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Proinflammatory Cytokine IL-1β Promotes Tumor Growth of Lewis Lung Carcinoma by Induction of Angiogenic Factors: In Vivo Analysis of Tumor-Stromal Interaction[1]

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Inflammatory conditions are associated with tumor development. IL-1β is a multifunctional and proinflammatory cytokine that affects nearly all types of cells. To investigate the role of IL-1β in tumor growth in vivo, we transduced the retroviral vector coding human *IL-1β* gene into mouse Lewis lung carcinoma (LLC) cells and subsequently inoculated the transformant (LLC/IL-1β) to syngeneic C57BL/6 mice. Tumors derived from LLC/IL-1β grew faster (240%, day 18, vs null-vector control LLC/neo; $p < 0.01$) and showed more abundant vasculature (250%, vs LLC/neo; $p < 0.05$), whereas LLC/IL-1β cells, LLC/neo cells, and wild-type LLC cells did not show any significant difference in the growth rate in vitro. As compared with LLC/neo cells, LLC/IL-1β cells secreted 2-fold the amount of vascular endothelial growth factor and >10-fold the amount of macrophage-inflammatory protein-2 (CXCL2), one of whose main functions is angiogenesis. Although LLC/IL-1β itself did not secrete hepatocyte growth factor (HGF), the tumor derived from LLC/IL-1β cells also contained a >4-fold higher concentration of HGF, another angiogenic factor. In situ hybridization of HGF mRNA in LLC/IL-1β tumor sections demonstrated that stromal fibroblasts and infiltrating cells overexpressed HGF mRNA. Moreover, when cultured in the presence of HGF in vitro, LLC/IL-1β cells secreted even larger amounts of vascular endothelial growth factor and macrophage-inflammatory protein-2. The antiangiogenic agent TNP-470 and anti-CXCR2 Ab inhibited the tumor growth of LLC/IL-1β cells in vivo. These results indicated that secreting IL-1β into the tumor milieu induces several angiogenic factors from tumor and stromal cells and thus promotes tumor growth through hyperneovascularization. ***The Journal of Immunology*, 2002, 169: 469–475.**

Interleukin-1β is a highly inflammatory and prototypical multifunctional cytokine that affects nearly all cell types, often in concert with other cytokines or small mediator molecules (1). It is produced mainly from activated monocytes and macrophages involved in inflammatory and immune responses. Nonmicrobial products as well as microbial products induce IL-1β expression in monocytes and macrophages (2, 3). IL-1β elicits important proinflammatory and immunological responses, such as fever, hypotension, increasing circulating NO, recruiting neutrophils, and costimulating T cell activation by increasing IL-2R expression and inducing IL-2 production (4). The basis of the various biologic properties of IL-1β depends on its regulatory effects on the expression of various genes and/or receptors. IL-1β induces the gene expression of the IL-1 family, other inflammatory cytokines, CSFs, and mesenchymal growth factors (5–7).

The natural history of certain diseases as well as epidemiology studies have revealed a strong association between particular chronic inflammatory conditions and carcinogenesis in several human cancers (8, 9). Sustained inflammation causes tissue damage, then increases the cellular proliferation activity, and finally develops tumors (10). It has been reported that inhibition of chronic inflammation can inhibit carcinogenesis in a murine tumor model (11). Mice deficient in TNF-α, a proinflammatory cytokine, are resistant to skin carcinogenesis (12). The effects of inflammation on tumor growth have been reported in human and murine tumors. Inflammatory breast cancer, which is fast growing, invasive, and angiogenic, overexpresses several genes involved in angiogenesis (13, 14). The proinflammatory cytokine TNF-α has been shown to enhance tumor growth in vivo (15).

IL-1β exhibited direct cytocidal effects and increasing nonspecific host immune responses, resulting in tumor regression in vitro in particular tumors (16, 17). In sharp contrast to the growth-inhibitory effects, administration of IL-1β to mice with a s.c. melanoma increases the tumor size and pulmonary metastasis (18, 19).

To investigate the controversial role of IL-1β in tumor growth in vivo, we constructed a retroviral vector expressing a hybrid gene of the signal sequence of human growth hormone and human *IL-1β* gene that enables the transduced cells to secrete efficient, mature IL-1β proteins. Murine Lewis lung carcinoma (LLC)[3] cells, infected with human IL-1β-expressing vector, grew rapidly in mice, despite the lack of a difference in the cell growth in vitro. This rapid growth was associated with hyperneovascularization

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[3] Abbreviations used in this paper: LLC, Lewis lung carcinoma; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; ISH, in situ hybridization; MIP-2, macrophage-inflammatory protein-2.

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induced by several angiogenic factors secreted from tumor cells and from stromal cells in the tumor milieu.

Materials and Methods

Tumor cell lines, mice, and chemicals

LLC cells were cultured in Eagle's MEM supplemented with 10% FBS. Male C57BL/6 mice (5 wk old) and male BALB/c *nu/nu* mice (5 wk old) were purchased from Charles River Breeding Laboratories (Kanagawa, Japan). All animal experiments were conducted in accordance with the institutional guidelines of the Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). TNP-470 (6-*O*-(*N*-chloroacetyl-carbamoyl)-fumagillol), a semisynthetic analog of fumagillin derived from *Aspergillus fumigatus*, was kindly provided by Takeda Chemical Industries (Osaka, Japan).

