Advances in Brief

Interleukin 9 Is Expressed by Primary and Cultured Hodgkin and Reed-Sternberg Cells

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Abstract

We show by mRNA hybridization analysis and immunostaining using a mouse monoclonal antibody (moAb) to recombinant human interleukin 9 (IL-9) that both primary and cultured Hodgkin and Reed-Sternberg (H-RS) cells produce IL-9 transcripts and protein and express surface binding sites for IL-9. In addition, the growth of H-RS cells obtained from the HDLM-2 line (abundantly producing IL-9 transcripts) was significantly inhibited when anti-IL-9 moAb or an IL-9 antisense oligodeoxynucleotide was added. Excess addition of recombinant human IL-9 relieved the effects of anti-IL-9 moAb on HDLM-2 growth. Growth of H-RS cells of the KM-H2 line, which displays only low amounts of IL-9, detectable upon hybridization of polyadenylic acid-selected RNA only, was not affected by anti-IL-9 moAb. The proliferative capacity of primary and cultured H-RS cells was, however, augmented at least 3-fold when cells were exposed to recombinant human IL-9. In conclusion, our results show that IL-9 is expressed by H-RS cells and point to a possible role of this molecule as a growth factor for these cells.

Introduction

The unbalanced expression of a variety of cytokines including IL-1α, IL-3, IL-5, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor β, leukemia inhibitory factor, and γ-interferon has been demonstrated in primary and cultured H-RS cells by several authors (1–7). Most of these molecules have been implicated in some of the unique histopathological and clinical alterations seen in patients with HD, such as eosinophilia and sclerosis in HD-involved tissues (4, 5); constitutional ("B") symptoms of patients with HD including fever, night sweats, generalized itching, and weight loss; and increases in acute-phase protein serum levels. Cytokines produced by H-RS cells may act as growth factors in an autocrine mode or may be secreted in a paracrine or endocrine fashion to modify the functioning and the activational or proliferative state of cells of surrounding or remote tissues. In this regard, previous notable studies demonstrate the production of collagen type I and C-reactive protein by bone marrow fibroblasts and hepatocytes, respectively, upon exposure to supernatants obtained from cultured H-RS cells.4 In this study we have examined the production of IL-9 in primary and cultured H-RS cells at the mRNA and protein levels and have explored its possible role as a growth factor for these cells. Interleukin-9 was also selected for our studies, because it has been shown to act as a growth factor for T-helper lymphocytes (8) and thus may be related to the numerous activated CD4-expressing lymphocytes seen in tissues involved by HD (9) and found in the circulation of these patients (10).

Materials and Methods

Cell Lines and Primary Tissues. HD-derived cell lines HDLM-2 and KM-H2 have been described previously (11, 12). Both lines are generally accepted to be representatives of the neoplastic component of HD based on many characteristics in common with primary H-RS cells (11, 12). HDLM-2 and KM-H2 cells were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with SCM [10% low-endotoxin fetal calf serum (Hazelton, Vienna, UT), 1% penicillin/streptomycin, and 2 mM l-glutamine (Sigma Chemicals, Munich, Germany)] at 37°C in a humidified, 5% CO2 atmosphere. Primary H-RS cells were analyzed in the pleural effusions of two patients with nodular sclerosing HD and the bone marrow aspirates of one patient with lymphocyte-depleted HD.

Complementary DNA Probes. For the hybridization of polyadenylated RNA from HDLM-2 and KM-H2 cells the 0.63-kilobase XhoI fragment of pXM-hIL-9 was used (kindly provided by Dr. M. Garnick, Genetics Institute, Cambridge, MA) (13). Control hybridization was performed with the 0.8-kilobase BamHI-PstI chicken a-actin fragment in pBR 322 (14) to ensure the integrity of RNA and comparative RNA loading in single lanes.

RNA Extraction and Northern Blot Analysis. Polyadenylated RNA was isolated by oligodeoxynucleotide cellulose chromatography, according to the protocol described by Aviv and Leder (15). Polyadenylated RNA from each sample was then electrophoresed in a 1% agarose gel containing 20 mM sodium borate, pH 8.3; 0.5 mM EDTA; and 3% formaldehyde. The RNA was transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) in 10× standard saline citrate (1.5 M sodium chloride and 150 mM sodium citrate) using capillary blotting overnight (16). The blots were then backed and prehybridized at 65°C in 20 mM sodium dodecyl sulfate, 10× Denhardt's (1× Denhardt's = 0.2% Ficoll, 0.2% bovine serum albumin, and 0.02% polyvinyl pyrrolidone), 5× standard saline citrate, and 20 mM salmon sperm DNA (Sigma). The blots were probed with specific complementary DNA probes radiolabeled by random priming with a [α-32P]CTP (>6000 Ci/mmol) (Amersham Buchler, Braunschweig, Germany). The blots were washed at 55°C in 1% sodium dodecyl sulfate/1× standard saline citrate and were autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

