Expression of interleukin-4 receptor alpha in human pleural mesothelioma is associated with poor survival and promotion of tumor inflammation

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STATEMENT OF TRANSLATIONAL RELEVANCE

Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleura that arises in a background of chronic inflammation. The overall survival of patients with MPM is poor despite multimodality treatment strategies that include surgery, chemotherapy, and radiation. In this study, we have identified interleukin-4 receptor alpha (IL-4Rα), the major subunit of the IL-4 receptor, as a biomarker with prognostic correlation in MPM. In patients undergoing resection for MPM, high tumor IL-4Rα expression was an independent predictor of decreased survival. Furthermore, freshly isolated T cells infiltrating human MPM tumors were polarized to produce IL-4, and IL-4 had direct biologic affects on MPM tumor cells that included promotion of STAT-6 phosphorylation and inflammatory cytokine production. Therefore, we believe that IL-4 endogenously produced in the tumor microenvironment may promote cancer inflammation and progression, and that interruption of the IL-4/IL-4 receptor axis may be a promising therapeutic target in human MPM.
ABSTRACT

Objectives: The origin and pathogenesis of malignant pleural mesothelioma (MPM) are closely aligned with inflammation. MPM tumors express interleukin-4 receptor alpha (IL-4Rα), the principal subunit of the IL-4 receptor. We set out to determine the biologic function and clinical relevance of IL-4Rα in human MPM.

Methods: Expression of IL-4Rα by human MPM tumors was determined by RT-PCR (n=37) and immunohistochemistry (n=52). Intracellular cytokine analysis of T cell-derived IL-4 was performed on matched tumor and blood samples from 8 MPM patients. Four human MPM cell lines were used to determine the direct effects of IL-4 on MPM tumor cells.

Results: High tumor mRNA expression of IL-4Rα was an independent predictor of poor survival in patients with epithelial MPM [HR 36(5-492), p=0.0002]. Ninety-seven percent of epithelial MPM tumors and 95% of non-epithelial MPM tumors expressed IL-4Rα protein by immunohistochemistry, and strong IL-4Rα staining correlated with worse survival in patients with epithelial histology (p=0.04). A greater percentage of tumor-infiltrating T cells produced IL-4 compared with matched blood T cells (21±7% vs. 4±2%, p=0.0002). In response to IL-4, human MPM cells demonstrated increased STAT-6 phosphorylation and increased production of IL-6, IL-8, and VEGF, without effect on proliferation or apoptosis.

Conclusions: Tumor expression of IL-4Rα is inversely correlated with survival in patients undergoing surgical resection for epithelial MPM. Tumor-infiltrating T cells in MPM are polarized to produce IL-4 and may provide endogenous activation signals to MPM tumor cells in situ. The IL-4/IL-4 receptor axis is a potential therapeutic target in human MPM.
INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive tumor that arises from the mesothelial lining of the pleura. It is a unique malignancy whose primary mode of spread is invasion of surrounding tissues and whose proclivity for distant metastases is low. MPM is a highly fatal tumor that is resilient to single modality treatment with surgery, chemotherapy, or radiation. With multimodality therapy in selected patients, median survival is 19 months (1). Approximately two-thirds of MPM patients have epithelial histology. The remaining third of patients have non-epithelial histology (sarcomatoid or biphasic) which are more aggressive tumors with significantly worse survival (2).

It recently has become evident that chronic inflammation may predispose individuals to develop cancer. Inflammation promotes the proliferation and survival of malignant cells, supports angiogenesis and metastasis, and subverts immune surveillance and responses to chemotherapy (3). MPM, in particular, is a cancer whose origin and pathogenesis are closely aligned with inflammation. Occupational exposure to asbestos is identified as the etiology of MPM in 80% of cases (4). Asbestos fibers are thought to initiate a carcinogenic inflammatory reaction marked by an influx of phagocytes into the tumor and the release of an array of pro-inflammatory cytokines such as TNF-α that promote malignant transformation of the mesothelium via NK-κβ-dependent mechanisms (5). In human MPM tumors, ongoing inflammation is represented by massive leukocyte infiltrates of T lymphocytes and macrophages whose densities are correlated with survival (6, 7).