Transduction of human IL-1β gene into LLC cells

A plasmid pSV1003 containing a hybrid gene coding for the signal sequence of human growth hormone and the mature form of IL-1β was kindly provided by Dr. J. Lupker (Sanofil Recherche, Centre de Labege, Labege Cedex, France) (20). This hybrid gene was subcloned into a retroviral vector PLXIN (Clontech Laboratories, Palo Alto, CA) containing neo^R cDNA. Retroviral supernatants were generated using these proviral constructs and a ϕ CRIP packaging cell line. LLC cells (5×10^5 per 6-cm diameter dish) were infected with these viral supernatants of the producer cell lines in the presence of polybrene (8 μg/ml). As a negative control, a retroviral vector PLXIN carrying only the neo^R gene was used. Infected cells were subsequently selected by G418 (600 μg/ml), and G418-resistant colonies were collected as a mass population and designated as LLC/IL-1β and LLC/neo. Secretion of human IL-1β from these infected LLC cells was measured with an ELISA kit (R&D Systems, Minneapolis, MN).

Growth of LLC cells in vitro and in vivo

The in vitro growth of LLC/IL-1β, LLC/neo, and LLC cells was analyzed by seeding 5×10^5 cells in 6-cm diameter dishes in 10% FBS medium and in 2% FBS medium. Viable cell numbers were counted every day for 6 days in triplicate.

LLC/IL-1β cells (5×10^5 per mouse) were inoculated s.c. into syngeneic C57BL/6 male mice and BALB/c *nu/nu* male mice, and tumors were measured with calipers in two perpendicular diameters every 2 or 3 days. Tumor volumes were calculated from the length (a) and width (b) by using the following formula: volume (mm^3) = $ab^2/2$.

Histology of the tumors

For histological evaluation, the tumors were surgically removed when they reached almost 1 cm in diameter. For conventional histology, tumors were fixed with 10% buffered formaldehyde and embedded in paraffin. Tissue sections were stained with H&E. For immunohistochemistry, the tumor tissues were embedded in Tissue-Tek OCT embedding medium (Sakura Finetechnical, Tokyo, Japan) and stored at −80°C until use. The cryostat sections were fixed in acetone at room temperature for 10 min and then stained with rat anti-mouse CD31 (PECAM-1, a specific marker for vascular endothelial cells) mAb (1/200 dilution; BD PharMingen, San Diego, CA) and visualized with a streptavidin-peroxidase complex (HISTOFINE; Nichirei, Tokyo, Japan). The intratumoral microvessel density was determined as described previously (21). Briefly, in the area of most intense neovascularization (hot spots), individual microvessel counts were made on a ×200 field. Any brown-staining endothelial cells clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements were considered to constitute a single, countable vessel.

Secretion of VEGF, MIP-2, and HGF from LLC/IL-1β cells in vitro

To determine the secretion of vascular endothelial growth factor (VEGF), macrophage-inflammatory protein-2 (MIP-2; functional homolog to human IL-8), and hepatocyte growth factor (HGF) from LLC/IL-1β cells in vitro, LLC/IL-1β cells (1×10^6/2 ml) were cultured for 24 h in 1 and 10% FBS medium. The production of VEGF, MIP-2, and HGF was determined by ELISA kits (R&D Systems) and expressed as nanograms per 1×10^6/24 h.

HGF concentration in the tumor tissues

When the tumors reached ~1 cm in diameter, they were removed and washed three times with PBS to remove blood. A 4× volume of solution containing 20 mM Tris-HCl (pH 7.5), 2 M NaCl, 0.1% Tween 80, 1 mM PMSF, and 1 mM EDTA was added to the tumors and homogenized vigorously. This homogenized solution was then centrifuged at 15,000 rpm at 4°C for 30 min. The second layer of centrifuged solution was applied for determination of the HGF contents by an ELISA kit (Institute of Immunology, Tokyo, Japan).

ISH of HGF mRNA in tumor tissues

RNA-RNA in situ hybridization (ISH) was performed with a riboprobe (cRNA) of a 603-bp murine HGF cDNA fragment (575–1177 nt). After linearization of the template cDNA, single-stranded antisense RNA probes labeled with digoxigenin-UTP were generated using a non-radioactive labeling kit (Genius 4; Boehringer Mannheim, Indianapolis, IN). Frozen tissue sections of the tumors were fixed with 4% paraformaldehyde and incubated with RNA probe (200 ng/ml) overnight at 42°C. For signal amplification, a HRP rabbit anti-digoxigenin Ab (DAKO, Carpinteria, CA) was used to catalyze the deposition of biotin-tyramide in a GenPoint kit (DAKO). Further amplification was achieved by adding HRP rabbit anti-biotin (DAKO), biotin-tyramide, and then alkaline-phosphatase rabbit anti-biotin (DAKO). Signals were detected with an alkaline-phosphatase substrate Fast Red TR/Napthol AS-MX (Sigma-Aldrich, St. Louis, MO).

Growth inhibition of LLC tumors in vivo by TNP-470 and CXCR2 Ab

To determine the in vivo inhibitory effect of the angiogenesis inhibitor TNP-470, TNP-470 (30 mg/kg) was s.c. administered every other day from day 1 to 9 after tumor inoculation as described previously (22). TNP-470 was dissolved in 0.5% ethanol plus 5% Arabic gum in saline as recommended by the manufacturer (Takeda Chemical Industries, Osaka, Japan). Tumor volumes were determined on day 15.

