Interleukin 23 regulates proliferation of lung cancer cells in a concentration-dependent way in association with the interleukin-23 receptor

Jun Li, Le Zhang, Jie Zhang, Yuyan Wei, Kai Li, Lugang Huang, Sen Zhang, Bo Gao, Xiujie Wang and Ping Lin

Division of Geriatrics, Center for Medical Stem Cell Biology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China and 1Department of Pediatric Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

© The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

A proinflammatory cytokine, interleukin 23 (IL-23), plays a role in tumor progression by inducing inflammation in the tumor microenvironment, although there is debate about its role in carcinogenesis. Direct effects of IL-23 on tumor cells have been reported rarely, and contradictory effects have been observed. Here, we studied such effects of IL-23 in lung cancer cells in vitro and in vivo and explored the underlying mechanism. We found IL-23 receptor expression in tissues from lung adenocarcinoma and small cell carcinoma but not in lung squamous cell carcinoma tissue. Interestingly, different concentrations of IL-23 had opposite effects in the same types of cells. We confirmed that the different effects could be explained by differences in binding to the IL-23 receptor (subunits IL-23r and IL-12Rβ1). Low concentrations of IL-23 promoted the proliferation of IL-23 receptor-positive A549 and SPCA-1 lung cancer cells by binding to IL-23r, whereas high concentrations of IL-23 inhibited proliferation of these cells by binding to both IL-23r and IL-12Rβ1. In contrast, IL-23 had no effect on IL-23 receptor-negative SK-MES-1 cells. IL-23 regulated the growth of human lung cancer cells through its effects on STAT3 expression and phosphorylation in a concentration-dependent way; the ki-67 gene was involved in these processes. Our findings demonstrate for the first time that IL-23 affects the proliferation of IL-23 receptor-positive lung cancer cells and that this effect is dependent on the IL-23 concentration. This can explain at least part of the inconsistent reports on the role of IL-23 in the progression of carcinogenesis.

Introduction

Lung cancer is one of the most lethal cancers throughout the world and kills at least 1 million people each year (1). Conventionally, inflammation is regarded as a strong promoter of carcinogenesis and malignancy of many different types of cancer, suggesting that inflammation provides the cancer cells a hospitable microenvironment in which they grow. Expression of interleukin 32 (IL-32) in human lung cancer is related to the histotype and metastatic phenotype (2). Yamaguchi et al. (3) reported that the serum IL-6 level was higher in 37.8% of 339 patients with lung cancer than in normal persons. Expression of IL-1 receptor-associated kinase-1, which leads to nuclear factor-κB activation and plays a key role in inflammation, has also been identified in non-small-cell lung cancer (4). Inflammation seems to be an important mediator in the development of lung cancer. Because IL-23 is known to be involved in some types of pulmonary diseases (5), we reasoned that the IL-23 and the IL-23 receptor may also be associated with lung cancer.

The proinflammatory cytokine IL-23 participates in and sustains the progression of chronic inflammation. IL-23 is a heterodimer comprising IL-12p40 and IL-23p19 subunits. The IL-23 receptor comprises the IL-23r and IL-12Rβ1 subunits (6). IL-23 appears to play contradictory roles in carcinogenesis. Some studies have shown that, similar to IL-12, IL-23 inhibits tumor growth, but recent studies suggest that IL-23 promotes carcinogenesis. IL-23 is a potent antimetastatic agent in a variety of murine tumor models. It causes regression of established tumors (7–9) and inhibits the formation of experimental metastases (7,8) and spontaneous metastases (10,11). Exogenously overexpressed IL-23 inhibits cancer progression. Murine colon carcinoma CT26 and B16F10 tumor cells transfected with the IL-23 gene exert potent antitumor and antimitastatic effects similar to those of IL-12 (12–14). In contrast, Langowski et al. (15) reported that genetic deletion or antibody-mediated elimination of IL-23 leads to increased infiltration of cytotoxic T cells into the transformed tissue, rendering a protective effect against chemically induced carcinogenesis. Expression of IL-23 and its receptors is detectable mainly in activated macrophages, dendritic cells and keratinocytes in healthy skin (16).

