Interleukin-6 Induced Basic Fibroblast Growth Factor-Dependent Angiogenesis in Basal Cell Carcinoma Cell Line via JAK/STAT3 and PI3-Kinase/Akt Pathways

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We have previously demonstrated a xenograft of interleukin-6 (IL-6) overexpressing basal cell carcinoma (BCC) cell line induced tumors with high vasculature in nude mice. Here we asked whether IL-6 could induce angiogenic activity in BCC cell line. Tenfold concentrated conditioned medium (CM) from IL-6 overexpressing BCC cells exhibited higher angiogenic activities in chorioallantoic membrane and Matrigel plug assays, when compared with CM from vector control or parental BCC cells. The level of basic fibroblast growth factor 2 (bFGF) mRNA and secreted bFGF increased in IL-6 overexpressing BCC cells as shown by RT-PCR and ELISA, respectively. Concordantly, recombinant IL-6 treatment caused the elevation of bFGF mRNA and protein levels in parental BCC cells in a time-dependent manner. Neutralizing bFGF function by anti-bFGF antibody significantly inhibited CM-induced human umbilical vein endothelial cells (HUVEC) tube formation and Matrigel plug formation. Meanwhile, cyclooxygenase 2 (COX-2)-specific siRNA markedly abolish HUVEC tube formation. These data indicated both bFGF and COX-2 play an essential role for IL-6-induced angiogenesis in BCC cell line. Treatment with AG490 (Janus tyrosine kinase [JAK] inhibitor) and LY294002 (PI3-Kinase inhibitor) inhibited IL-6-mediated upregulation of bFGF mRNA and protein secretion. Consistently, transfection with dominant negative mutants of signal transducer and activator of transcription 3 (STAT3) and acutely transforming retrovirus AKT8 in rodent T cell lymphoma (Akt) effectively abolished IL-6-mediated expression of bFGF mRNA and protein. Our data suggest that under *in vitro* experimental condition, bFGF and COX-2 are downstream effectors of IL-6-induced angiogenic activity in BCC cell. The IL-6-mediated bFGF upregulation is through activation of JAK/STAT3 and PI3-Kinase/Akt pathways.

Key words: angiogenesis/basal cell carcinoma cells/bFGF/IL-6/JAK/STAT3/PI3-Kinase/Akt
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Basal cell carcinoma (BCC) is the most common skin cancer in humans. The incidence of BCC increases with exposure to ultraviolet (UV) light, and BCC favors sun-exposed sites of the body (Kricker *et al*, 1994, 1995). Both of these facts indicate that UV light is the causal agent of BCC. Although BCC generally shows a relatively benign course, growing slowly and expanding only locally, some cases are aggressive and can rapidly infiltrate deeper structures and sometimes give rise to metastatic spreads (Miller, 1991).

It has been demonstrated that the *in vivo* expression of interleukin-6 (IL-6) accompanies vascularization in reproductive system, during wound healing, in psoriasis (in which tortuous capillary in dermal papilla is a characteristic finding), and during tumor growth (Hirano *et al*, 1990; Motro *et al*, 1990; Elder *et al*, 1992; Mateo *et al*, 1994). These data suggest that IL-6 may induce angiogenesis.

Tumor growth and metastasis depend upon the ability to induce its own blood supply (Folkman and Shing, 1992). A number of studies have reported an association between intratumor microvessel density and tumor aggressiveness (Weidner, 1995). The tumor vasculization was also found to correlate with the aggressive (malignant) phenotype in human BCC (Staibano *et al*, 1996).

UV irradiation is considered to be a major etiological factor for pathogenesis of BCC (Kricker *et al*, 1994, 1995). Interestingly, UV irradiation can trigger the release of IL-6 and tumor necrosis factor α (TNF-α from human epidermal keratinocytes; Avalos-Diaz *et al*, 1999). Our previous study revealed that increased tumorigenicity and vascularity in nude mice transplanted IL-6 overexpressing BCC cells. In addition, vascular endothelial growth factor (VEGF) and cyclooxygenase 2 (COX-2) have been found to be expressed in IL-6 overexpressing BCC cells (Jee *et al*, 2001). A recent study has shown that basic fibroblast growth factor 2 (bFGF) was overexpressed in epithelial neoplasm of skin (Arbiser *et al*, 2000), suggesting that bFGF may have a role in neoplastic alteration of skin. This study is thus aimed to

Abbreviations: Akt, acutely transforming retrovirus AKT8 in rodent T cell lymphoma; BCC, basal cell carcinoma; bFGF, basic fibroblast growth factor 2; CAM, chorioallantoic membrane; CM, conditioned medium; COX-2, cyclooxygenase 2; HUVEC, human umbilical vein endothelial cells; IL-6, interleukin-6; JAK, Janus tyrosine kinase; STAT3, signal transducer and activator of transcription 3; UV, ultraviolet; VEGF, vascular endothelial growth factor

S.-H. Jee and C.-Y. Chu contributed equally to this work.

investigate whether bFGF would be regulated by IL-6 and the possible role of bFGF in IL-6 induced angiogenesis in BCC cells. We found that IL-6 indeed upregulated bFGF mRNA and promoted the secretion of bFGF protein in BCC cells. In addition, IL-6-mediated angiogenic activity was strongly attenuated by treatment with anti-bFGF antibody.