Interleukin-4 receptor alpha (IL-4Rα) is the major subunit of the IL-4 receptor. In addition to being present on immune cells such as B cells, T cells, and macrophages, IL-4Rα is expressed on a variety of cancer cell lines (8), and in situ on a number of solid human tumors including non-small cell lung cancer, pancreatic cancer, ovarian cancer, breast cancer, prostate cancer, and head and neck cancer.
Furthermore, IL-4 production is augmented in the immune cells of cancer patients, and serum and tissue levels of IL-4 are correlated with tumor progression in man (13). In MPM, Beseth et al. have demonstrated IL-4Rα expression on mesothelioma tumors from 13 patients using immunohistochemistry (14). We set out to determine the clinical significance and biologic function of IL-4Rα in human MPM.
MATERIALS AND METHODS

Patients, tissues, and cell lines

Patients. Human tumor, blood, and effusion were acquired from patients undergoing extrapleural pneumonectomy (EPP) in accordance with Institutional Review Board policy and following informed consent. Required criteria for EPP include disease confined to the ipsilateral hemithorax and sufficient predicted postoperative pulmonary reserve. At our institution, EPP is more commonly performed over pleurectomy/decortication for MPM and likely provides more complete resection, especially for patients with bulky disease. Patient cohorts for PCR and immunohistochemistry experiments were based on previously published data sets constructed to represent institutional experience (15) and their clinicopathologic characteristics are shown in Supplemental Tables 1 and 2. For flow cytometry experiments, tissues were obtained from the operating room in patients undergoing cytoreductive surgery for biopsy-proven MPM.

Tissues. The pathologic diagnosis of MPM was confirmed in all cases. For PCR experiments, tumor samples were snap frozen on the day of resection. For flow cytometry experiments, tumor and matched blood samples were obtained fresh and used on the day of surgery. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll-Paque Plus (GE Healthcare). To isolate tumor-infiltrating mononuclear cells, tumor tissues were minced into fine fragments in cold Hank’s balanced salt solution (HBSS) and digested in RPMI (ATCC, Manassas, VA) containing 10% fetal calf serum (FCS, Gibco, Grand Island, NY), 1 mg/ml Collagenase D (Roche Diagnostic, Indianapolis, IN), and 100 μg/ml DNAse (Qiagen, Valencia, CA) at 37 degrees Celsius for 3 hours. The tissue slurry was passed through 70 μm and 40 μm filters and washed with cold HBSS. The cells were then layered over a Ficoll-Paque density gradient and centrifuged at 1000g for 20 minutes at which time the interface layer containing tumor mononuclear cells was harvested.
MPM cell lines. Four human MPM cell lines were maintained in RPMI media containing 10% FCS (Gibco). H2052 cells (epithelial origin) were obtained from the American Type Culture Collection (ATCC). MS924 (epithelial) and MS428 cells (sarcomatoid) were kindly provided by Dr. Jonathan A. Fletcher, M.D., Department of Pathology, Brigham and Women’s Hospital. JMN cells (biphasic) were a gift from Dr. James Rheinwald (16).

Quantitative PCR

Quantitative PCR was performed on MPM tumor tissues as previously described (17). Briefly, tumors were selected for portions with highest tumor density (greater than 50% tumor cells per high power field) and stored snap frozen. Total RNA (2 μg) was isolated using Trizol reagent (Invitrogen Life Technologies, Camarillo, CA) and reverse transcribed using Taq-Man Reverse Transcription reagents (Applied Biosystems, Warrington, UK). Real-time quantitative PCR (RT-PCR) was performed using a SYBR-Green fluorometric-based detection system (Applied Biosystems) with a Stratagene MX 3000P device (La Jolla, CA). The primer sequences for IL-4Rα (synthesized by Invitrogen Life Technologies) used for RT-PCR were as follows: 5′-TCATGGATGACGTGGTCAGT-3′ (forward) and 5′-GTGTCGGAGACATTGGTGTG-3′ (reverse). PCR amplification of cDNA samples were performed in triplicate using appropriate negative controls and the melting curves were in agreement with the expected amplified fragments. Ribosome 18S RNA was used as an internal control for normalization of data [5′-ATGGCCGTTCTTAGTGTGTG-3′ (forward) and 3′-CGCTGAGCCAGTCAGTGTAG-5′ (reverse)].