In our earlier study, we found that the IL-23 receptor is expressed in SW-480 colon cancer cells and that IL-23 is involved in colorectal cancer progression (17). Our data suggested that IL-23 can affect tumor cells directly. There are only two reports on the direct effects of IL-23 on tumor growth, and the results in these reports are completely opposite. Cocco et al. reported that IL-23 acts as an antitumor agent in childhood acute B-lymphoblastic leukemia cells (18). In contrast, Fukuda et al. (19) reported that IL-23 promotes growth and proliferation in human squamous cell carcinoma (SCC) of the oral cavity. The reasons for these opposing effects and the mechanism underlying any effect of IL-23 on lung cancer proliferation are unclear. The aim of this study was to determine whether IL-23 can increase or inhibit lung cancer cell proliferation, and, if so, to identify the underlying mechanism.

Materials and methods

Cells, mice and reagents

Human lung adenocarcinoma (AC) cell line A549, SPCA-1 and human lung SCC line cells SK-MES-1 were obtained from the China Center for Type Culture Collection (Wuhan, China), maintained in RPMI-1640 medium (Gibco) or Dulbecco’s modified Eagle’s medium (high glucose; Gibco) containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma, St Louis, MO). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2.

Recombinant human IL-23 (hrIL-23) was bought from R&D Systems (Minneapolis, MN). For IL-23r and IL-12Rβ1 neutralization experiments, the human anti-IL-23r antibody (anti-IL-23r) and human anti-IL-12Rβ1 antibody (anti-IL-12Rβ1) were bought from R&D Systems, also. Anti-IL-23p19 neutralization antibody (anti-IL-23p19) was purchased from eBiocience. Cells were treated with various concentrations of IL-23 with or without anti-IL-23p19 (0.3 μg/ml) or anti-IL-12Rβ1 (1 μg/ml) or anti-IL-12Rβ1 (1 μg/ml) and anti-IL-23p19 (1 μg/ml) AG490 (Sigma) was dissolved in ethanol (5 mg/ml), diluted with the culture medium and then used as 20 μM for experiments. BALB/c male nude mice, used at 6–8 weeks, were bred and maintained under standard housing conditions in the animal facility of Sichuan University. All experiments in this study were performed in accordance with nation’s relevant laws and animal welfare requirements.

Tissues

Forty lung SCC, 40 lung AC and 37 small-cell lung carcinoma (SCLC) tissues archived formalin-fixed, paraffin-embedded samples were obtained from West China Hospital. The stages and histological grades of these tumors were
established according to the criteria of the Tumor Node Metastasis (TNM). Prior written and informed consent was obtained from each patient and the study was approved by the ethics committee of the Medical Faculty of Sichuan University. Clinical and pathological data relating to the clinical samples are presented in Supplementary Table 1, available at Carcinogenesis Online.

**Immunohistochemistry**

Lung cancer tissue and the tumor from BALB/c nude mice embedded in paraffin were sliced and were pretreated with hydrogen peroxide. The lung cancer tissue slides were incubated with rabbit-antihuman IL-23r antibody (1:100 dilution; Boaoesen, Beijing, China) and rabbit-antihuman IL-12Rβ1 antibody (Boaoesen, 1:100 dilution) overnight at 4°C. The tumor from BALB/c nude mice was incubated with rabbit-antihuman ki-67 antibody (1:100 dilution; Boaoesen). Subsequently, the tissues were incubated with biotinylated secondary anti-rabbit immunoglobulin (IgG) (1:800 dilution; Zhongshan, Beijing, China). Diaminobenzidine was used as a substrate chromogen, and slides were counterstained with hematoxylin. Rabbit isotype IgG (1:300) was designed to be the corresponding native control. The positively stained tumor cells were assessed in 40 fields selected randomly, at a final magnification of ×400. The expression of IL-23r and IL-12Rβ1 was scored by proportion and intensity, according to Allred’s procedure (20). In brief, the proportion score represented the proportion of tumor cells staining positive, which was as follows: 0 (none), 1 (1–10%), 2 (11–50%), 3 (51–100%) and 4 (>100%). The intensity score represented the average intensity of the positive cells, which was as follows: 0 (none), 1 (weak), 2 (intermediate) and 3 (strong). The total score was proportion add intensity scores, which range from 0 to 8. Tumors with total score of 0–2 were identified as negative staining and tumors with score of 3–8 were estimated as positive staining in this study (Supplementary Table 2, available at Carcinogenesis Online).