Results

IL-6 enhances angiogenesis in *in vivo* models We previously demonstrated that BCC/IL-6 cells were more tumorigenic and induced more vascularization in nude mice (Jee *et al*, 2001). To clarify whether IL-6 could induce the angiogenic activity in BCC cells, we collected conditioned medium (CM) from cells and then performed chorioallantoic membrane (CAM) and Matrigel plug assay. CM from IL-6 overexpressing BCC cells (BCC/IL-6 or BCC/IL-6/8D cells) produced profound neo-capillaries 3 days after loading onto CAM as compared with that from parental BCC cells or BCC/neo cells (Fig 1). Matrigel plug assay showed that CM from IL-6 overexpressing cells displayed more vascularization in plugs on the seventh day after inoculation (data not shown). The degree of vascularization was quantitatively measured based on the hemoglobin contents (Fig 4*B*). These biological assays confirmed that IL-6 may increase angiogenic activity in human BCC cells.

Upregulation of bFGF by IL-6 in BCC cells To dissect whether bFGF or other possible angiogenic factor(s) may be regulated by IL-6, we used RT-PCR to examine the expression level of various angiogenic factors (bFGF, platelet-derived growth factor (PDGF), and IL-8) that have previously been reported to be important in activating angiogenesis. Only bFGF mRNA was elevated in IL-6 overexpressing BCC cells (Fig 2*A*). Correlating with the mRNA level, IL-6 overexpressing cells secreted a significant amount of bFGF protein (Fig 2*B*). We further confirm exogenous IL-6 treatment enhanced bFGF expression in the parental BCC cells. RT-PCR analysis showed a time-dependent increase in bFGF mRNA in BCC cells after treatment with IL-6 (Fig 3*A*). ELISA analysis revealed that IL-6 stimulation gradually increased the level of secreted bFGF at 4–24 h and peaked (3.3-fold) at 48 h in BCC cells (Fig 3*B*). Secreted VEGF was, however, upregulated to 1.36-fold only. Supportively, Western blot analysis of cell lysates from IL-6-treated BCC cells revealed that either high molecular weight (HMW) or low molecular weight (LMW) bFGF was significantly elevated at 4–16 h after IL-6 treatment (Fig 3*C*).

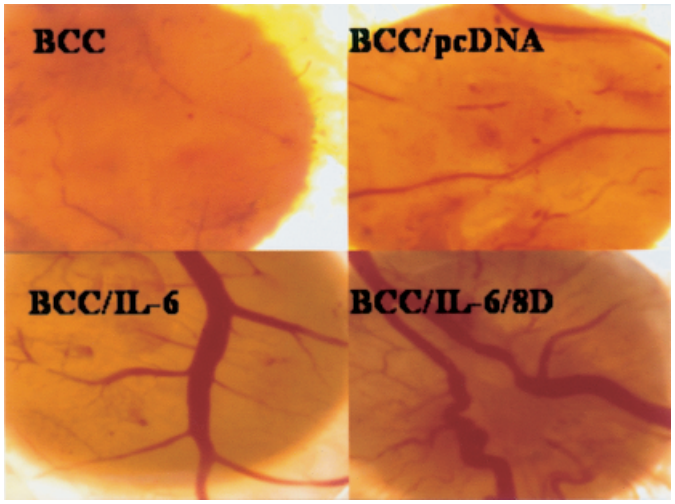

Figure 1
Increase in angiogenic activity in basal cell carcinoma (BCC) cells overexpressing interleukin-6 (IL-6). The condition media collected from parental BCC cells (BCC), control vector transfectant (BCC/Neo), pooled IL-6 transfectant (BCC/IL-6) and IL-6 transfectant with highest IL-6 expression (BCC/IL-6/8D) for chorioallantoic membrane (CAM) assay on Leghorn chicken eggs for 3 d. This represents one of three experiments.