Neutralizing polyclonal rabbit anti-CXCR2 Ab (CXCR2 Ab) was prepared as described previously (23). Mice inoculated with LLC/IL-1β cells (5×10^5/mouse) at day 0 received i.p. injections of 200 μg of either anti-CXCR2 Ab or control IgG from day 2 to 6 every other day. Tumor volumes were determined on day 9.

Results

Production of IL-1β from LLC/IL-1β cells and its cell growth in vitro

LLC cells transduced with a hybrid gene of human growth hormone signal sequence and human *IL-1β* gene (LLC/IL-1β) secreted human IL-1β protein into the supernatant at 1.6 ± 0.23 μg (10^6 cells/24 h), whereas neither LLC cells nor LLC/neo secreted any detectable human IL-1β protein (<10 pg/10^6 cells/24 h). No secretion of mouse IL-1β protein from LLC, LLC/neo, or LLC/IL-1β cells was detected by the mouse IL-1β ELISA kit. The in vitro cell growth and morphology of LLC/IL-1β cells were not substantially different from those of LLC/neo and LLC cells in 10% FBS (Fig. 1) and 2% FBS medium (data not shown).

Gene transfer of IL-1β promotes tumor growth in both syngeneic and nude mice

In contrast to the absence of any significant change of the in vitro cell growth, tumors of LLC/IL-1β cells transplanted to the syngeneic C57BL/6 male mice grew strikingly faster than those of LLC/neo and LLC cells as shown in Fig. 2*A*. The tumor weight of LLC/IL-1β reached 3.63 ± 0.69 g, while those of LLC/neo and LLC reached 1.53 ± 0.4 and 1.45 ± 0.51 g at day 18 after inoculation. The tumor weight of LLC/IL-1β was 2.4 times greater than those of LLC/neo and LLC ($p < 0.01$). Consistently, this acceleration of tumor growth in LLC/IL-1β cells was also observed in BALB/c *nu/nu* male mice (Fig. 2*B*). The tumor volume of LLC IL-1β cells was more than three times greater than the control tumors ($p < 0.01$). These results suggested that this promotion of tumor growth was not associated with T cell-mediated immune responses against tumor cells.

Histological analysis of the LLC/IL-1β tumor

To investigate the mechanisms of the enhanced tumor growth of LLC/IL-1β cells in vivo, tumor sections were first stained with H&E. As shown in Fig. 3*A*, abundant blood vessels and RBCs

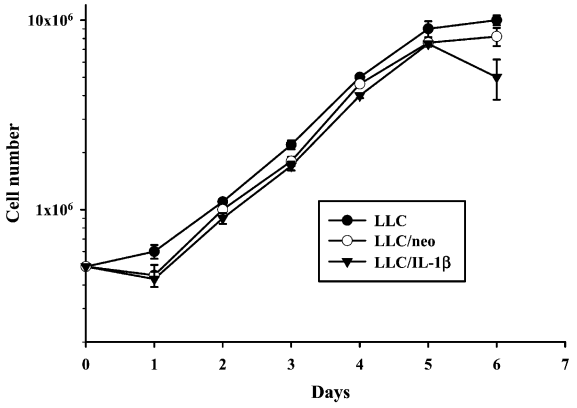

FIGURE 1. In vitro growth of LLC cells transduced with human IL-1β gene. The LLC cells transduced with a hybrid gene of human growth hormone signal sequence and human IL-1β gene by a retroviral vector were seeded at 5×10^5 cells in 6-cm dishes. The viable cells were counted every day for 6 days. Data represent the mean $\pm$ SD in triplicate experiments. ●, LLC parental cells; ○, LLC cells transduced by control retroviral vector PLXIN-neo (LLC/neo); ▼, LLC cells transduced with IL-1β gene by retroviral vector (LLC/IL-1β).

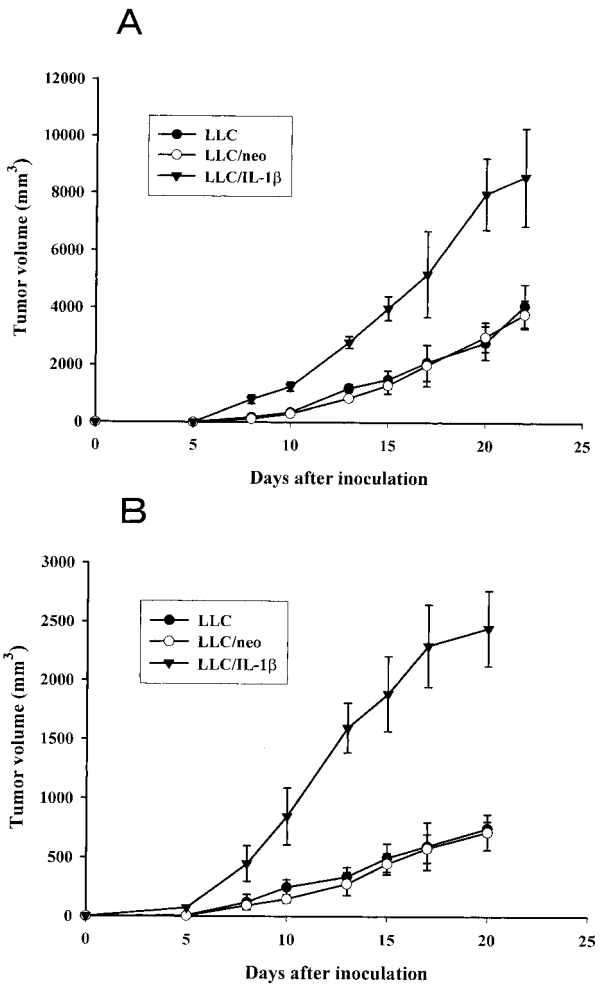

FIGURE 2. Effect of IL-1β gene transduction into LLC cells on tumor growth in mice. The LLC, LLC/neo, and LLC/IL-1β cells (5×10^5/mouse) were inoculated s.c. into syngeneic male C58BL/6 mice (*A*) or male BALB/c *nu/nu* mice (*B*) in the left flank at day 0. Tumors were measured with calipers in two perpendicular diameters every 2 or 3 days. The tumor volumes were calculated as described in *Materials and Methods.* Data represent the mean $\pm$ SD ($n = 5$). ●, LLC cells; ○, LLC/neo cells; ▼, LLC/IL-1β cells.