Immunohistochemistry and tissue microarray

Immunostaining was performed on a tissue microarray (TMA) of 58 resected MPM tumors as previously described (7). Each tumor was represented in duplicate or quadruplicate cores. Six patients were
excluded because of damage to the tissue cores, resulting in a sample size of 52. In brief, the TMA slides were soaked in xylene, passed through graded alcohols, and then placed in distilled water. The slides were next treated with citrate buffer in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) and then washed in distilled water. All further steps were performed at room temperature in a hydrated chamber. To quench endogenous peroxidase activity, the slides were pre-treated with peroxidase block (DAKO USA, Carpinteria, CA) for 5 minutes. A mouse monoclonal anti-human IL-4Rα antibody (H-4, SC-28361, Santa Cruz biotechnology, Santa Cruz, CA) was applied at 1:100 in DAKO diluent for 1 hour. Afterward, the slides were washed in 50mM Tris-Cl, pH 7.4, and detected with Mouse Envision kit (DAKO) as per manufacturer’s instructions. After another washing step, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. For image analysis, TMA slides stained with IL-4Rα were scanned at 200X magnification using an Aperio ScanScope XT workstation (Aperio Technology, Inc., Vista, CA). The images were visualized and annotated using ImageScope software (version 10.0.35.1800, Aperio Technology). For each core, three regions of interest (ROI) were selected by a pathologist (S.R.) based on areas containing predominantly tumor cells and thereby excluding the stromal compartment which harbors most of the immune infiltrate (Supplemental Figure 1). Aperio software (positive pixel count v9 algorithm) was used to deconvolute the brown (positive staining for the antigen) and blue (hematoxylin stain) colorations. The percent of the annotated area was scored by a software algorithm based upon a scale of pixel intensity ranging from 0 to 256, with 0 representing black and 256 representing white. Staining intensity thresholds were set as follows: strong (0-100), not strong (101-256). A representation of strong staining is shown in Supplemental Figure 1A and a representation of not strong staining is shown in Supplemental Figure 1B. Data were collected for each ROI and the results were averaged. Data from duplicate cores were similarly averaged.
Flow cytometry and intracellular cytokine detection

Flow cytometry was performed on mononuclear cell suspensions and cancer cell lines with a LSR II cytometer (BD Biosciences, Palo Alto, CA) using the following monoclonal antibodies: CD4-FITC, CD45-PE-Cy5, CD8-PE-Cy7, CD3-Pacific Blue (all from BD Biosciences), and IL-4Rα-APC [R&D Systems (Minneapolis, MN)]. Matched immunoglobulin isotype antibodies and unstained cells were used as controls to set gates. Detection of intracellular IL-4 was performed on blood and tumor mononuclear cell suspensions 4 hours following activation with 5 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) and 1 μM ionomycin (Sigma), in the presence of 10 μg/ml Brefeldin A (BD Biosciences). Surface staining was completed and then the cells were fixed and permeabilized. Intracellular IL-4 was assayed using an APC-conjugated anti-IL-4 antibody (intracellular cytokine detection kit, BD Biosciences). Flow cytometry data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Cell based assays

Five thousand cells were plated on 96 well cell culture plates in RPMI containing 10% FCS. Twenty-four hours later, recombinant human IL-4 (Invitrogen, Carlsbad, CA) was added at a concentration of 20 ng/ml. Cellular proliferation was measured at indicated timepoints using a MTT cell proliferation assay (ATCC) and apoptosis was assessed via measurement of caspase 3/7 activity with a luminescence-based assay (Promega, Madison, WI). ELISA was used to assay the supernatant for cytokines IL-6, IL-8, and VEGF, 24 hours after the addition of IL-4. In-Cell ELISA assays were used to quantify changes in the concentration of STAT-6 and phosphorylated STAT-6, following 24 hours of culture in 20 ng/ml of IL-4. Final values were normalized to cell number using Janus Green reagent. To detect IL-4 in tumor tissues, snap frozen MPM tumors were homogenized in buffer containing Iscove's Medium (Gibco), 10% FCS, 0.5 mM Amino-n-caproic acid (Sigma), and 0.05 mM EDTA (Sigma). An ultrasensitive ELISA
detection kit was used to detect IL-4 in the tissue homogenate. All ELISA kits were purchased from Invitrogen.