**Immunofluorescence**

Cells were plated onto sterile round microscope slides in 24-well plates and grown to 70% confluence. After washing, cells were fixed with 4% paraformaldehyde for 30 min at 4°C. After three washes, cells were blocked with 5% non-fat milk for 1 h at room temperature followed by incubation with rabbit-antihuman IL-23r antibody (dilution, 1:100) and rabbit-antihuman IL-12Rβ1 antibody (1:100 dilution) at 4°C for overnight. After several washes, cells were incubated with an Alexa fluor® 488-conjugated secondary antibodies (1:100 dilution; Boaoesen) for 1 h. 4′,6-diamidino-2-phenylindole (5 μg/ml) (Invitrogen) was used to stain nuclei. Rabbit isotype IgG (1:300) was designed to be the corresponding native control. The positively stained tumor cells were assessed in 40 fields selected randomly, at a final magnification of ×400.

**MTT assay**

Cells proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549, SPCA-1 and SE-MES-1 cells were plated at density of 5 × 10^4 cells per well in 96-well plates treated with different concentrations of IL-23 (0, 5, 10, 20 or 40 ng/ml) or IL-23 together with anti-IL-23p19, anti-IL-23r and IL-12Rβ1. After 72 h of treatment, MTT assay was performed. Finally, the optical density was determined at 570 nm using the enzyme-linked immunosorbent assay plate reader (Model 550; Bio-Rad, Hercules, CA). At least three independent experiments were performed.

**Colony formation assay**

A549, SPCA-1 and SE-MES-1 cells were plated at a density of 300 cells per well in 24-well plates and treated with IL-23 (0, 5, 10, 20 or 40 ng/ml) or IL-23 together with anti-IL-23p19, anti-IL-23r and IL-12Rβ1. When the plates appeared visibly clone, the clones were fixed with methanol and stained by crystal violet for 20 min. The number of clones in wells, which is over 50 cells, was counted using microscope. At least three independent experiments were performed.

**Quantitative real-time PCR**

Total RNA was isolated from cells by using the TAKARA kit (Takara, Dalian, China), according to the manufacturer’s protocol. Each 1 μg of total RNA derived from cells was subjected to reverse transcription with a PrimeScript TMRT reagent kit (Takara), and PCR protocol consisted of one cycle at 95°C for 10 s followed by 40 cycles at 95°C for 5 s and at 60°C for 45 s. Gene-specific primers used to determine the relative expression levels of STAT3 and β-actin were as follows: human STAT3, forward 5′-CAG TGA ATG TGG GAA ATA ATG GTG A-3′ and reverse 5′-CAT GTC GTA ACG GGA GGT GTC CTC-3′; human ki-67, forward 5′-CAT GGA TCA TAC ACG CCG TAG TGC CTC-3′ and reverse 5′-TGC GAT AGA CAC TCT CTT TTG-3′; and β-actin, forward 5′-AAC GTG ACA GCA GTC GTG-3′ and reverse 5′-GGT TGG GTT-3′. The expression of β-actin was used to normalize for transcription. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. All assays were performed in triplicates.

**Western blot analysis**

Whole-cell protein extracts from cells were prepared with lysis buffer for 30 min on ice. Protein concentrations were determined using an assay kit (Bio-Rad). Fifty micrograms of protein lysates were loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were incubated in blocking buffer (Tris-buffered saline containing 5% skim milk) for 1 h at 37°C, followed by hybridization with rabbit-anti-p-STAT3 (tyr-705) antibody, rabbit-anti-STAT3 antibody (1:1000 dilution; Cell Signaling Technology), anti-Ki-67 (1:500 dilution; Boaoesen) or rabbit-anti-β-actin antibody (1:100 dilution; Lab Vision, Fremont, CA) at 4°C overnight. After washing, the membranes underwent hybridization with horseradish peroxidase-conjugated secondary antibody rabbit IgG (Santa Cruz Biotechnology, 1:5000 dilution) for 1 h at room temperature. After washing signals were detected by chemiluminescence using western blotting luminol reagent (Santa Cruz Biotechnology). Protein levels were quantified by scanning blots on a Gel Doc EZ imager (Bio-Rad) and analysis with Quantity One 1D image analysis software 4.4.0 (Bio-Rad).

**Apoptosis assay**

A549 cells were cultured with recombinant human IL-23 (5, 10, 20 or 40 ng/ml) for 3 days. Apoptosis was assessed by flow cytometry (FACSAnia, Becton Dickinson) using the annexin V/fluorescein isothiocyanate kit (Keygen, China), according to the manufacturer’s protocol. Statistical analysis was performed using unpaired Student’s t-test, with P values 0.05 considered statistically significant.