were noted in the LLC/IL-1β tumor. In higher magnification (Fig. 3*A*, insets), inflammatory cells such as leukocytes and monocytes were massively infiltrated into the LLC/IL-1β tumor compared with the LLC and LLC/neo tumors, suggesting that the tumor growth enhancement was associated with hyperneovascularization. As a next step, immunohistochemical staining of CD31 vascular endothelial adhesion protein was performed to analyze the neovascularization in the tumors. As shown in Fig. 3*B*, the number of CD31-positive vessels was strikingly increased in the LLC/IL-1β tumor, whereas the density of vessels in the LLC/neo tumor was similar to that of the LLC tumor. In the quantification of the vascular density in the tumor, the LLC/IL-1β tumor developed 2.5-fold more microvessels than LLC and LLC/neo tumors ($p < 0.05$) (Fig. 3*C*).

Up-regulation of VEGF and MIP-2 secretions from LLC/IL-1β cells

Because IL-1β is known to affect almost all types of cells and induce multiple factors, including angiogenic factors, we analyzed crucial angiogenic factors for tumor neovascularization, VEGF, and CXC chemokine MIP-2 (CXCL2, mouse functional homolog to human IL-8) secretion from LLC/IL-1β cells using ELISA kits. Although LLC cells constitutively secreted VEGF (0.9 ± 0.1 ng/10^6 cells/24 h), LLC/IL-1β cells secreted 1.8-fold more VEGF (1.6 ± 0.09 ng/10^6 cells/24 h) in 1% FBS containing medium (Table I). Furthermore, over 10 times the amount of MIP-2 was secreted from LLC/IL-1β cells (1.8 ± 0.21 ng/10^6 cells/24 h) than from LLC (0.08 ± 0.01 ng/10^6 cells/24 h) and LLC/neo cells (0.1 ± 0.02 ng/10^6 cells/24 h). Similar results were obtained in the 10% FBS medium (data not shown).

HGF in the LLC/IL-1β tumor tissue

Regarding tumor angiogenesis, HGF is known as another major angiogenic factor. In contrast to control LLC cells, LLC/IL-1β cells did not secrete HGF protein in vitro that was detectable by ELISA. The expression of HGF mRNA was also not detected in LLC/IL-1β cells even by RT-PCR (data not shown). However, the LLC/IL-1β tumors in mice contained a concentration of HGF protein over four times higher than did the control LLC tumors ($p < 0.05$) (Fig. 4*A*). Based on the observation that LLC/IL-1β cells themselves did not express HGF, we performed ISH of HGF mRNA to investigate what type of cell secreted HGF in the LLC/IL-1β tumor (Fig. 4*B*). A positive signal of HGF mRNA was detected in the LLC/IL-1β tumor (Fig. 4*Ba*), whereas no significant signal was detected in the LLC/neo tumor (Fig. 4*Bb*). In high magnification, with the data of H&E staining, infiltrating cells other than LLC/IL-1β cells overexpressed HGF mRNA in the LLC/IL-1β tumor (Fig. 4*B*, *c* and *d*). In low magnification, although a positive signal of HGF mRNA was detected inside of the LLC/IL-1β tumor, a stronger signal was observed in interstitial cells surrounding the tumor tissue (Fig. 4*Be*). In contrast, only a fine signal of HGF mRNA was detected in the cells surrounding the LLC/neo tumor (Fig. 4*Bf*). Taken together, analyses of in vitro and in vivo revealed that stromal cells consisting of fibroblasts, monocytes, and macrophages overexpressed HGF mRNA in the LLC/IL-1β tumor tissue.

To mimic the in vivo milieu of the LLC/IL-1β tumor, we added recombinant HGF protein (R&D Systems) to the LLC/IL-1β cells in vitro and examined whether HGF could alter the growth capacity of LLC/IL-1β cells and induce the secretion of angiogenic factors from LLC/IL-1β cells. Although LLC cells expressed HGF

