C** and **D** are means of triplicate cultures of four independent experiments.

Effects of IL-3 on Apoptosis of Cultured H-RS Cells

To analyze whether IL-3 was able to rescue H-RS cells from apoptosis induced by serum starvation, exponentially growing HD-cell lines (L428, KMH2, HDLM2, L1236) were cultured for 72 hours in low-serum media in the presence or absence of IL-3 (50 ng/ml), and the fraction of apoptotic cells was then assessed by determining the percentage of APO2.7⁺ and Annexin-V⁺ cells.³⁰ With the only exclusion of L428 cells, which seemed resistant to apoptosis induced by low-serum conditions (<20% of APO2.7⁺ and Annexin V⁺ cells; not shown), in all of the other HD-derived cell lines the switch to 2% serum-containing cultures yielded an increase of both APO2.7⁺ (KMH2 = 38 ± 4 ; HDLM2 = 40 ± 5 ; L1236 = 87 ± 9) and

Annexin V⁺ (KMH2 = 56 ± 8 ; HDLM2 = 61 ± 5 ; L1236 = 63 ± 4) cells (Figure 9A). Addition of recombinant IL-3 inhibited apoptosis in HDLM2 and L1236 cells, as detected by a significant ($P < 0.05$) reduction of APO2.7 expression, while showing a less marked effect on the KMH2 cell line (Figure 9A). Similarly, binding of Annexin-V protein was inhibited by 56% and 52% on addition of IL-3 to HDLM2 and L1236 serum-deprived cultures ($P < 0.05$), and by only 17% in KMH2 cells (Figure 9A). A representative experiment documenting the IL-3-induced inhibition of apoptosis in L1236 cells, as evidenced by APO2.7 staining, is shown in Figure 9B.

Because L428 and L1236 cells were recently shown to be resistant to apoptosis induced by CD95 ligand,³³ we selected the HDLM2 cell line as a model to assess the effects of IL-3 on CD95-mediated programmed cell death

Figure 9. Effects of IL-3 on apoptosis of HD-derived cell lines. **A:** Exponentially growing HD-derived (KMH2, HDLM2, L1236) cell lines were cultured in IMDM containing 2% FCS in the absence (filled bars) and presence (shaded bars) of IL-3 (50 ng/ml). Percentages of apoptotic cells were assessed by flow cytometry analysis of cells stained with the APO2.7 mAb (top) and Annexin V-FITC (bottom) after 48 hours and 72 hours of culture, respectively. Asterisks refer to experimental points in which a statistical significance ($P < 0.05$) was reached. **B:** Representative flow cytometry dot plots showing changes in APO2.7 staining of L1236 cells. Cells were cultured for 48 hours in 2% FCS in the absence (medium) and presence of IL-3 (50 ng/ml) and double-stained with PE-conjugated APO2.7 mAb and FITC-conjugated anti-CD30 mAb BerH2.

of H-RS cells. In this regard, kinetic analysis of CD95-mediated apoptosis indicated a time-dependent increase of APO2.7⁺ and Annexin-V⁺ cells, which was maximal after 12 to 24 hours of culture with the agonistic anti-CD95 CH11 mAb. Addition of IL-3 to anti-CD95-treated HDLM2 cells inhibited CD95-mediated apoptosis between 25% and 20% as detected by APO2.7 and Annexin-V staining, respectively (Figure 10).

Discussion

Tumor cell proliferation and survival in HD is regulated, via autocrine and paracrine mechanisms, by different

Figure 10. Effects of IL-3 on CD95/Fas-mediated apoptosis of the HD-derived cell line HDLM2. HDLM2 cells were cultured for the indicated time points in complete medium containing 5.0% FCS in the presence of the agonistic anti-CD95 mAb CH11 (100 ng/ml; open circles), anti-CD95 mAb CH11 plus IL-3 (50 ng/ml; filled squares), an irrelevant isotype-matched (IgM) mouse mAb (CNT; open squares), and IL-3 alone (50 ng/ml; filled circles). Percentages of apoptotic cells were assessed by staining cells with the APO2.7 mAb (top) and Annexin V-FITC (bottom) and analyzed by flow cytometry.

cytokines whose receptors are functionally expressed on H-RS cells.^{3-6,9-18} To further define the cytokine circuitries underlying cellular expansion in HD, we have investigated the expression of the IL-3R in a series of HD samples and a panel of HD-derived cell lines. We have shown that the great majority (>90%) of tissue H-RS cells of cHD constantly expressed IL-3R α irrespective of the histological subtype (MC and NS) and of their antigenic phenotype (B cells, T cells, undetermined). In addition, L & H cells from two cases of nodular lymphocyte predominance HD were also reactive with anti-IL-3R α mAbs. Consistently, all HD-derived cell lines expressed the IL-3R α/β_c complex at protein and/or mRNA level, although different expression levels among the various cell lines analyzed were observed.