Separation of H-RS Cells from Primary HD-involved Tissues. H-RS cells were separated from HD-involved pleural effusions and bone marrow aspirates of three patients with HD by a multistep procedure including the use of plastic adherence followed by an immune rosette technique and two-color FACS cell sorting. To this end, cell suspensions were first subjected to plastic adherence for 2 h (37°C, 5% CO2 in air) to remove monocytes and mesothelial cells. Nonadherent cells were then depleted of T-cells, B-cells, NK cells, mature and immature...
myeloid cells, and erythroid cells by using a panel of murine mAbs including CD2, CD3 (T-cells), CD10, CD19 (B-cells), CD16 (NK cells and neutrophils), CD17, CD34 (early myeloid cells), and anti-glyco-phenin A (nucleated erythroid cells) and affinity-purified antibody to mouse Ig that were previously coupled to sheep RBC by chromium chloride (17). The resulting cell populations were then subjected to two-color cell sorting using fluoresceinated CD15 and phcoerythrin-conjugated CD30 mAb and a FACS V cell sorter ( Coul- ter Electronics, Krefeld, Germany). All mAbs were purchased from Becton Dickinson (Heidelberg, Germany). The separation procedure yielded populations containing ≥85% H-RS cells in the CD2,3,10,19,16,17,34, glycophorin A-negative, CD15,30-positive fraction. This cell fraction and the CD2,3,10,16,17,34, glycophorin A, CD15,30-negative population were then subjected to reverse transcriptase-polymerase chain reaction to analyze ft-microglobulin mRNA expression (Krefeld, Germany). All mAbs were purchased from Becton Dickinson (Heidelberg, Germany) with 10 nmol of each deoxynucleotide triphosphate (Boehringer Mannheim, Mannheim, Germany) and 100 pmol of random hexamer (Pharmacia, Freiburg, Germany). After incubation for 1 h at 42°C, the reaction mixture was denaturated by heating to 95°C for 10 min and immediate chilling on ice. The reaction mixture was diluted with 80 ml of PCR reaction buffer containing 5' and 3' amplification primers (12.5 pmol/αl) and 1 unit AmpliTaq rTap DNA polymerase (Perkin Elmer Cetus, Emeryville, CA). Sense and antisense primers were located in different exons to distinguish between DNA- and RNA-derived PCR products, thus ruling out false-positive results due to RNA preparations contamnated with genomic DNA (sense primer for ft-microglobulin, 5'-TCCATTCTTCAGGAGGAGATG-3', antisense primer for ft-microglobulin, 5'-AGGAATGCCACCAAGAGGACT-3', or a randomly generated nonsense oligomer (5'-CAAGATGCTTCTGGCCATG-3'). Cycles of PCR consisted of denaturing by heating to 94°C for 30 s, annealing of primers at 55°C for 15 s, and primer extension at 72°C for 30 s. This cycle was repeated 33 times by using a programmable heat block (Perkin Elmer Cetus) with an increment of each primer extension step (5 s/cycle). As a final step, incomplete amplicons and products were extended at 72°C for 10 min. One-fourth of each reaction mixture was run on 4% NuSieve Agarose Gels. PCR products were 180 base pairs for IL-9 mRNA and 158 base pairs for ft-microglobulin mRNA. The PCR product of the DNA template was 380 base pairs in size.