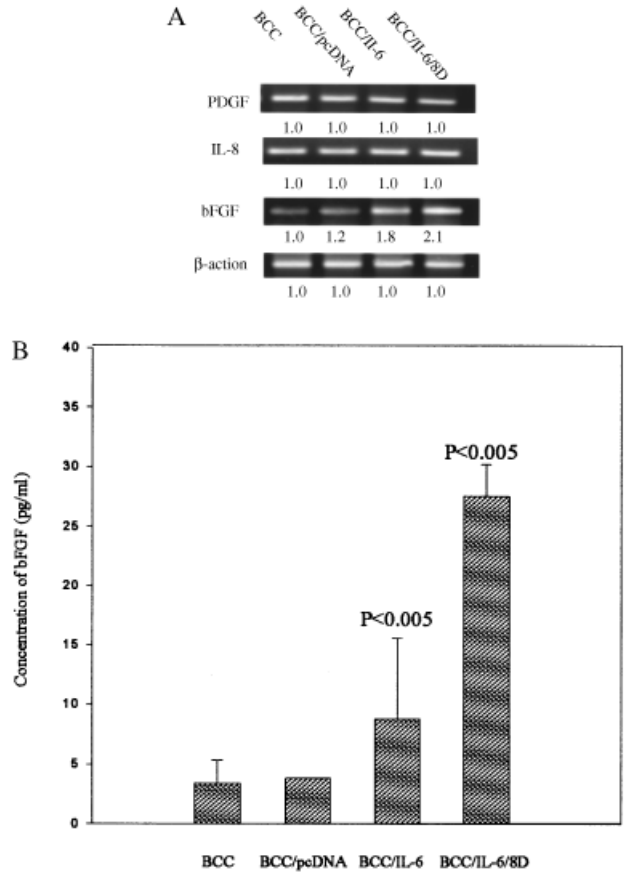

Figure 2
Upregulation of basic fibroblast growth factor 2 (bFGF) in basal cell carcinoma (BCC) cells over-expressing interleukin-6 (IL-6). (*A*) After serum starvation, the mRNA of various cell lines as indicated were extracted and semiquantified by RT-PCR of bFGF, platelet-derived growth factor (PDGF), and IL-8. The number below each lane indicates the relative densitometric intensity to control (defined as 1.0). This represents one of three experiments. (*B*). Cell-free culture supernatant was analyzed for bFGF by ELISA. Concentration of bFGF was expressed as mean ± SD of five experiments. Student's *t* test was used for comparison.

Neutralization of bFGF in CM and transfection of COX-2-specific siRNA into IL-6 over-expressing BCC cells inhibits IL-6-induced angiogenic activities Our previous study shows that IL-6 induces VEGF and COX-2 in BCC cells (Jee *et al*, 2001). To clarify the biological function of bFGF, VEGF, and COX-2 in IL-6-mediated angiogenesis, we used anti-bFGF and anti-VEGF neutralizing antibody and transfection of COX-2 siRNA, respectively, to block their function and examined the angiogenic activity by using