The presence of IL-3R α on H-RS cells is in keeping with the current view supporting a B-lineage derivation for most cases of cHD.³⁴⁻³⁶ CD19⁺ and CD20⁺ B cells co-expressing IL-3R α and β_c circulate in human peripheral blood,^{37,38} whereas B cells bearing IL-3R α have

been identified in T-cell-rich areas of normal lymphoid tissues and, at a lower frequency, in secondary lymphoid follicles.³⁸ In addition, functional IL-3Rs have been detected on normal B-cell precursors in the bone marrow³⁹ and on tumor cells from B-cell malignancies reflecting early and late stages of B-cell lymphopoiesis such as B-lineage acute lymphoblastic leukemias,^{40–42} follicular lymphomas,^{43,44} chronic lymphocytic leukemias,⁴⁵ and malignant plasma cells precursors.⁴⁶

H-RS cells, along with immunoglobulin gene rearrangements,^{34–36} display impressive functional and phenotypic overlaps with dendritic cells (DCs), including antigen-presenting capacity,^{3,4,47} expression of specific co-stimulatory molecules,^{3–5,17,47,48} cytoskeleton-associated proteins,^{49,50} and cytokines/chemokines.^{3–5,7,8,51,52} Interestingly, IL-3R α s have been also found to be expressed on a specific subset of human DC precursors of T-cell derivation, originally described as plasmacytoid T cells or plasmacytoid monocytes.^{53,54} The present demonstration of IL-3R α on tumor cells of HD, beside strengthening the relationship of H-RS cells with some DC subsets, raises the possibility of an allegedly lymphoid DC of B-cell origin as the normal counterpart of H-RS cells.^{52–54}

We have also shown here that IL-3 stimulates the proliferation both in semisolid and liquid cultures of H-RS cells and enhances colony cell survival, these effects being potentiated by IL-9, another cytokine specifically involved in the proliferation of H-RS cells.^{11,12,17} Remarkably, IL-3 was strikingly effective, with and without IL-9 cooperation, in stimulating the clonogenic growth of L1236 cells, the only available cell line whose actual H-RS cells derivation was formally demonstrated.^{26,55} Finally, we showed that IL-3 is able to partially rescue HDLM2 and L1236 cell lines from apoptosis induced by serum deprivation and to reduce the fraction of HDLM2 cells undergoing CD95-mediated programmed cell death.

Taken together our results indicate that IL-3 may have a role in the cellular growth of HD given its ability to promote proliferation and survival of H-RS cells. In agreement with such views it has been recently demonstrated that H-RS cells overexpress the basic leucine zipper transcription factor NF-IL-3,¹⁵ which transduces anti-apoptotic signals from the IL-3 receptor and plays a pivotal role in IL-3-mediated survival of neoplastic B cells.^{56,57} Because IL-3 represents an important survival and proliferation factor for normal and neoplastic B lymphocytes^{58,59} and DCs of myeloid and lymphoid origin,^{52–54} it seems conceivable that H-RS cells, which are thought to derive from an unusual B-cell lineage^{34–36} associated to a DC-like functional phenotype,^{3–5,47–49,51} might have maintained IL-3 responsiveness for growth and survival.

In agreement with previous reports,²⁵ we have shown that, with the only exception of L540 cells, HD-derived cell lines do not constitutively express IL-3 transcripts. We did not analyze IL-3 production by tissue H-RS in our patients' series, but Merz and colleagues⁶⁰ have previously demonstrated that tumor cells of HD usually lack IL-3 mRNA, whereas Trümper and colleagues⁶¹ detected IL-3 transcripts in only five of seven and four of seven

single H-RS cells from two analyzed HD cases. Thus, the occurrence of an autocrine loop involving IL-3 seems unlikely in HD. It is rather conceivable that the large numbers of activated CD4⁺ T cells and eosinophils, typically present in HD-involved tissues,^{1–5,48} may represent the more likely cellular sources of exogenous IL-3 for H-RS cells.^{22,62} Even though IL-3 is produced by CD4⁺ T cells irrespective of their functional polarization,^{63,64} higher levels of this cytokine can be found in Th2 than in Th1 responses,⁶⁵ and IL-3 itself is able to amplify the expansion of Th2-type T cells.^{66,67} Because T-cell reaction around H-RS cells is mainly associated with a polarized Th2-like response,^{3,4,48} it is therefore conceivable that large amounts of IL-3 can be released within the HD microenvironment. Our results demonstrating for the first time that H-RS cells constitutively express functional IL-3Rs capable of transducing proliferative and anti-apoptotic signals, suggest that IL-3 may contribute to the abnormal cytokine network associated with cellular growth and expansion of HD.

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