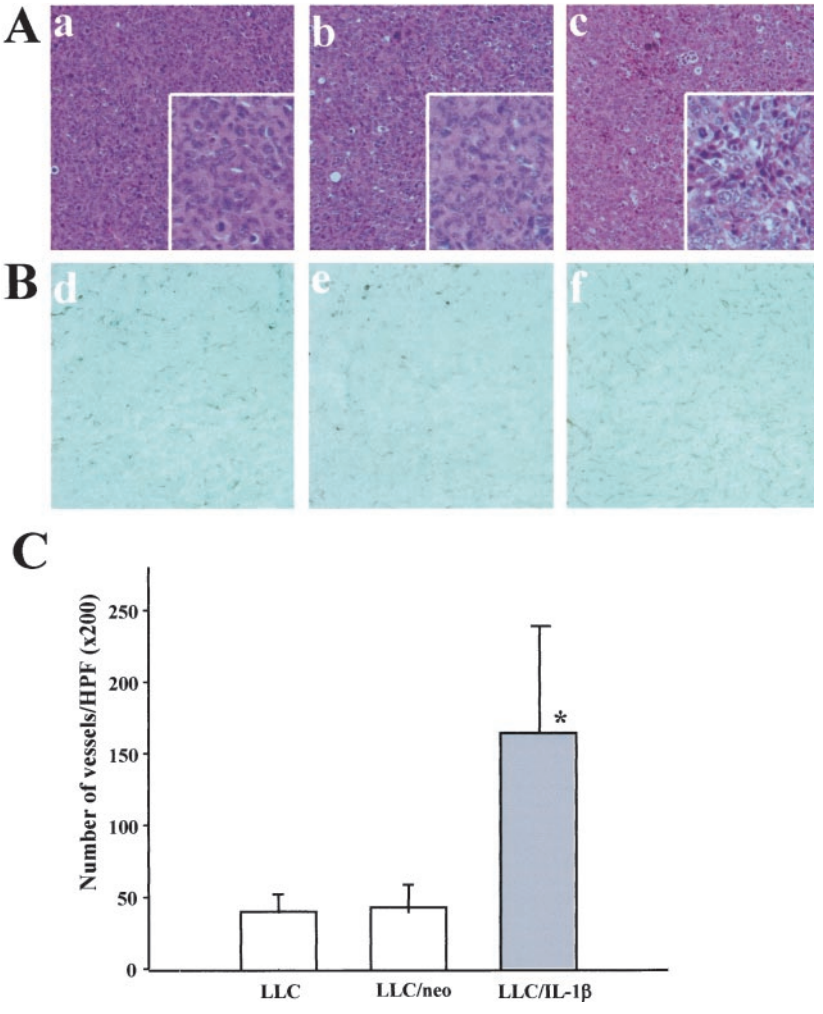

FIGURE 3. Hyperneovascularization in the LLC/IL-1β tumor. When the diameter of the tumors reached ~1 cm, these tumors were taken and fixed with 10% buffered formaldehyde or embedded in Tissue-Tek OCT embedding medium. *A*, Histology of the LLC/IL-1β tumor stained with H&E at lower magnification (×200) and higher magnification (insets, ×400). *a*, LLC tumor; *b*, LLC/neo tumor; *c*, LLC/IL-1β tumor. *B*, Immunohistochemical staining of CD31 in the LLC/IL-1β tumor (×100). Frozen sections of the tumors (LLC tumor, *left panel*; LLC/neo tumor, *middle panel*; LLC/IL-1β tumor, *right panel*) were stained with rat anti-mouse CD31Ab to visualize the vascular endothelial cells as described in *Materials and Methods*. *C*, Quantitative analysis of microvessel density in the LLC/IL-1β tumor. CD31-positive vessels were counted in the four hottest areas of the tumor as described in *Materials and Methods*. Data represent the mean ± SD ($n = 4$). *, $p < 0.05$.

receptor c-Met, no significant stimulation of growth capacity was observed in vitro at the different HGF concentrations (20 and 40 ng/ml) in 2% FBS medium (data not shown). In contrast, the secretion of VEGF and MIP-2 from LLC cells and LLC/neo cells was induced by HGF (40 ng/ml) (Table I). In addition, of interest, the secretion of VEGF and MIP-2 from LLC/IL-1β cells was further induced by HGF (Table I). These data suggested that LLC/IL-1β cells induced angiogenic factors by autocrine and paracrine loops in the in vivo condition.

Growth inhibition of the LLC/IL-1β tumor by TNP-470 and by neutralizing anti-CXCR2 receptor homolog Ab (CXCR2 Ab)

Because the LLC/IL-1β tumors showed hyperneovascularization, the LLC/IL-1β tumors were treated with the antiangiogenic agent TNP-470 to investigate whether hyperneovascularization in LLC/IL-1β tumor was directly associated with their accelerated growth in vivo. As shown in Fig. 5*A*, the growth of the LLC/IL-1β tumors was strongly inhibited by TNP-470 compared with that of the control tumors (81% compared with 12% inhibition). Moreover, neovascularization in the LLC/IL-1β tumors was decreased significantly by 60% with the TNP-470 treatment.

Because the LLC/IL-1β cells secreted over 10 times more MIP-2 than did the control cells, the LLC/IL-1β tumors were treated with CXCR2 Ab. As shown in Fig. 5*B*, CXCR2 Ab partially inhibited the tumor growth of the LLC/IL-1β cells (44% inhibition). However, after termination of the CXCR2 Ab treatment, the inhibitory effect disappeared and the LLC/IL-1β tumor began to grow faster (data not shown).

Table I. *Secretion of VEGF and MIP-2 from LLC/IL-1β cells in vitro in the absence or presence of HGF*[a]

	VEGF		MIP-2	
HGF	−	+	−	+
LLC	0.9 ± 0.1	1.24 ± 0.04	0.08 ± 0.01	0.16 ± 0.03
LLC/neo	0.81 ± 0.07	0.98 ± 0.11	0.1 ± 0.02	0.19 ± 0.01
LLC/IL-1β	1.6 ± 0.09	2.24 ± 0.22	1.80 ± 0.21	3.74 ± 0.76

[a] All cells were cultured in 1% FBS-containing medium. Data (measured in nanograms/1 × 10^6 cells/24 h) are presented as mean ± SD in triplicate experiments.