**Fig. 1.** IL-23r and IL-12Rβ1 are expressed in human lung cancer tissues and cells. Immunohistochemistry showed that IL-23r (left panel) and IL-12Rβ1 (middle panel) positive expressed in AC tissues and SCLC tissues, but not in SCC tissues. Rabbit IgG was used as negative control (right panel). The positive cells were brown. The antigenic determinant of these receptors were located in the intracellular domain and membrane, as a result, the positive cells were cytoplasmic positive and membranous positive. The IL-23r and IL-12Rβ1 were highly expressed in AC tissues than in SCLC tissues. Immunofluorescence showed that IL-23r (left panel) and IL-12Rβ1 (middle panel) positive expressed in AC cells A549 and SPCA-1, but not in SCC tissues SK-MES-1. 4′,6-Diamidino-2-phenylindole was used to stain nuclei. Magnification, ×400. (micron bar = 20 μm).
In vivo experiments

To test the effect of IL-23 on tumor growth, $1 \times 10^7$ A549 cells were mixed with 500 µl of liquid Matrigel (BD Biosciences). BALB/c nude mice were randomly divided into eight groups as follows (10 mice each group): IL-23 and Matrigel (IL-23); A549 cells and Matrigel (A549); A549 and 10 ng/ml IL-23 (A549 + IL-23/10); A549 and 40 ng/ml IL-23 (A549 + IL-23/40); A549, 10 ng/ml IL-23 and anti-IL-23p19 (A549 + IL-23/10 + Ab); A549, 40 ng/ml IL-23 and anti-IL-23p19 (A549 + IL-23/40 + Ab); A549, 10 ng/ml IL-23 and IgG (A549 + IL-23/10 + IgG) and A549, 40 ng/ml IL-23 and IgG (A549 + IL-23/40 + IgG). SK-MES-1 cells were treated in the same way for the eight groups.

A549 cells were also subjected to other experiments using four other groups of 10 mice each: A549, 10 ng/ml IL-23 and anti-IL-23r (A549 + IL-23/10 + Abr); A549, 40 ng/ml IL-23 and anti-IL-23r (A549 + IL-23/40 + Abr); A549, 10 ng/ml IL-23 and anti-IL-12Rβ1 (A549 + IL-23/10 + Abβ1) and A549, 40 ng/ml IL-23 and anti-IL-12Rβ1 (A549 + IL-23/40 + Abβ1). The cell mixture was injected subcutaneously into the flanks of nude mice. The tumor dimensions were measured using a linear caliper, and tumor volume $V$ was calculated using the formula: $V (cm^3) = a \times b^2/2$, where $a$ is the larger diameter and $b$ is the shorter diameter. We recorded the time as the tumor-forming time when the tumor volume reached 0.5 cm$^3$. Two weeks later, the tumor mass was obtained by measuring the volume and weight. All tissues were formalin fixed and paraffin embedded for histological examination.

Statistical analysis

Data were expressed as mean/median ± SD. To evaluate the significant differences between two groups, the means were compared using Student’s $t$-test. Multiple group comparisons were performed using one-way analysis of variance. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL).

Results

IL-23r and IL-12Rβ1 are expressed in human lung cancer tissues and cells

To study whether IL-23 acts on lung cancer cells, we first measured the expression of the IL-23 receptor subunits IL-23r and IL-12Rβ1 in human lung cancer tissues and cells. As shown in Figure 1, positive expression of IL-23r and IL-12Rβ1 was observed in carcinoma cells in AC tissues. Weaker expression of IL-23r and IL-12Rβ1 was observed in SCLC tissues, whereas no expression of IL-23r and IL-12Rβ1 was detected in SCC tissues. IL-23r was expressed in 90.0% of AC tissues (36/40) and in 86.5% of SCLC tissues (32/37); IL-12Rβ1 was expressed in 82.5% of AC tissues (33/40) and in 81.1% of SCLC tissues (30/37) (Supplementary Table 2, available at Carcinogenesis Online). Strong positive expression of the IL-23r and IL-12β1 was observed in A549 and SPCA-1 cells (AC cells) but was not detected in SK-MES-1 cells (SCC cells) (Figure 1). Expression of the IL-23 receptor in lung cancer cells suggests that IL-23 can act directly on lung cancer cells.
IL-23 and proliferation of lung cancer cells

Low concentration of IL-23 increases and a high concentration of IL-23 inhibits the proliferation of human lung cancer cells