**Statistical analysis**

Statistical analysis was performed using JMP statistical software (version 8.0, SAS, Cary, NC). Distributions were plotted to examine compliance with distributional assumptions. Nonparametric tests were used to compare dichotomous variables in univariate analyses. The Wilcoxon test was used to compare two groups of continuous variables and one-way ANOVA was used to compare three groups of continuous variables. Kaplan-Meier plots were used to illustrate the cumulative proportion surviving as a function of months since surgery and differences in survival were tested for significance by the log-rank test. Cox proportional hazards regression was used to test the predictive capacity of IL-4Rα expression and overall survival in univariate and multivariate models, and to estimate 95% confidence intervals influencing survival. P values less than 0.05 were considered significant.
RESULTS

Increased expression of the IL-4Rα message is predictive of poor survival in patients undergoing resection for epithelial MPM.

Tumor expression of IL-4Rα was determined by RT-PCR of MPM tumors from 37 patients undergoing EPP. Greater than median expression levels of IL-4Rα were associated with significantly worse survival for patients with epithelial (n=22), but not non-epithelial MPM (n=15; Figure 1A). Univariate cox proportional hazards modeling identified increased IL-4Rα expression as a predictor of worse survival in patients with epithelial MPM; when adjusted for age, gender, and stage, IL-4Rα expression was retained as an independent predictor of survival (Table 1). Interestingly, a slightly higher level of IL-4Rα expression was found in non-epithelial tumors compared with epithelial tumors (Figure 1B). The expression level of IL-4Rα did not correlate with pathologic stage in the epithelial group, but could not be assessed in the non-epithelial group owing to the high distribution of stage III tumors in these patients (Figure 1C).

Increased expression of the IL-4Rα protein is predictive of poor survival in patients undergoing resection for epithelial MPM.

To confirm the expression of IL-4Rα on tumor cells from human MPM tumor specimens, we performed flow cytometry on the CD45 negative cell fraction of tumor mononuclear cell suspensions, where high frequencies of IL-4Rα positive cells were found (Figure 2A). To determine the degree of expression of the IL-4Rα protein on epithelial and non-epithelial MPM, and if expression of the IL-4Rα protein was associated with survival in MPM, we stained our TMA of tumors from 52 patients with an anti-IL-4Rα antibody (Figure 2B). IL-4Rα staining was present on the tumors of 31 of 32 patients with epithelial tumors (97%), and 19 of 20 patients with non-epithelial tumors (95%). Although a small, scattered fraction of cells in the stromal compartment of the tumor stained positive for IL-4Rα, the great majority
of IL-4Rα staining was found on the tumor cells themselves. Similar to the RT-PCR data, strong IL-4Rα staining was associated with worse overall survival following EPP in patients with epithelial MPM (Figure 2C). A greater fraction of epithelial tumors demonstrated strong staining for the IL-4Rα protein compared with non-epithelial tumors (27±4% vs. 10±2%, Figure 2D), and strong IL-4Rα staining was not associated with pathologic stage (Figure 2E). Because IL-4Rα is expressed on tumor-infiltrating macrophages in human MPM (7), we compared IL-4Rα and CD68 staining on epithelial and non-epithelial mesothelioma tumors (Supplemental Figure 1). Whereas IL-4Rα strongly stained the tumor cell compartment, there was less marked staining in the tumor stroma. Conversely, CD68 staining was predominantly found on stromal cells.