Monoclonal Antibodies and Immunostaining. We used a modified peroxides-antiperoxidase technique (18) to examine the expression of IL-9 protein in H-RS cells in pleural effusions and bone marrow aspirates from three patients with HD. To this end, cells were attached to positively charged adherence slides (Superior, Bad Magengheim, Germany) for 10 min in a humidified chamber. Nonviable cells that had lost their membrane charge were removed by washing with phosphate-buffered saline (Gibco). Cells were then fixed with a mixture of 80% acetone and 20% ethanol for 5 min in ice, air dried, and refixed with 100%, 80%, and 50% acetone for 1 min each. A mAb 9 to IL-9, kindly provided by Dr. M. Garnick (Genetics Institute), was layered on top of the slides overnight at 4°C and a concentration of 4 μg/ml. Sandwich antiserum [rabbit anti-mouse IgG (1:500) and swine anti-rabbit IgG (1:50)] and peroxides-antiperoxidase (Dakopatts, Copenhagen, Denmark) were then added for 90 min. The subsequent substrate reaction was done with 0.03% diaminobenzidine (Sigma) for 10 min. Cells were postfixed with osmium tetroxide (2%) for 5 min, and slides were mounted with glycerol gelcine (Dakopatts). As a control for staining specificity, anti-IL-9 was replaced with mouse nonimmune ascites at equivalent concentrations. In selected experiments cells were also stained for the presence of cytoplasmic CD3 using anti-CD3 mAb (Coulter Electronics, Hialeah, FL). To identify the presence of IL-9 receptors, HDLM-2 and KM-H2 cells were incubated with biotin- labeled IL-9 (5 μg/ml) for 60 min at 4°C. Cells were then washed twice in phosphate-buffered saline and treated with avidin fluorescein isothiocyanate (Becton Dickinson, Sunnyvale, CA) (1 μg/ml). Control staining was performed with an irrelevant nonbinding biotin-labeled mouse IgG (850 μg/ml) antibody (19). The binding of IL-9 to cells was analyzed by flow cytometry (FACS Star, Becton Dickinson).

Effect of Anti-IL-9 and rh IL-9 on the Growth of Cultured H-RS Cells. We determined the effect of anti-IL-9 and rh IL-9 on the proliferation of HDLM-2 and KM-H2 cells by measuring the capacity of these cells to incorporate [3H]thymidine (20) and to form colonies of these cells to incorporate [3H]thymidine (20) and to form colonies in semisolid agar. To this end we added rh IL-9 (1–100 units/ml) or anti-IL-9 (0.5–2 μg/ml), or both compounds to cells cultured either in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) in 5% CO2 or in 24-well flat-bottomed culture plates (Nunc, in SCM supplemented with 1% (v/v) fetal calf serum and 20% (v/v) in SCM 50 μM 2-mercaptoethanol (Sigma) at cell densities as indicated. Liquid suspension cultures (humidified atmosphere of 5% CO2 in air) were performed over a period of 24 to 96 h in the presence of [3H] thymidine (1 μCi/well) during the last 12 h. The cells were then harvested, and [3H]thymidine uptake was assessed by liquid scintillation counting. Agar cultures were performed over a period of 10 days in a humidified atmosphere of 7% CO2 in air. Thereafter colonies were enumerated by using an inverted microscope.

Effect of an Antisense Oligodeoxioribonucleotide on the Growth of HDLM-2 Cells. Serum was withdrawn from logarithmic phase HDLM-2 cells 16 h prior to assay. Cells were resuspended in RPMI 1640 supplemented with 1% (v/v) bovine serum albumin at 106 cells/ml. Sense (5'-ACCATGCTTCTGGCCATG-3'), antisense (5'-CATCGGCCAAAGTCTTG-3'), and internalized by the H-RS cells, H-RS cells were separated from HD-involved tissues by a multistep procedure involving a programmable heat block (Perkin Elmer Cetus) with an increment of each primer extension step (5 s/cycle). As a final step, incomplete amplicons and products were extended at 72°C for 10 min. One-fourth of each reaction mixture was run on 4% NuSieve Agarose Gels. PCR products were 180 base pairs for IL-9 mRNA and 158 base pairs for ft-microglobulin mRNA. The PCR product of the DNA template was 380 base pairs in size.

Results

Primary and Cultured H-RS Cells Express IL-9 Protein. As shown in Fig. 1 both primary H-RS cells and cells of the HDLM-2 and KM-H2 lines produce IL-9. IL-9 was detected in the paranuclear region of H-RS cells by immunostaining with anti-IL-9 mAb. The staining intensity in primary H-RS cells (Fig. 1, E and F) was similar to that in HDLM-2 (Fig. 1C) and KM-H2 (Fig. 1D) cells and was detectable in almost 50–60% of H-RS cells investigated. Five to 10% of lymphocytes were also stained with anti-IL-9 mAb. Cultured H-RS Cells Synthesize IL-9 Transcripts. Expression of IL-9 by H-RS cells was also analyzed at the mRNA level. HDLM-2 cells abundantly synthesized IL-9 transcripts. IL-9 expression levels of mRNA isolated from KM-H2 cells were much weaker than those detectable in HDLM-2-derived mRNA and required the use of polyadenylated RNA (Fig. 2).