The proliferation of lung cancer cells was analyzed by MTT assay and a colony-forming assay. Cells were treated with IL-23 (0, 5, 10, 20 or 40 ng/ml) for 3 days. The MTT assay (Figure 2A–C) indicated that a low concentration of IL-23 (10 or 20 ng/ml) promoted the proliferation of A549 and SPCA-1 cells (P < 0.05), whereas a high concentration of IL-23 (40 ng/ml) inhibited the proliferation of these IL-23 receptor-positive cells (P < 0.05). However, IL-23 had no effect on IL-23 receptor-negative SK-MES-1 cells (P > 0.05). Similar results were obtained for the colony-forming assay (Figure 2D–F). To confirm that IL-23 affected the growth of lung cancer cells, cells were treated with anti-IL-23p19 antibody (Ab). As shown in Figure 2, blocking IL-23 with anti-IL-23p19 antibody (Ab) eliminated the effect of exogenous IL-23 on cell growth. This result suggests that IL-23 affects the proliferation of lung cancer cells, but that the effect depends on the concentration of IL-23 and the expression of the IL-23 receptor on the cells.

The proliferation of lung cancer cells is regulated by binding of IL-23 to the IL-23 receptor

Why did different concentrations of IL-23 have a completely opposite effect? We suspected that this difference was caused by the different affinity of the two receptor subunits: high-affinity IL-23r and low-affinity IL-12Rβ1 (21,22). We used anti-IL-23r (Abr) and anti-IL-12Rβ1 antibodies (Abβ1) to block IL-23r and IL-12Rβ1, respectively. As shown in Figure 3, blocking IL-23r and IL-12Rβ1 with both antibodies did not affect lung cancer cell proliferation and clone formation. With blockade of only IL-12Rβ1, a low concentration of IL-23 could still promote IL-23 receptor-positive cell growth, whereas the inhibiting effect of a high concentration of IL-23 was reversed (P < 0.05). This indicated that IL-12Rβ1 is the major mediator of growth inhibition in the presence of a high concentration of IL-23. When IL-23r only was blocked, IL-23 had no effect on the proliferation of lung cancer cells at all concentrations tested; in other words, both the promoting and inhibiting effects of IL-23 were cancelled. This implies that IL-23r is essential for IL-23 function and that IL-12Rβ1 must combine with IL-23r to acquire its biological activity. We conclude that at low concentrations, IL-23 binds to the higher affinity IL-23r and promotes cell proliferation, whereas at higher concentrations, IL-23 binds to both IL-23r and IL-12Rβ1 and inhibits cell proliferation.

Expression and phosphorylation of STAT3 in A549 cells are influenced by IL-23 and the IL-23 receptor

STAT3 is constitutively activated in diverse cancers (23,24) and IL-23 could activate the JAK–STAT3 pathway by binding to the IL-23 receptor in the tumor microenvironment (25). To explore the
Effect of STAT3 on the proliferation of lung cancer cells induced with IL-23, we detected the messenger RNA (mRNA) and protein expression of STAT3 and phosphorylated STAT3 (pSTAT3) in A549 cells treated with or without IL-23 for 48 h. Consistent with the tumor cell proliferation pattern, the expression of mRNA (Figure 4A) and protein, and the phosphorylation of STAT3 (Figure 4B) in A549 cells were increased by low concentrations of IL-23 (5, 10 or 20 ng/ml) but were inhibited by a high concentration of IL-23 (40 ng/ml) (**P < 0.05). The increase in the expression and activation of STAT3 induced by low concentrations of IL-23 was prevented by blocking IL-23r with anti-IL-23r antibody (Abr). The decrease in the expression and activation of STAT3 induced by a high concentration of IL-23 was attenuated by blocking IL-12Rβ1 with anti-IL-12Rβ1 antibody (Abβ1). These results suggest that IL-12Rβ1 inhibits the activation of the STAT3 pathway induced by a high concentration of IL-23.

Our findings indicated that the influence of IL-23 on cell growth is associated with the STAT3 pathway. To confirm the effect of STAT3 on tumor proliferation induced by IL-23, we used AG490 (20 µM), an inhibitor of JAK–STAT3, to inhibit the phosphorylation of STAT3 in A549 cells stimulated with IL-23 (10 ng/ml). Figure 4C shows that STAT3 phosphorylation was blocked by AG490. Figure 4D and E show that AG490 attenuated the effect of various concentrations of IL-23 on the proliferation of A549 cells. AG490 cancelled the promotion of colony formation by A549 cells by low concentrations of IL-23 and the inhibition of colony formation by a high concentration of IL-23. These findings suggest that the phosphorylation of STAT3 is an indispensable event for the IL-23-induced stimulation of the proliferation of lung cancer cells.