**Tumor-infiltrating T cells in human MPM are polarized to produce IL-4.**

Because T lymphocytes are a chief source of IL-4 in tumors, we performed intracellular cytokine analyses to determine whether tumor-infiltrating T cells in MPM were a potential source of endogenous IL-4. Mononuclear cells were freshly isolated from matched tumor and blood samples from 8 patients undergoing EPP for MPM (epithelial n=4, non-epithelial n=4). We found that high frequencies of tumor-infiltrating CD3 T cells produced IL-4 after stimulation (21±7%) when compared with matched blood CD3 T cells from the same patients (4±2%; Figure 3A-B), suggesting that T cells within MPM tumors are skewed to produce IL-4. Whereas both CD4 and CD8 tumor-infiltrating T cells produced IL-4 (Figure 3C), CD8 T cells were the major T cell subset responsible for IL-4 production within the tumor, representing 63±12% of tumor CD3^+^IL-4^+^ T cells, and CD4 T cells were the major T cell subset responsible for IL-4 production in the blood, representing 56±14% of blood CD3^+^IL-4^+^ T cells (Figure 3D). This difference was due mainly to a greater number of CD8 than CD4 T cells within the tumor, as a similar number of CD8 and CD4 T cells in each compartment stained positive for IL-4 (Figure 3E). Similar numbers of IL-4^+^ tumor-infiltrating T cells were found in epithelial and non-epithelial tumors (not shown). To further characterize the phenotype of tumor-infiltrating T cells in human MPM, we assayed
for the production of interferon-gamma and IL-10. Interestingly, we found that tumor-infiltrating T cells in human MPM were significant producers of interferon-gamma in response to PMA/ionomycin when compared to matched circulating T cells (Supplemental Figure 2A, 47±4% tumor, 20±12% blood, p=0.02). A small frequency of both tumor-infiltrating T cells and matched circulating T cells produced IL-10 in response to PMA/ionomycin stimulation (Supplemental Figure 2B, 5±1% tumor, 3±1% blood, p=0.2). To determine if the ligands for the IL-4 receptor are present in MPM tumors, we used ELISA to detect IL-4 and IL-13 concentrations within the tumor tissue. Low levels of IL-4 were measured in tumor tissues (n=8), whereas IL-4 was undetectable in the serum (n=10) and effusion (n=8) of MPM patients (Figure 3F). IL-13 was present in all compartments, with higher levels found in tumors and effusions than in serum (Figure 3G).

**STAT-6 signaling is activated in human MPM cancer cells in response to IL-4.**

Because human MPM tumor cells express high in situ levels of IL-4Rα, and tumor-infiltrating T cells in MPM are skewed towards an IL-4 producing phenotype, we asked whether IL-4 had a biologic effect on MPM tumor cells. To investigate the function of IL-4Rα on human MPM cancer cells, we studied four human MPM cell lines. We first confirmed that each of these cell lines demonstrated cell surface expression of IL-4Rα using flow cytometry (Figure 4A). We then cultured MPM tumor cells in the presence of IL-4 to determine if IL-4 caused activation of STAT-6 signaling. Culture with IL-4 increased the levels of phosphorylated STAT-6 in each of the four MPM cells lines by 29 to 46%, suggesting that the IL-4Rα receptor complex on MPM tumor cells is functional (Figure 4B).

**Human MPM cancer cells respond to IL-4 via production of inflammatory and angiogenic cytokines.**

IL-4 is a pleiotropic cytokine that has been shown to have varied and sometimes opposing effects on tumors that include both increasing and decreasing the proliferation and survival of malignant cells (18).
To determine whether IL-4 could provide a direct survival benefit to human MPM cells, we cultured MPM tumor cells in the presence of IL-4 and found no change in proliferation or apoptosis (Figure 5A-B). To determine whether IL-4 had pro-inflammatory or anti-inflammatory effects on MPM tumors cells, we assayed cell culture supernatants for production of various cytokines including IL-6, IL-8, and VEGF. Addition of IL-4 resulted in statistically significant increases in production of the inflammatory cytokine IL-6 on each of the four MPM cell lines (Figure 5C). MS924 and JMN cells similarly increased production of the inflammatory cytokine IL-8, whereas no change in IL-8 production was seen in H2052 or MS428 MPM cells. Culture in IL-4 resulted in increased production of the angiogenic cytokine, VEGF in H2052, MS924, and JMN cells. Although all MPM cell lines made significant quantities of transforming growth factor beta (TGF-β), no increase in production was found in response to IL-4, and no MPM cell lines made significant quantities of tumor necrosis factor-alpha (TNF-α), macrophage colony-stimulating factor (MCSF), or monocyte chemotactic protein 1 (MCP-1) while resting or in response to IL-4 stimulation (not shown).
DISCUSSION