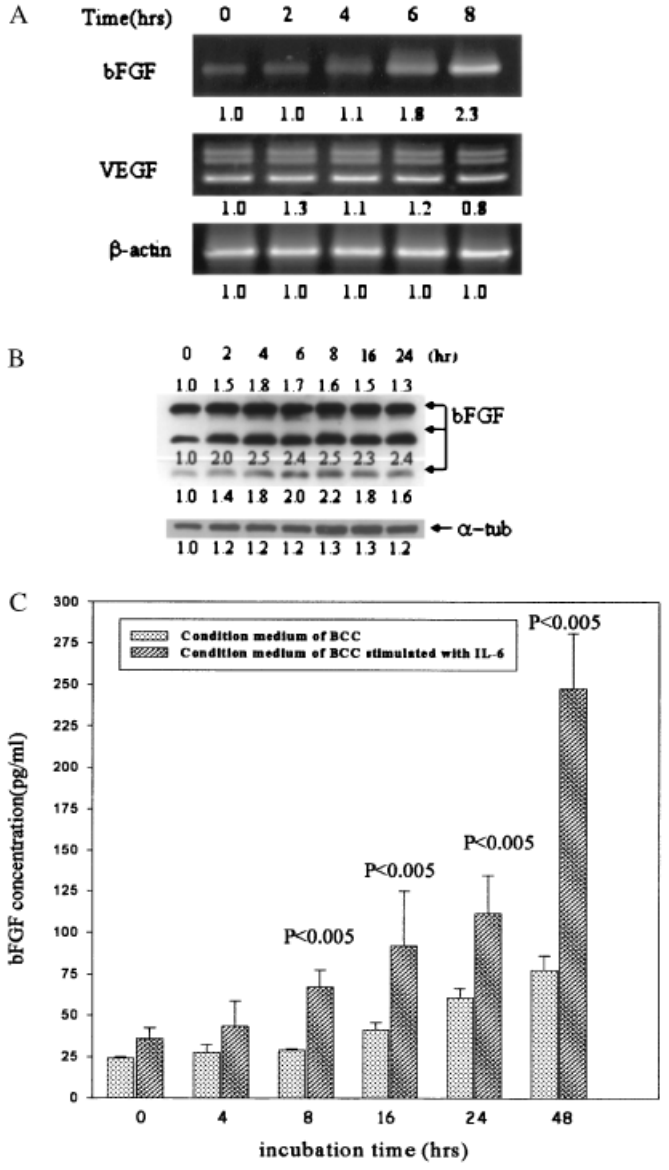

Figure 3
Time dependent upregulation of mRNA, protein and secretion of basic fibroblast growth factor 2 (bFGF) in basal cell carcinoma (BCC) cells stimulated with interleukin-6 (IL-6). (*A*) After serum starvation, the parental BCC cells were incubated in the presence of 100 ng per mL human recombinant interleukin-6 (rIL-6) for different times as indicated. The mRNA extracted was semiquantified by RT-PCR for bFGF and vascular endothelial growth factor (VEGF). (*B*) The protein extracted from cell lysates were quantified by Western blotting for bFGF. The number below each lane indicates the relative densitometric intensity to control (defined as 1.0). This represents one of three experiments. High molecular weight (HMW: 24, 22.5 kDa) and low molecular weight (LMW: 18 kDa) bFGF are shown in upper, mid-, and lower row, respectively. (*C*) The secretion of bFGF in the culture supernatant was measured by ELISA. Concentration of bFGF was expressed as mean ± SD of five experiments. Student's *t* test was used for comparing the paired cultured cells with and without stimulation with IL-6 at various time as indicated.

human umbilical vein endothelial cells (HUVEC) capillary tube formation on Matrigel. Exposure of HUVEC to CM from BCC/IL-6 or BCC/IL-6/8D resulted in tube formation that was significantly inhibited by neutralizing with anti-bFGF but not anti-VEGF antibody (83.1% and 14.9% inhibition, respectively). (Fig 4*A*, *left*). The protein lysate from BCC/IL-6/8D cells transfected with COX-2-specific siRNA was collected for western blotting to ensure the abrogation of 50% of Cox-2 (data not shown). The CM collected from COX-2-specific siRNA transfectant caused 62% inhibition of capillary tube formation compared with that of BCC/IL-6/8D cells. (Fig 4*A*, *right*). The results suggest that bFGF and COX-2 are possibly the main downstream effectors induced by IL-6 in the angiogenesis of BCC.

Direct treatment of HUVEC with IL-6 cytokine did not induce tube formation significantly (data not shown). In addition, anti-IL-6 antibody also failed to affect the tube formation induced by CM from IL-6 overexpressing cells (data not shown), suggesting that tube-forming activity of CM from IL-6 overexpressing cells was not directly caused by IL-6.

Using Drabkin method for quantitation of the total hemoglobulin in Matrigel gel plug assay, we showed that treatment with anti-bFGF antibodies caused 85% inhibition on the total amount of hemoglobin (Fig 4*B*). This confirmed that bFGF contributed to the IL-6-induced angiogenic activity in BCC cells.

JAK/STAT3 and PI3-Kinase/Akt signal pathways are involved in IL-6 induced upregulation of bFGF Three major signaling pathways including the JAK/STAT3 pathway, PI3-Kinase/Akt pathway, and mitogen-activated protein kinase (MAPK) pathway (Heinrich *et al*, 2003) have been reported to be involved in the IL-6-mediated cellular functions. Initially, pharmacological inhibitors such as AG490 (a JAK inhibitor, 50 μM), LY294002 (a PI3-Kinase inhibitor, 25 μM), or PD98059 (a MEK inhibitor, 50 μM) were used to examine which pathways were involved in IL-6-mediated bFGF upregulation in BCC cells. The optimal doses used to specifically inhibit the signaling pathways by these inhibitors were described in our previous study (Jee *et al*, 2002). RT-PCR analysis revealed that IL-6-mediated increase of bFGF mRNA was significantly attenuated by AG490 and LY294002 but not by PD98059 (Fig 5*A*). Both AG490 and LY294002 effectively reduced the amount of secreted bFGF protein in BCC cells stimulated by IL-6, as determined by ELISA (Fig 5*B*). These data suggest that PI3K/Akt and JAK/STAT3 are coordinately involved in IL-6-mediated bFGF gene expression in BCC cells. Consistently, transfection with dominant-negative STAT3 mutants (STAT3F and STAT3D) and Akt mutant (dnAkt) into BCC cells caused a strong inhibition on IL-6-stimulated bFGF mRNA and protein level. (data not shown).

Together, our study revealed that IL-6 induced angiogenesis in BCC by upregulation of secreted bFGF via both JAK/STAT3 and PI3-Kinase/Akt pathways and by COX-2.

Discussion

IL-6 has been implicated in the pathogenesis of multiple myeloma and some other malignancies, including renal cell carcinoma, prostate cancer, and Kaposi's sarcoma (Akira and Kishimoto, 1992; Aoyagi *et al*, 1996; Adler *et al*, 1999; Aoki *et al*, 1999; Kinoshita *et al*, 1999). Our previous study has demonstrated that transplantation of IL-6 overexpressing BCC cells into nude mice resulted in the enhancement of tumor growth accompanied by high vascularization (Jee