Phosphorylation of STAT3 regulates Ki-67 expression

To explore the mechanism by which IL-23 regulates lung cancer cells proliferation, we assessed apoptosis of A549 cells and the expression of Ki-67 as a representative gene for cell proliferation. Different concentrations of IL-23 (5, 10, 20 or 40 ng/ml) had no obvious effect on the apoptosis of A549 cells (Supplementary Figure 1, available at Carcinogenesis Online), but the expression of Ki-67 increased significantly. We detected STAT3 and Ki-67 mRNA expression at different time points (0, 12, 24, 36, 48, 60 and 72 h) after A549 cells were treated with IL-23 at 10 ng/ml. The expression of STAT3 and Ki-67 increased significantly. The expression peak of Ki-67 occurred later than the peak of STAT3 expression (Figure 5A and B), suggesting that Ki-67 expression is regulated by STAT3 in A549 cells treated with IL-23. We detected Ki-67 expression at the genetic and protein levels in A549 cells treated with or without IL-23 for 48 h. Figure 5C shows similar trends: the expression of Ki-67 in A549 cells was increased by IL-23 at 10 ng/ml but was decreased by IL-23 at 40 ng/ml (**P < 0.05). To explore further the relationship between Ki-67 expression and phosphorylation of STAT3, we measured Ki-67 expression in A549 cells treated with IL-23 and AG490 (Figure 5C). AG490 inhibited the effect of IL-23 on the expression of Ki-67, indicating that...
IL-23 and proliferation of lung cancer cells

Phosphorylation of STAT3 is related to the effects of IL-23 on Ki-67 expression.

IL-23 regulates A549 cells growth in vivo

Four measures of tumor growth were obtained: volume, weight, formation time and expression of Ki-67 in tumor tissues (Figure 6). The tumor mass grew in the Matrigel + A549 cell mixture groups but not in the IL-23 groups. All four parameters indicated that tumor growth was greater in the A549 + IL-23/10 group but lower in the A549 + IL-23/40 group compared with the A549 group (P < 0.05). Tumor growth was lower in the A549 + IL-23/10 + Ab group compared with the A549 + IL-23/10 + IgG group but was greater in the A549 + IL-23/40 + Ab group compared with the A549 + IL-23/40 + IgG group (P < 0.05). Tumor growth did not differ significantly between the A549 + IL-23/10 + Abr, A549 + IL-23/40 + Abr and IgG groups. In contrast, tumor growth was greater in the A549 + IL-23/10 + Abβ1 and A549 + IL-23/40 + Abβ1 groups compared with the IgG groups. These results suggest that the growth of A549 tumor cells increased at a low dose of IL-23 but was inhibited at a high dose of IL-23.

This dose-dependent effect of IL-23 appears to reflect differences in the affinity of binding to IL-23r and IL-12Rβ1. Such effect can be eliminated by IL-23-neutralizing antibody. We measured the in vivo growth of IL-23 receptor-negative cell SK-MES-1 cells. Interestingly, the growth of SK-MES-1 cells differed between in vivo and in vitro conditions. The tumors were larger in the SK-MES-1 + IL-23/10 and SK-MES-1 + IL-23/40 groups than in the control group (Supplementary Figure 2, available at Carcinogenesis Online). This suggests that even when mixed with Matrigel, IL-23 may have influenced the immune cells, which then affected the growth of SK-MES-1 cells in vivo.

Discussion

The relationship between chronic inflammation and tumor development has been debated intensely in recent years. The IL-12 family of cytokines plays an important role in chronic inflammation and has been reported to be associated with tumor development (7,26). IL-12 induces antitumor immune responses, whereas IL-23 has been identified recently as a link between tumor-associated inflammation and tumor immune escape (15,27). Endogenous IL-23 promotes tumor progression, and anti-IL-23 monoclonal antibody can suppress tumor growth and metastasis (28). Some studies have reported that exogenously overexpressed IL-23 can facilitate antitumor activity (12–15). However, direct actions of IL-23 on tumor cells have been reported rarely. And the direct role of IL-23 in carcinogenesis seems to have two aspects.