Discussion

In this study, we show that secretion of the proinflammatory cytokine IL-1β from tumor cells accelerates the growth rate of LLC transplanted in mice. A direct effect of IL-1β on the in vivo proliferation of LLC cells seems unlikely because LLC/IL-1β cells exhibited similar proliferation properties to control cells in vitro even in the presence of HGF. This promoting of the tumor growth of LLC/IL-1β cells in vivo can be attributed to the hyperneovascularization. Secretion of IL-1β from tumor cells into the tumor milieu induces several angiogenic factors, not only from tumor cells but also from stromal cells, including fibroblasts and macrophages, and thus promotes tumor neovascularization. Antiangiogenic agent TNP-470 strongly inhibited the growth of the LLC/

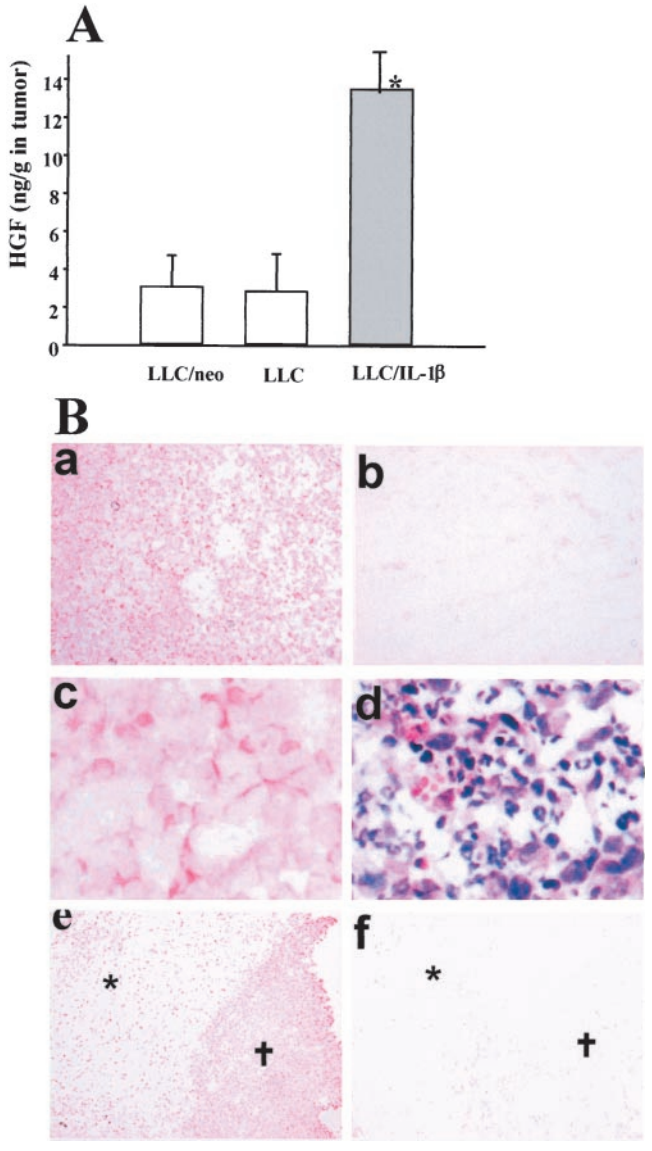

FIGURE 4. HGF in the LLC/IL-1β tumor. *A*, High concentration of HGF protein in the LLC/IL-1β tumor. When the tumor reached 1 cm in diameter, the tumor was extracted and used for the mouse HGF ELISA kit. The concentration of HGF in the tumor was determined and expressed as nanograms per gram of tissue as described in *Materials and Methods*. Data represent the mean ± SD ($n = 5$). *, $p < 0.05$. *B*, Increased expression of HGF mRNA inside and in the surrounding tissues of the LLC/IL-1β tumor. When the tumors reached ~1 cm diameter, tumors were removed and embedded in Tissue-Tek OCT embedding medium. The frozen sections were subjected to ISH of HGF mRNA as described in *Materials and Methods*. *a*, ISH of HGF mRNA of the LLC/IL-1β tumor; *b*, ISH of HGF mRNA of the LLC/neo tumor; *c*, high-power field (HPF) in ISH of the LLC/IL-1β tumor; *d*, high-power field in H&E staining of LLC/IL-1β tumor; *e*, low-power field of ISH of HGF mRNA of the LLC/IL-1β tumor (*) and surrounding interstitial cells (†); *f*, low-power field of ISH of HGF mRNA of the LLC/neo tumor (*) and surrounding interstitial cells (†). Original magnification: ×100 (*a* and *b*), ×200 (*c* and *d*), and ×40 (*e* and *f*).