IL-4Rα is the major subunit of the IL-4 receptor and a primary IL-4 binding protein. Following binding of IL-4 and receptor complex formation, intracellular signaling ensues through phosphorylation of STAT-6 (19, 20). IL-4Rα is expressed on a number of cancer cell lines derived from non-hematologic human malignancies that include pancreatic adenocarcinoma, renal cell carcinoma, melanoma, ovarian carcinoma, Kaposi’s sarcoma, glioblastoma, colon carcinoma, gastric carcinoma, breast carcinoma, lung carcinoma, and head and neck cancers (8, 21). Moreover, elevated expression of IL-4Rα has been found on several human tumors in situ. For example, IL-4Rα has been detected in 66% to 79% of non-small cell lung cancer tumors (9), 60% of ovarian carcinomas (10), 77% of head and neck cancers (11), and is also present on pancreatic, breast, bladder, and prostate carcinomas (12).

In addition to its expression on tumor cells, IL-4Rα is detected on circulating and tumor-infiltrating myeloid immune cells with immunosuppressive properties in cancer-bearing hosts. IL-4Rα is a marker of the recently described myeloid-derived suppressor cells in tumor-bearing mice, and also identifies a fraction of circulating mononuclear and polymorphonuclear cells with T cell suppressive function in patients with melanoma and colon cancer (22, 23). In human MPM, we have found that IL-4Rα is highly expressed on a population of tumor-infiltrating macrophages that displays an immunoregulatory phenotype (7). Given the high expression of IL-4Rα on multiple human cancers, and the tumor-promoting effects of IL-4 on human cancer cells (18), it is likely that IL-4Rα plays a role in tumor progression in humans. For example, IL-4Rα competent murine tumors exhibit increased tumor growth when compared with tumors from IL-4Rα deficient counterparts (24). Herein, we show that patients with epithelial MPM whose tumors demonstrate high IL-4Rα expression have a significant survival disadvantage following surgical resection. The reason for this difference between epithelial and non-epithelial MPM is not exactly clear, however, non-epithelial MPM is a particularly aggressive tumor and the immune response to epithelial and non-epithelial MPM is distinct (7). It should be noted that our
PCR data represents IL-4Rα expression of the entire tumor mass (tumor cells and stromal cells that include macrophages), whereas our immunohistochemistry experiments were designed to focus on tumor cell expression of IL-4Rα. To our knowledge, this is the first study to equate tumor expression of IL-4Rα with survival indices in patients with cancer.

The distinction of Th1 and Th2 immune responses has important implications for patients with cancer. Antitumor immune responses are sustained principally by tumor-infiltrating lymphocytes consisting primarily of T cells. Whereas Th1 responses typically signify the production of IL-2 and interferon-gamma with activation of cytotoxic T cell responses, Th2 responses generally represent a cytokine profile including IL-4, IL-5, IL-6, and IL-13 (25), of which IL-4 is the prototypical Th2 cytokine. IL-4 has differential affects on immunity that could be advantageous to tumor growth. These include inhibition of Th1 cell differentiation and cellular immunity, abrogation of CD8 T cell cytotoxicity, accumulation of Th2 cytokines, and facilitation of Th2 CD4 T cell development (26, 27). Most clinical studies support the finding of an abnormal Th1 to Th2 ratio in patients with various cancers and it has been shown that the nature of the immune response (Th1 vs. Th2) can affect outcome in cancer patients. Specifically, high serum and tumor IL-4 levels, and high frequencies of IL-4 secreting T cells are elevated in cancer patients and parallel worse prognosis (28-30). Our data demonstrate that the natural immune reaction to human MPM involves tumor-infiltrating T cells skewed towards an IL-4 producing phenotype.