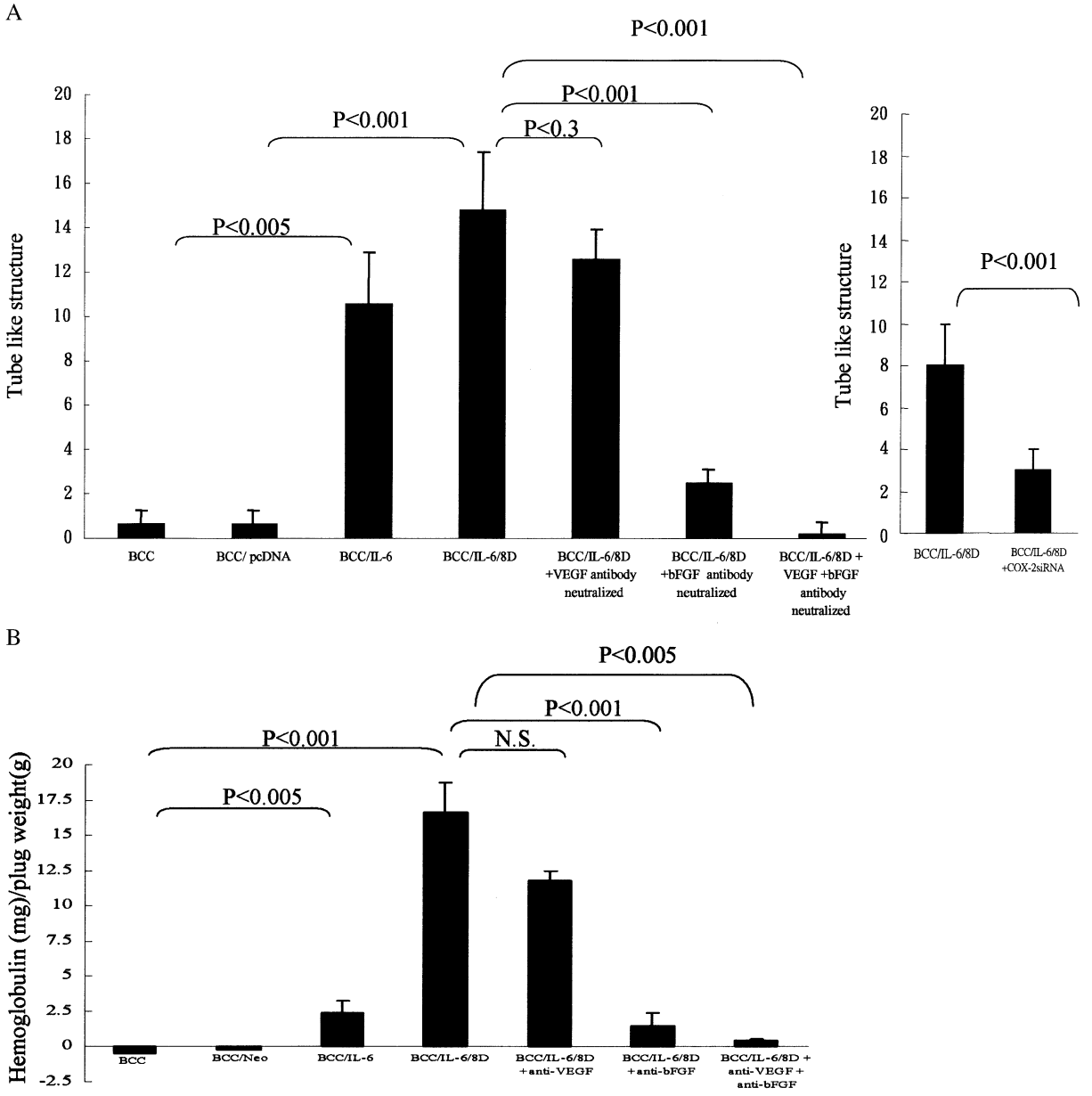

Figure 4
Blockage of basic fibroblast growth factor 2 (bFGF) function or cyclooxygenase 2 (COX)-2 inhibited angiogenesis induced by interleukin-6 (IL-6). (A) 1 μg anti-bFGF or anti-vascular endothelial growth factor (VEGF) antibody was pre-incubated with 150 μL condition medium (CM) of BCC/IL-6/8D for 30 min. before incubation with human umbilical vein endothelial cells (HUVEC) cells. CM collected from BCC/IL-6/8D cells transfected with vector containing COX-2 siRNA was incubated with HUVEC cells. (The number of tube-like structures was expressed as mean ± SD of randomly counting five 9.7 mm^2 microscopic fields. $n = 3$) (*B*) CM neutralized with anti-bFGF antibody was served for Matrigel blood plug assay in C57BL6j mice (five per group). The blood plugs were extracted and quantization of hemoglobulin was performed using the Drabkin method. The mean amount of hemoglobulin was calculated. Student's *t* test was used for comparison. NS: non-significant.

et al, 2001). Here we provide evidence to show that exogenous IL-6 stimulation can upregulate the bFGF mRNA expression and protein secretion in BCC cells whereas IL-8 and PDGF are not affected by IL-6. Blockage of bFGF function by neutralizing antibody strongly prevented IL-6-mediated angiogenic activities as seen in HUVEC tube formation, and Matrigel plug assay. Our data revealed that bFGF is a downstream effector of IL-6-induced angiogenesis in BCC cell line cells under *in vitro* condition.