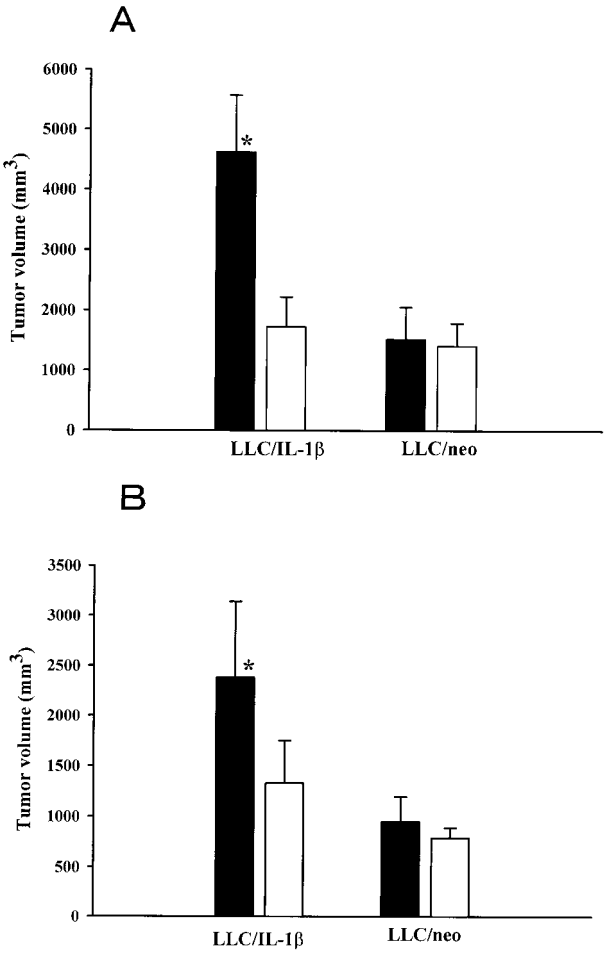

FIGURE 5. Growth inhibition of the LLC/IL-1β tumor by TNP-470 and by neutralizing anti-CXCR2 Ab (CXCR2 Ab). *A*, After inoculation of LLC/neo and LLC/IL-1β cells (5×10^5/mouse) into mice, TNP-470 (30 mg/kg) was s.c. administered every other day from day 1 to 9. Data represent the mean ± SD of tumor volume at day 15 ($n = 5$). Filled bars, Control; open bars, TNP-470 treatment. *, $p < 0.05$. *B*, Mice inoculated with the LLC/IL-1β cells (5×10^5) were injected i.p. with 200 μg of either anti-mouse CXCR2 Ab or preimmune IgG as a control three times on days 2, 4, and 6. Data represent mean ± SD of the tumor volume on day 9 ($n = 3$). Filled bars, Treatment with preimmune IgG; open bars, treatment with anti-mouse CXCR2 Ab. *, $p < 0.05$.

IL-1β tumor. Although we used human IL-1β expression system in this murine model that expresses mouse IL-1R, it is known that human IL-1β was able to bind and stimulate mouse IL-1R as well as murine IL-1β (24).

The progressive growth of a malignant solid tumor is dependent on the development of new blood vessels that provide oxygen and nutrients to the tumor cells (25). The extent of angiogenesis is determined by a balance between positive- and negative-regulating molecules, which are released by tumor cells and/or host cells in the tumor microenvironment. Although many factors are considered to be angiogenic, VEGF, IL-8, and HGF are all exceptionally important and powerful inducers of angiogenesis for solid tumors and are constitutively or inductively secreted from tumor cells and/or mesenchymal stromal cells (26–31). The stromal fibroblasts are induced to secrete HGF and VEGF by the stimulation of IL-1, basic fibroblast growth factor, and platelet-derived growth factor derived from tumor cells and macrophages in the tumor tissue (32–36).

In our system, the transduction of IL-1β into LLC cells leads to hyperneovascularization due to the overexpression of VEGF, MIP-2 (CXCL2, mouse functional homolog of human IL-8), and HGF by cross-talk between tumor cells and stromal cells. LLC/IL-1β cells overexpress VEGF and MIP-2 by themselves, but not HGF. However, high amounts of HGF in the LLC/IL-1β tumor were found by ELISA, and HGF mRNA was overexpressed in stromal fibroblasts and infiltrating macrophages as demonstrated

by ISH. IL-1β from tumor cells could stimulate the secretion of HGF from stromal cells. Furthermore, HGF further induces VEGF and MIP-2 production from LLC/IL-1β cells (37).

Direct evidence of a correlation between angiogenic factors and tumor promotion has been reported. Inhibition of IL-8 by neutralizing Abs abrogated the tumor size of human non-small cell lung cancer in SCID mice (38). VEGF overexpression in murine hepatocellular carcinoma cells by tetracycline-regulated retrovirus vector revealed the direct effect of tumor promotion by VEGF (39). Another report, using $VEGF^{null}$ fibrosarcoma cells, concluded that, in addition to the VEGF derived from stromal cells, VEGF derived from tumor cells is a critical factor in tumor expansion and vascular function (26).

HGF, originally identified as a mitogen of mature hepatocytes, is a multifunctional cytokine that stimulates mitogenesis, motogenesis, or morphogenesis in a wide variety of epithelial and endothelial cells in vitro (40–42). In vivo, HGF is a strong inducer of angiogenesis as well as invasion and metastasis of tumor cells. A recent report provides evidence that HGF promotes angiogenesis indirectly through the induction of other angiogenic factors, VEGF and CXC chemokine, as well as directly (39). A specific receptor of HGF, c-MET protein, is frequently overexpressed in human tumor cells and is recognized as an oncogene (43). HGF is produced mainly from stromal fibroblasts and macrophages in solid tumors. NK4, an antagonist of HGF, inhibits tumor growth by the suppression of tumor angiogenesis (44, 45). We have demonstrated that the growth of LLC/IL-1β tumors was inhibited by the antiangiogenic agent TNP-470 and by neutralizing CXCR2 Ab.