The direct effects of IL-4 on tumor cells are varied and sometimes opposing. In human colon cancer cell lines, treatment with IL-4 results in dose-dependent increases in cellular proliferation (31), and IL-4 is an autocrine growth factor made by pancreatic cancer cells (21). IL-4 protects human prostate, breast, and cancer cell lines from chemotherapy and death receptor-induced apoptosis through upregulation of anti-apoptotic proteins cFLIP/FLAME-1 and Bcl-xL (12). Similar results are seen in murine in vivo tumor models where IL-4 increases tumor growth, reduces apoptosis, and promotes...
metastases (24, 32). Conversely, IL-4 is required for the development of effective antitumor immunity (33). For instance, IL-4 can have a direct inhibitory effect on proliferation of human cancer cells (34, 35), and IL-4 can be a potent anti-tumor agent in some animal models (36, 37). It has been suggested that the differential positive and negative effects of IL-4 on tumor development may be explained by the source of IL-4, with exogenously delivered IL-4 exerting tumor suppressing effects and endogenously produced IL-4 exerting tumor promoting effects (18).

Our data demonstrate that IL-4 did not have a direct effect on MPM tumor cell line proliferation or apoptosis. Activation of human MPM cells with IL-4 resulted in upregulation of STAT-6 phosphorylation and an increase in production of inflammatory and angiogenic cytokines including IL-6, IL-8, and VEGF. Each of these cytokines has been shown to be produced by MPM cell lines where they act as potent autocrine growth factors, and are each detected at significant levels in the effusions of MPM patients (38-42). IL-6 is a multi-functional, pro-inflammatory cytokine involved in acute phase inflammatory responses and additionally stimulates VEGF production by MPM cells (43). It has been suggested that IL-6 may be responsible for the systemic manifestations of MPM that include fever, cachexia, and thrombocytosis. (44). IL-8 is a member of the C-X-C chemokine family and has direct growth-potentiating and angiogenic effects on tumors. VEGF is a powerful mitogen for the vascular endothelium and in MPM patients, elevated serum levels of VEGF correlate with advanced disease stage and worse survival (45). It is reasonable to surmise that each of these factors has tumor-promoting effects in the tumor microenvironment.

Taken together, the high expression of IL-4Rα on human tumor cells, its association with pro-tumor function, and its correlation with survival reported here make it a promising therapeutic target. Puri’s group has targeted tumor IL-4Rα with a chimeric protein consisting of a circularly permuted IL-4 molecule that is fused to a truncated from of Pseudomonas exotoxin. This agent has been shown to
have potent cytotoxic activity in vitro against numerous IL-4Rα expressing cell lines and in vivo in xenograft models of various human cancers including MPM (8, 14, 46, 47). Ultimately, this molecule has been brought to clinical trials and has shown encouraging results in patients with glioblastoma (48).

In summary, MPM is a malignancy whose origin and development are deeply rooted in inflammation. We have found that IL-4Rα is highly expressed in situ by tumor cells in human MPM tumors, tumor expression of IL-4Rα is predictive of survival in patients undergoing cytoreductive surgery for epithelial MPM, and that MPM tumor cells respond to IL-4 via upregulation of STAT-6 signaling and inflammatory cytokine production. We hypothesize that IL-4 endogenously produced by MPM tumor-infiltrating T cells provides stimulus to the IL-4 receptor on MPM tumor cells in vivo, and promotes inflammatory and angiogenic signals that stimulate tumorigenesis. Taken together, it seems that mesothelioma tumors with high IL-4Rα expression are clinically more aggressive cancers that have worse outcome after surgical resection. These data have important implications for future research and clinic applications. IL-4Rα is a potentially useful marker of prognosis in patients undergoing surgical resection, and possibly also for patients undergoing definitive chemotherapy, however this requires further investigation. Moreover, the IL-4-IL-4Rα axis represents a promising target for directed therapy in man, with potential application for IL-4 and IL-4 receptor blocking agents and IL-4Rα specific cytotoxins.
REFERENCES