bFGF is a multifunctional protein with well-studied mitogenic and angiogenic properties (Basilico and Moscatelli, 1992). It is a protein devoid of secretory signal sequence. At variance with VEGF, bFGF is released via an alternative ATP-dependent secretion pathway independent of the endoplasmic reticulum–Golgi complex (Florkiewicz *et al*, 1998). During the development of fibrosarcoma in a transgenic mouse model, the appearance of an angiogenic phenotype is correlated with the export of bFGF (Kandel *et al*, 1991). A secreted FGF binding protein that mobilizes stored extracellular bFGF can serve as an angiogenic switch for different cell lines including squamous cell carcinoma (SCC) and colon cancer cells (Czubayko *et al*, 1996). Interestingly, bFGF has been shown to affect early tumor angiogenesis but without any effect in established

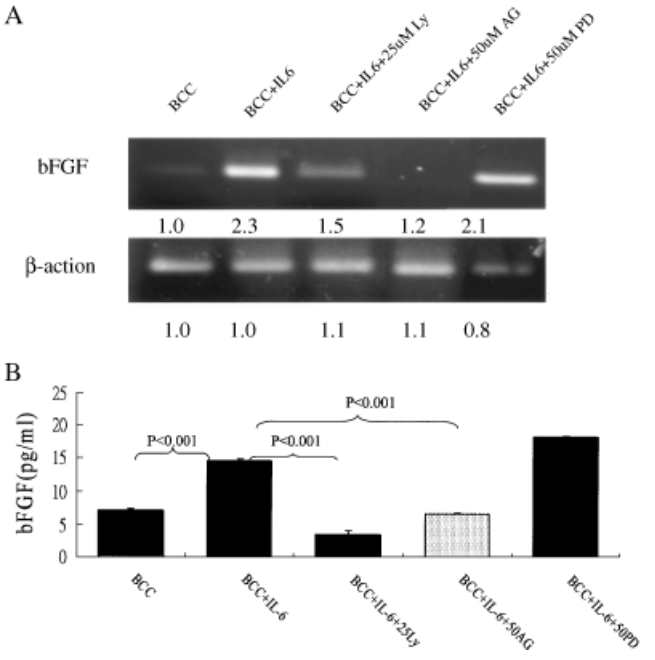

Figure 5
AG490 (JAK inhibitor) and LY294002 (PI3-Kinase inhibitor) reduced interleukin-6 (IL-6) upregulated expression and secretion of basic fibroblast growth factor 2 (bFGF) in basal cell carcinoma (BCC) cells. After pre-treating with human recombinant interleukin-6 (rIL-6) (100 ng per mL) for 15 min, BCC cells were serum starved for 72 h and treated with AG490 or LY294002 or PD98059 (MEK inhibitor) for 8 more hours. (*A*) The mRNA of BCC cells with various treatment were semi-quantified by RT-PCR for bFGF. The number below each lane indicated the relative intensity of bFGF mRNA to controls (defined as 1.0). This is one representative experiments of three. (*B*) The culture supernatants were subjected to ELISA with bFGF antibody. The concentration of bFGF was expressed as mean ± SD of five experiments. Student's *t* test was used for comparison. NS: non-significant.

tumors (Giavazzi *et al*, 2001). Our study reveals that the secreted bFGF was responsible for IL-6-induced angiogenesis; however, whether the elevation of bFGF contributes to IL-6-induced tumor growth in BCC is unknown and needs to be clarified. The paracrine tumor–stromal cell interaction of VEGF and IL-6 in multiple myeloma has been reported (Dankbar *et al*, 2000). This study indicates the crucial role of bFGF in IL-6-induced angiogenesis in BCC cells.

bFGF has been detected in normal skin, benign epidermal proliferation, and malignant neoplasm of skin (Yaguchi *et al*, 1993; Arbiser *et al*, 2000). Sun exposure is the essential causal factor of BCC. A single exposure to UVB irradiation can induce erythema, epidermal hyperplasia, and increased cutaneous vasculization. IL-6 is one of the pro-inflammatory cytokines induced by UV irradiation in keratinocytes (Schwarz and Luger, 1989; Chung *et al*, 1996). UVB induced cutaneous angiogenesis in C3H/HeN mice via upregulation of bFGF, but not VEGF, on day 1 of UVB irradiation (Bielenberg *et al*, 1998). Concordantly, our data demonstrated that bFGF was markedly upregulated by IL-6 in BCC cells.