We first analyzed the expression of the IL-23 receptor subunits, IL-23r and IL-12Rβ1, in human lung cancer tissues and cells. Interestingly, expression of the IL-23 receptor was not detected in all pathological types of lung cancer: IL-23r and IL-12Rβ1 were expressed in AC (90%/82.5%) and in SCLC (86.5%/81.1%) tissues, but not in SCC tissues. We considered the clinical and pathological paring, tumor stage, tumor size and patient age, but have not drawn the corresponding conclusion. Perhaps, it was not enough due to our samples. We will study this phenomenon in our future work. Regardless of the reason, our data raise the possibility that IL-23 acts
directly on some types of lung cancer cells. In the *in vitro* proliferation assay, at low concentrations, IL-23 promoted the proliferation of A549 and SPCA-1 cells. In contrast, a high IL-23 concentration inhibited the proliferation of these IL-23 receptor-positive cells, and IL-23 had no effect on IL-23 receptor-negative SK-MES-1 cells. The growth of A549 cells *in vivo* was similar to that *in vitro*. Our findings suggest that the IL-23 receptor is necessary for the activation of IL-23 function and that IL-23 affects IL-23 receptor-positive cell growth.

Fig. 6. IL-23 regulated A549 growth *in vivo*. Two weeks after A549 cells were injected, the tumor mass was measured. The tumor mass grew in the Matrigel + A549 cell mixture groups but not in the IL-23 group. (A) The volume and the weight of the tumor. (B) The time for the tumor volume to reach 0.5 cm³. (C) The samples were stained for immunohistochemical analysis with anti-human Ki-67 antibody, and the positive cells were counted. The positively stained tumor cells were assessed in 20 fields selected randomly at a final magnification of ×400. Bar = 20 µm. Each column and bar represents the median ± SD of three independent experiments. And the photograph is one represent of three independent experiments.
IL-23 and proliferation of lung cancer cells

directly in a concentration-dependent manner. The growth of IL-23 receptor-negative SK-MES-1 cells displayed different results between in vitro and in vivo conditions. Both low and high concentrations of IL-23 promoted the growth of SK-MES-1 cells in vitro, although the effect was smaller than that in A549 cells. This difference may be explained by the fact that IL-23 also affects immune cells in the tumor microenvironment, so that even if tumors do not express IL-23R, IL-23 may still be able to promote tumor growth indirectly.

Why did the different concentrations of IL-23 induce completely opposite effects? We suspect that this difference reflects the different affinities of the two receptors. IL-12Rβ1 is a subunit with low affinity to IL-23, whereas IL-23R has high affinity (21,22). IL-12Rβ1, which is thought to be an affinity-converting molecule with Tyk2 kinase-binding activity, lacks an intracellular tyrosine phosphorylation site; it forms a functional receptor for IL-12 in combination with a possible signaling subunit IL-12Rβ2 or for IL-23 with IL-23R (29). In this study, we confirmed that low concentrations of IL-23 bound to the higher affinity IL-23R results in promotion of cell proliferation, whereas a high concentration of IL-23 bound to both IL-23R and IL-12Rβ1 results in inhibition of cell proliferation. Our in vitro and in vivo results indicated that IL-12Rβ1 was the major mediator of the growth inhibition with a high dose of IL-23 and that IL-23R is required for IL-23 function.

The STAT transcription factors have been shown to be essential for signaling by most of the interleukins (30). IL-12 and IL-23 are members of the IL-12 family, and both these cytokines activate STAT1, STAT3, STAT4 and STAT5. However, STAT4 is key to IL-12-specific signaling and activated STAT3 is key to IL-23 signaling (31,32). STAT3 is constitutively activated in various tumors (23,24) and plays an important role in tumor cell proliferation (33), suggesting that STAT3 might play an oncogenic role in various human cancers. Generally, IL-23 can activate the JAK–STAT3 pathway by binding to the IL-23 receptor in macrophages (25). Our data indicate that the expression and phosphorylation of STAT3 changed following the action of IL-23, leading to alteration in the proliferative ability of IL-23 receptor-positive lung cancer cells; these effects were negated by the JAK–STAT3 inhibitor AG490. Our data show that IL-23 and the IL-23 receptor promote lung cancer cell proliferation via STAT3 and upregulation of pSTAT3.