FIGURE LEGENDS

Figure 1. Expression of the IL-4Rα message predicts survival in human MPM. (A) IL-4Rα mRNA expression was determined by RT-PCR in MPM tumor specimens obtained following EPP in patients with all (n=37), epithelial (n=22), and non-epithelial histology (n=15). Patients were divided into groups of either greater than, or less than median tumor IL-4Rα expression. Expression of tumor IL-4Rα was compared between epithelial and non-epithelial tumors (B) and among tumors of varying pathologic stage (C).

Figure 2. Expression of the IL-4Rα protein is associated with survival in human MPM. (A) IL-4Rα expression on tumor cells from human MPM tumors was confirmed by flow cytometry on CD45 negative tumor mononuclear cells (n=4). IL-4Rα protein expression was determined by immunohistochemistry on MPM tumor specimens obtained following EPP for patients with all (n=52), epithelial (n=32), and non-epithelial histology (n=20). (B) Representative cores from epithelial and non-epithelial MPM stained with IL-4Rα. (C) Kaplan-Meier survival curves are shown for patients with either high or low tumor expression of IL-4Rα. (D) The percentage of patients with high tumor expression of IL-Rα was compared between epithelial and non-epithelial histology. (E) The percentage of patients with high tumor expression of IL-4Rα was stratified by pathologic stage.

Figure 3. Tumor-infiltrating T cells in human MPM are polarized to produce IL-4. Mononuclear cells were freshly isolated from matched blood and tumor specimens from patients undergoing surgical resection of MPM. Intracellular IL-4 production was determined in CD3 T cells in by intracellular cytokine analysis following 4 hours of non-specific stimulation with PMA and ionomycin. (A) Flow cytometry dot plots from one representative patient. (B) Summary data of matched tumor and blood CD3^+IL-4^+ cells from 8 patients. (C) Representative contour plot of intratumoral CD4 and CD8 T cell...
subsets and intracellular IL-4 production. (D) CD4 and CD8 subset composition of CD3^IL-4^ T cells (n=5). (E) The percentage of CD4 and CD8 T cells that stain positive for IL-4 (n=5). IL-4 concentrations (F) and IL-13 concentrations (G) in tumor (n=8), effusion (n=8), and serum (n=10) of patients with MPM was determined by ultrasensitive ELISA. Values represent pg/mg of tissue and pg/ml of effusion and serum.

**Figure 4. Human MPM cells respond to IL-4 by activation of STAT-6 signaling.** (A) The presence of IL-4Rα on 4 human MPM cell lines was determined by flow cytometry. Shaded histograms represent IL-4Rα staining and empty histograms represent isotype controls. (B) Each human MPM cell line was cultured for 24 hours in the presence of 20 ng/ml of IL-4, and the level of STAT-6 and phosphorylated STAT-6 was determined by intracellular ELISA. These experiments were performed a minimum of three times.

**Figure 5. Human MPM cells produce pro-inflammatory and angiogenic cytokines in response to IL-4.** Four human MPM cell lines were cultured in the presence of 20 ng/ml of IL-4. On days 1, 3, and 5 thereafter, cellular proliferation was assayed by MTT (A), and apoptosis was assayed by determination of caspace 3/7 activity measured by relative light units (B). Twenty-four hours after the addition of IL-4, supernatants were assayed for IL-6, IL-8, and VEGF via ELISA (C). These experiments were performed a minimum of three times. RLU-relative light units.
ACKNOWLEDGEMENTS

The authors would like to thank Dr. Christina Wei for her assistance with PCR.
Table 1. Association of IL-4Rα message with survival in MPM.

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<th>Variable</th>
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<td></td>
<td>Hazards Ratio (95% CI)</td>
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</tbody>
</table>

*Footnote: Adjusted for age, gender, stage.
Figure 1
Figure 4

A.

B.
Expression of Interleukin-4 Receptor Alpha by Human Pleura Mesothelioma is Associated with Poor Survival and Promotion of Tumor Inflammation

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