Accumulating evidence is defining a critical role for STAT3 in oncogenesis (Bowman *et al*, 2000; Catlett *et al*, 1999a, b). Constitutive activation of STAT3 signaling contributes to oncogenesis by preventing apoptosis and enhancing cell proliferation (Grandis *et al*, 1998, 2000; Catlett *et al*, 1999a, b; Bowman *et al*, 2000). Similarly, PI3-Kinase/Akt pathway has been reported to play a major role in agonist-induced cell survival and growth (del Peso *et al*, 1997; Blume-Jensen, 1998; Vanhaesebroeck and Alessi *et al*, 2000). We previously demonstrated the anti-apoptotic activity of both STAT3 and PI3-Kinase/Akt in BCC cells or BCC cells stimulated with IL-6. We reported previously that cervical tumor growth promotion by IL-6 in VEGF-dependent angiogenesis via a STAT3 pathway (Wei *et al*, 2003). In this study bFGF was upregulated via JAK/STAT3 and PI 3-Kinase/Akt pathways in BCC cells stimulated with IL-6. It has been shown that 5(*S*)-hydroxyeicosatetraenoic acid stimulates autocrine growth of HUVEC via activation of JAK/STAT and PI3-Kinase/Akt signal leading to activation of bFGF (Zeng *et al*, 2002). Our study demonstrated that IL-6 stimulates secretion of bFGF via similar pathways to act on HUVEC cells in a paracrine mode. These data suggest that activation of JAK/STAT/bFGF and PI3-Kinase/Akt/bFGF pathways in either tumor cells (paracrine mode) or endothelial cells (autocrine mode) leads to angiogenesis.

Finally, our study revealed that IL-6 induced angiogenesis in BCC by upregulation of secreted bFGF via both JAK/STAT3 and PI3-Kinase/Akt pathways. This finding suggests prevention of IL-6 production (e.g., avoid exposure to UV light or usage of anti-inflammatory drugs), inhibition of bFGF production or secretion, or blockage of the JAK/STAT3 and PI3-Kinase/Akt signal pathways may be the feasible management for prevention or treatment of skin cancer. In addition, our data also showed that blockage of Cox-2 by siRNA reduced angiogenic activity in IL-6 overexpressing BCC cells, suggesting that Cox-2 also play a role in IL-6-induced angiogenesis.

Materials and Methods

All the described study was approved by the medical ethical committee of National Taiwan University College of Medicine and was conducted according to Declaration of Helsinki Principle.

Cell origin, cell culture, and establishment of IL-6 transfectants The human BCC cell line (parental BCC cells), originally named BCC-KMC-1, was originated from an undifferentiated BCC derived from a trauma scar. Using keratinocytes cultured in serum-free medium as positive control, we performed RT-PCR to measure the expression of K14 mRNA in BCC cells. The basaloid keratinocytes nature of this BCC cell line is verified by the presence of keratin 14 mRNA by RT-PCR method in these BCC cells, but not in Hela cells.

The 105–108th passages of this cell line were used in this study. The pCMV-IL-6, a constitutive expression vector, carries 0.64 kb full-length human IL-6 cDNA under the control of the CMV promoter/enhancer sequence and with a neomycin selection marker. The BCC cells were transfected with pCMV-IL-6 or control pcDNA3 vector (GIBCO Invitrogen, Grand Island, New York), containing a CMV promotor and a neomycin selection marker, using the TransFast transfection reagent (Promega, Madison, Wisconsin). After 24 h, cells were replated in RPMI 1640 medium (GibcoBRL, Rockville, Maryland) with 10% fetal calf serum (FCS) and 500 ng per mL G418 (Sigma, St Louis, Missouri). G418-resistant clones were selected and expanded. For this study, we used the pooled transfectants (BCC/IL-6) and the highest expression transfectant (BCC/IL-6/8D), with IL-6 secretion of about 832 and 1427 pg per mL, respectively. The parental BCC cells and BCC cells transfected with control vector, BCC/Neo cells, served as controls. All these cells were grown at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS).

Antibodies and reagents Affinity-purified monoclonal mouse anti-bFGF antibody was purchased from BD Bioscience (San Diego, California). Anti-α-tubulin antibody was from NeoMarkers (Fremont, California). Neutralizing antibodies (mouse monoclonal anti-IL-6 antibodies and goat polyclonal anti-bFGF antibody) were from R&D Systems (Minneapolis, Minnesota). AG490 (JAK inhibitor), LY294002 (LY, PI3-Kinase inhibitor), and PD98059 (MEK inhibitor) were from Calbiochem (San Diego, California).

Preparation of CM Parental BCC, BCC/Neo, BCC/IL-6, or BCC/IL-6/8D cells were plated in 1 mL culture medium without serum at 2×10^5 cells per well in 24-well 18 mm culture dishes. The culture supernatants were collected 24 h later and centrifuged sequentially at 12,500 g with Microcon YM-3 centrifugal filter devices (cut-off molecules smaller than 3000 Da; Millipore, Bedford, Massachusetts) for 10 min to obtain a 10-fold concentrate culture supernatant.

CAM assay Nine-day-old fertilized White Leghorn chicken eggs were incubated at 37°C at constant humidity. On incubation day 3, a square window was opened on the shell and sealed with a glass. On day 11, 1 mm^3 filter papers loaded with 30 μL CM were implanted on top of the CAM. Capillary tube formations were examined 3 days later, when the angiogenic response peaked. The blood vessels entering the paper were recognized macroscopically and photographed.