Primary H-RS Cells Synthesize IL-9 Transcripts. To demonstrate that IL-9 detectable in the cytoplasm of primary H-RS cells (Fig. 1) was actually produced by these cells themselves rather than being secreted by neighboring cells and then bound and internalized by the H-RS cells, H-RS cells were separated from HD-involved tissues by a multistep procedure involving plastic surface adherence, cell depletion by immune rosetting,

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Fig. 1. Staining of HD involved tissues with anti-IL-9 mAb using the peroxidase-antiperoxidase method. A, negative control (in this case, staining of HD-involved pleural effusion with nonimmune mouse ascites). In B, the same cells are stained with anti-CD3 mAb to detect cytoplasmic CD3. Note the negative staining of a Hodgkin cell and the positive staining of T-lymphocytes. C (KM-H2) and D (HDLM-2), staining of cultured H-RS cells. E, positive staining of Hodgkin cells in a pleural effusion of a HD patient. Note the negative staining of lymphocytes and a neutrophil. F, staining of cells of a pleural effusion with anti-IL-9 mAb from a second patient. Note the weak cytoplasmic staining of some lymphocytes around a strongly stained Hodgkin cell.

and positive selection by two-color FACS sorting and then subjected to IL-9 mRNA analysis using RT-PCR. As shown in Fig. 3, cell fractions highly enriched for H-RS cells exhibited IL-9 transcripts.

Effect of rh IL-9 and Anti-IL-9 mAb on the Growth of Cultured H-RS Cells. Recombinant human IL-9 or neutralizing anti-IL-9 mAb was added to cultures of HDLM-2 and KM-H2 cells to examine the effects of these reagents on the proliferation of H-RS cells. To this end 5 x 10⁴ cells/ml were exposed to rh IL-9 (1–100 units/ml), anti-IL-9 mAb in concentrations (1–2 µg/ml) sufficient to neutralize 1000 units/ml rh IL-9 in pilot experiments, or a combination of anti-IL-9 mAb (2 µg/ml) and IL-9 (250 units/ml). After 72 h of culture the proliferation of cells was assessed by measuring their capacity to incorporate [³H]thymidine into DNA. As shown in Fig. 4, proliferation of KM-H2 cells was dose-dependently enhanced upon exposure to IL-9. In contrast, proliferation of HDLM-2 was not affected by IL-9 treatment, unless anti-IL-9 mAb was present in cultures.

Cultured H-RS Cells Express IL-9 Surface Binding Sites. To analyze surface binding of rh IL-9 to KM-H2 and HDLM-2 cells, biotin/avidin-fluorescein isothiocyanate-conjugated ligand and flow cytometry were used. Fig. 5 shows that about 73% and 69% of KM-H2 and HDLM-2 cells, respectively, expressed IL-9 surface receptors.

Cultured H-RS Cells Use IL-9 as an Autocrine Growth Factor. Given the finding that excess addition of rh IL-9 relieved the effects of anti-IL-9 mAb on HDLM-2 growth, the possibility was investigated that HDLM-2 cells used IL-9 as an autocrine growth factor. To that end HDLM-2 cells were exposed to anti-IL-9 mAb (0.5–2 µg/ml) for a period of 24–96 h and were subjected to [³H]thymidine uptake analysis. As shown in Fig. 5, growth of HDLM-2 cells was inhibited by >80% when cells were treated with anti-IL-9 mAb for 96 h at a concentration of 2 µg/ml anti-IL-9 mAb. The effect of anti-IL-9 was not toxic, since a murine mock antiserum (preimmune serum) and neutralizing mAbs to other cytokines, including anti-lymphotoxin and anti-IL-6, had no significant effect on HDLM-2 growth (not shown). Furthermore, excess addition of rh IL-9 (250 units/ml) abrogated the inhibitory activity on anti-IL-9
Effect of an IL-9 Antisense Oligodeoxyribonucleotide on the Proliferation of HDLM-2 Cells. In the next set of experiments the possibility was examined that IL-9 serves as an autocrine growth factor for HDLM-2 cells by using an IL-9 antisense oligodeoxyribonucleotide. As shown in Fig. 7 exposure of HDLM-2 cells to the IL-9 antisense oligomer and thus inhibition of IL-9 protein synthesis were associated with the inhibition of proliferation of these cells, while treatment of HDLM-2 cells with a sense or nonsense oligomer had no effect on their growth.