Changes in cell growth are related to apoptosis in some cases. To explore further the mechanism responsible for the regulation of lung cancer cell proliferation by IL-23, we examined apoptosis and expression of Ki-67 in A549 cells induced with IL-23. Ki-67, a marker of cell proliferation, is involved in lung cancer cell proliferation (34). Coco et al. reported that high concentrations of IL-23 (>100 ng/ml) could induce cancer cell apoptosis (18). However, our data demonstrate that lower doses of IL-23 (5, 10, 20 or 40 ng/ml) could not induce apoptosis of A549 cells, implying that the inhibition of A549 cell proliferation induced by 40 ng/ml of IL-23 did not occur because of apoptosis. In our study, Ki-67 expression was influenced by IL-23, and Ki-67 expression followed that of STAT3 and pSTAT3. We also found that the peak of Ki-67 expression occurred after the expression peak of STAT3 and that inhibition of phosphorylation of STAT3 by AG490 diminished IL-23-induced Ki-67 expression. Yang et al. (35) also reported that pSTAT3 expression was related to Ki-67 expression in cervical intraepithelial neoplasia. Taken together, these findings suggest that the expression of IL-23-induced expression of Ki-67 is regulated by STAT3.

Is there any other mechanism associated with IL-23 that might regulate lung cancer cell proliferation? We found that some soluble factors were produced by A549 cells following incubation with IL-23, in particular vascular endothelial growth factor (VEGF) and IL-6 (data not shown). Some reports have confirmed that both VEGF and IL-6 can regulate STAT3 expression and the growth of cancer cells (36,37). It has been suggested that STAT3 can be activated by IL-23 via the JAK–STAT3 pathway and by other soluble factors such as IL-6 and VEGF in tumor cells. We are studying this phenomenon at present (a paper on VEGF is in preparation). IL-23 binding to both IL-23R and IL-12Rβ1 might activate another inhibitor signal pathway, which may control IL-23R signaling. Suppressor of cytokine signaling (SOCS) family members have been reported to have a strong regulatory function in the STAT signaling pathway (38). Inflammatory factor-induced phosphorylation of STAT might increase SOCS expression, which could act as a negative feedback loop to restrict the inflammatory reaction. Although some reports have shown that the SOCS family is attenuated in tumor cells, there is no obvious evidence to rule out that the SOCS family is involved in the inhibition of tumor cell proliferation caused by high-dose IL-23 (39). We plan to analyze further the role of the SOCS family in the IL-23 signaling pathway in tumor cells and to determine whether this family is involved in IL-23-associated inhibition of tumor cell proliferation.

Preclinical and clinical studies have demonstrated that IL-12 has potent antitumor activity (40–42), but that IL-12 administration leads to severe toxicity that is associated with extremely high levels of interferon-γ induced by IL-12 (43). IL-23 has a similar structure and bioactivities to IL-12 but induces a lower level of interferon-γ than that induced by IL-12. Our data have demonstrated that a high concentration of IL-23 can inhibit lung cancer cell growth, suggesting that IL-23 may be a potential antitumor agent with lower toxicity (21). Our study provides preliminary data for later clinical trials to test IL-23 toxicity. The findings that a low dose of IL-23 can promote tumor growth and that IL-23 can also induce immune cells to secrete cytokines, which can promote tumor growth in vivo, are important to consider when planning the clinical use of IL-23. Clinical applications of IL-23 may require combination with other methods to prevent stimulation of other cytokines.

In conclusion, our study has demonstrated IL-23 receptor expression in lung cancer and has confirmed that exogenous IL-23 can influence human lung cancer cell growth in vitro and in vivo. At low concentrations, IL-23 promotes the proliferation of A549 and SPCA-1 cells via binding to the IL-23R subunit, whereas at a high dose, IL-23 binds to both IL-23R and IL-12Rβ1 and has the opposite effect. STAT3 and pSTAT3 are critical to these processes. Our data suggest that IL-23 participates in the formation of the tumor microenvironment and affects tumor cells directly. This provides a new explanation for the inconsistent effects of IL-23 on the cancer process. This study has provided the basic proof and further direction for research. Future work could involve demonstrating whether IL-23 at a low dose can induce the proliferation of primary lung ACs. For example, a large sample size of primary lung ACs should be analyzed to study the relationships between IL-23 level and clinical stage, tumor size and expression of genes related to proliferation.

Supplementary material
Supplementary Tables 1 and 2 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding
Startup Foundation of the West China Hospital of Sichuan University (13605132).

Acknowledgements
We thank Department of Pathology of the West China Hospital for providing the paraffin-embedded samples.

Conflict of Interest Statement: None declared.

References


*Received July 4, 2012; revised November 23, 2012; accepted December 4, 2012.*

666