***In vivo* Matrigel blood plug assay** Matrigel blood plug assay was performed as described previously (Plunkett and Hailey, 1990; O'Reilly *et al*, 1997). Briefly, C57BL6j mice (five per group) were each injected subcutaneously with 50 μL of CM from various cell lines mixed with 450 μL Matrigel L (growth factor reduced; Becton Dickinson Labware, Bedford, Massachusetts) and 40 U heparin per mL at 4°C. Mice were sacrificed 6 d later, and gels were recovered and processed for further studies. Neovessels was quantified by Drabkin method, measuring hemoglobin of the plug with Drabkin reagent kit 525 (Sigma). Experiment was authorized by animal experiment committee of National Taiwan University College of Medicine.

Cox-2-specific RNA interference Target sequence for the COX-2 siRNA was bases 291–313 of NM000963.1 (5′ aactgctcaacaccggaattttt3′). PRNA-U6/neo (GenScript, Piscataway, New Jersey) is a siRNA expression vector with neomycin as selection marker. We construct pRNA-U6/COX-2, a constitutively expressed vector that carries the COX-2 siRNA sequence under the control of U6 promotor. The BCC/IL-6/8D cells were transiently transfected with pRNA-U6/COX-2 overnight. The CM was collected for capillary tube formation assay.

***In vitro* capillary tube formation on Matrigel** Human umbilical vein was collected with consent form. HUVEC capillary tube formation was evaluated as follows. 24-well 18 mm tissue culture dishes were coated with Matrigel basement membrane matrix (300 μL per well; Becton Dickinson Labware) at 4°C and allowed to polymerize at 37°C for at least 30 min. HUVEC (2×10^5 cells per well) were grown in a final volume of 0.30 mL culture medium containing 150 μL M199 (GibcoBRL) and 150 μL CM. After 6 h incubation, tube formation was observed through a reverted, phase-contrast photomicroscope photographed and counted. The number of tube formations was measured by counting the number of tube like structures formed by connected endothelial cells in five randomly selected 9.7 mm^2 microscopic fields. The assay was performed in triplicate.

Construction of dominant-negative DNA and transient transfection For assay of JAK/STAT3 signal pathway, we constructed hemagglutinin (HA) epitope-tagged dominant-negative STAT3 mutants. An HA epitope tag was inserted into STAT3F (Nakajima *et al*, 1996) cDNA or STAT3D (Horvath *et al*, 1995) cDNA and cloned into a pcDNA3 vector (GIBCO Invitrogen). BCC cells were plated 12 h before transfection at a density of 1×10^5 cells per 6 cm Petri dish, followed by transfection with 1 μg of STAT3F plasmid or STAT3D plasmid using the Transfast Transfection Reagent (Promega) as per manufacturer's instructions. The amount of HA was concomitantly measured to confirm the transfection efficiency. Dominant-negative mutant of Akt was similarly constructed and transfected as described above.

RT-PCR Messenger RNAs from BCC cells treated with IL-6 were isolated using commercial kits (Promega). The total RNA was subjected to first-strand synthesis using Random Hexamer (Promega) and M-MLV Reverse Transcriptase (RNase H Minus) (Promega) at 37°C for 3 h. The cDNA was then diluted to a final volume of 50 μL and quantified. PCR reactions contained 5 μg cDNA, 0.5 U per μL Taq polymerase (Protech Technology, Taiwan), and 25 pmol each of the sense and the anti-sense primers in PCR buffer (10 mM Tris pH 9, 50 mM KCl, 6 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100). The reaction mixture was incubated for 5 min at 94°C and then amplified by 25 PCR cycles (denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 1 min at 72°C). Each PCR product was then analyzed on a 2% agarose gel stained with 1 μg per mL ethidium bromide and photographed (Digital Science SP700 camera; Kodak Scientific Imaging Systems, New Haven, Connecticut). Intensity of bands on the photographs was quantified by scanning laser densitometry.

Enzyme immunoassay (ELISA) The bFGF and VEGF levels of the cell culture supernatant were determined by using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Each measurement was repeated in triplicate, and the average value was recorded as pg per mL.

Western blot analysis The cellular lysates were prepared as described previously (Kuo *et al*, 1998). Lysate samples (50 μg from each lysate) were subjected to electrophoresis on 15% SDS-polyacrylamide gels and were electroblotted on nitrocellulose papers. After blocking, blots were incubated with horseradish peroxidase-conjugated mouse anti-bFGF antibody (BD PharMingen, San Diego, California) in PBST (phosphate-buffered saline containing Triton X-100) for 1 h followed by three washes (15 min each) in PBST. After washing, blots were incubated with the Western blotting reagent ECL (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) for 1 min. Chemiluminescence was detected by exposure of Kodak-BioMax films to the blots for 10 s and 5 min for HMW and LMW bFGF, respectively. Intensity of bands on autoradiograms were quantified by scanning laser densitometry.

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