Fig. 4. Effect of rh IL-9 and anti-IL-9 moAb treatment on [3H]thymidine uptake by KM-H2 and HDLM-2 cells. Cells were seeded in 96-well round-bottomed plates and exposed for a period of 72 h to rh IL-9 (1–100 units/ml), anti-IL-9 moAb (1–2 μg/ml), or a combination of both compounds. [3H]Thymidine was present in cultures during the last 12 h. A representative experiment is shown. Two additional experiments gave almost identical results.

Fig. 5. Expression of IL-9 receptors on the surface of KM-H2 and HDLM-2 cells. Receptor expression was assessed by using biotin/avidin-FITC-conjugated rh IL-9 and flow cytometry. control, background staining with an irrelevant biotin/avidin-FITC-conjugated protein.

(Fig. 6A). Similarly, clonogenic growth of HDLM-2 cells in soft agar was reduced by 50% with anti-IL-9 moAb (1 μg/ml), which could also be reversed when IL-9 (250 units/ml) was present in the cultures (Fig. 6B).
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Discussion

In the present article we have shown by mRNA hybridization and immunostaining with anti-IL-9 mAb that primary H-RS cells express IL-9 and bear surface binding sites for this molecule. IL-9 is a growth factor for helper T-cells (8). So far it appears that IL-9 secreted by H-RS cells may contribute in part to the hyperplastic lymphoid cell reaction seen in HD-involved tissues, which is mainly characterized by activated helper T-lymphocytes surrounding H-RS cells (9). Coexpression of ligand and ligand receptor also suggests autocrine growth stimulation. To address the role of IL-9 as a possible autocrine growth factor for H-RS cells, cultured H-RS cells (cell lines HDLM-2 and KM-H2) were used as a model. Both lines share with primary H-RS cells expression of a variety of characteristic cell surface antigens and display a morphology and cytochemical staining profile identical to those of H-RS cells (11). Similar to primary H-RS cells, HDLM-2 and KM-H2 cells express IL-9, as demonstrated by mRNA hybridization and cytoplasmic immunolocalization with anti-IL-9 mAb. While HDLM-2 cells expressed abundant amounts of IL-9 transcripts and protein, IL-9 was detectable, however, only in small amounts in KM-H2 cells. Surface binding of IL-9 was also detectable in both cell lines by using biotin/avidin-fluorescein isothiocyanate-conjugated rh IL-9. Moreover, addition of rh IL-9 to HDLM-2 and KM-H2 cells led to the stimulation of clonogenic growth in semisolid agar and increased [3H]thymidine uptake in liquid suspension cultures. Detection of the proliferation-inducing capacity of IL-9 on HDLM-2 cells required the presence of neutralizing concentrations of a mAb to rh IL-9, suggesting the autocrine use of IL-9 by these cells. This notion was substantiated by experiments demonstrating that anti-IL-9 inhibited [3H]thymidine uptake by HDLM-2 cells by >80% as compared to cultures performed in the absence of anti-IL-9. Similarly, clonogenic growth of HDLM-2 cells was reduced by 50% when anti-IL-9 mAb was present. Addition of an IL-9 antisense oligodeoxyribonucleotide to cultures of HDLM-2 cells was associated with a 63-70% reduction of spontaneous [3H]thymidine incorporation by these cells. Unlike in HDLM-2 cell cultures, anti-IL-9 mAb treatment failed to show growth inhibition in both culture types performed with KM-H2 cells. These results indicate that IL-9 may act as a growth factor for cultured H-RS cells and that IL-9 may serve as an autocrine stimulus for these cells when high levels of factor are expressed. It remains to be seen in future studies whether primary HD-involved tissues can be expanded in vitro when exposed to rh IL-9 and whether other factors may stimulate the growth of H-RS cells as well.

Fig. 6. Effect of anti-IL-9 mAb on [3H]thymidine DNA incorporation by HDLM-2 cells (A) and clonogenic growth of HDLM-2 cells in soft agar cultures (B). For experimental details see "Materials and Methods." A representative experiment is shown. Two additional experiments gave similar results.

Fig. 7. An IL-9 antisense oligodeoxynucleotide specifically inhibits the proliferation of HDLM-2 cells. HDLM-2 cells were withdrawn from serum and cultured with 7.5 μM of the indicated oligomers for 18 h in the presence of [3H] thymidine during the last 12 h of culture. [3H]Thymidine incorporation into DNA was assessed by scintillation counting. Results are expressed as the percentage of control cultures that received medium treatment only and represent the mean of two separate experiments, each performed in triplicate.

References


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