In contrast to our results, two previous studies showed tumor growth inhibition by human *IL-1β* gene transfer to murine tumor cells. Peplinski and colleagues (46, 47) demonstrated that intratumoral and i.v. injection of recombinant vaccinia virus-encoding IL-1β gene into murine pancreas tumors reduced the tumor growth in vivo. Another report revealed that constitutive expression of IL-1β gene in murine melanoma B16 resulted in reduced tumor growth in vivo (48). Immunohistochemical analyses have noted the severe infiltration of macrophages into B16 tumors transduced with IL-1β gene. A possible explanation for this discrepancy is the different biological effects of IL-1β according to the tumor type. In early reports using recombinant human IL-1β protein, IL-1β exhibited direct cytocidal or cytostatic effects on tumor cells and tumor regression in certain types of tumors by increasing the host immune responses (16, 17, 49), whereas it promoted tumor growth and metastases in other types of tumors (18, 19). These results indicate that the biological effects of IL-1β on tumor cells may depend on the type of tumor, doses of IL-1β, and a condition of IL-1β administration.

Numerous studies have shown the constitutive expression of IL-1β gene in human leukemia in which IL-1β acts as an autocrine and/or paracrine growth factor (50, 51). Although human solid tumors secrete a variety of molecules (52, 53), IL-1β is secreted from a limited number of tumors, i.e., melanoma, sarcoma, and hepatoblastoma, but not other common types of cancer because a proteolytic process from proIL-1β to mature IL-1β by IL-1-converting enzyme is necessary for the secretion of active IL-1β (54). IL-1β expression was not detectable by either RT-PCR or ELISA in human lung cancer cells, even the presence of inflammatory stimuli in our experiments.

Various hematological cells infiltrate and perform a wide variety of functions depending on the physiologic or pathophysiologic conditions in solid tumor tissues. Macrophages infiltrating into tumor tissues, referred to as tumor-associated macrophages (TAMs), are reported to correlate with angiogenesis and thus poor prognosis in several human cancers (55, 56). TAMs activated by TGF-β1 secreted from tumor cells produce both IL-1 and TNF-α, which in turn stimulate tumor cells to secrete IL-8 and VEGF, and stromal cells to secrete HGF (32). In another experiment, macrophage migration-inhibitory factor produced from tumor cells induced monocytes to increase their expression of CXC chemokine (57). Taken together, IL-1β might be secreted from TAMs activated by tumor cells and promote tumor angiogenesis through the stimulation of secretion of angiogenic factors from both tumor cells and stromal cells.

References

1. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood 87:2095.*
2. Wakabayashi, G., J. A. Gelfand, W. K. Jung, R. J. Connolly, J. F. Burke, and C. A. Dinarello. 1991. *Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. *J. Clin. Invest. 87:1925.*
3. Ghezzi, P., C. A. Dinarello, M. Bianchi, M. E. Rosandich, J. E. Repine, and C. W. White. 1991. Hypoxia increases production of interleukin-1 and tumor necrosis factor by human mononuclear cells. *Cytokine 3:189.*
4. Dinarello, C. A., J. G. Cannon, J. W. Mier, H. A. Bernheim, G. LoPreste, D. L. Lynn, R. N. Love, A. C. Webb, P. E. Auron, R. C. Reuben, et al. 1986. Multiple biological activities of human recombinant interleukin 1. *J. Clin. Invest. 77:1734.*
5. Ghezzi, P., and C. A. Dinarello. 1988. IL-1 induces IL-1. III. Specific inhibition of IL-1 production by IFN-γ. *J. Immunol. 140:4238.*
6. Koch, A. E., P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner S. G. Elner, and R. M. Strieter. 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science 258:1798.*
7. Tamura, M., N. Arakaki, H. Tsubouchi, H. Takada, and Y. Daikuhara. 1993. Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumor necrosis factor-α by fibroblasts in culture. *J. Biol. Chem. 268:8140.*
8. Wang, J. M., X. Deng, W. Gong, and S. Su. 1998. Chemokines and their role in tumor growth and metastasis. *J. Immunol. Methods 220:1.*
9. Spechler S. J. 1999. The role of gastric carditis in metaplasia and neoplasia at the gastroesophageal junction. *Gastroenterology 117:218.*
10. Nardone, G., S. Staibano, A. Rocco, E. Mezza, F. P. D'armiento, A. Insabato, A. Coppola, A. Salvatore, A. Lucariello, N. Figura, et al. 1999. Effect of *Helicobacter pylori* infection and its eradication on cell proliferation, DNA status, and oncogene expression in patients with chronic gastritis. *Gut 44:789.*
11. Tsutsumi, M., H. Kitada, K. Shiraiwa, M. Takahama, T. Tsujiuchi, H. Sakitani, Y. Sasaki, K. Murakawa, M. Yoshimoto, and Y. Konishi. 2000. Inhibitory effects of combined administration of antibiotics and anti-inflammatory drugs on lung tumor development initiated by *N*-nitrosobis (2-hydroxypropyl) amine in rats. *Carcinogenesis 21:251.*
12. Moore, R. J., D. M. Owens, G. Stamp, C. Arnott, F. Burke, N. East, H. Holdsworth, L. Turner, B. Rollins, M. Pasparakis, et al. 1999. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. *Nat. Med. 5:828.*
13. Kleer, C. G., K. L. van Golen, and S. D. Merajver. 2000. Molecular biology of breast cancer metastasis: inflammatory breast cancer: clinical syndrome and molecular determinants. *Breast Cancer Res. 2:423.*
14. Shirakawa, K., H. Tsuda, Y. Heike, K. Kato, R. Asada, M. Inomata, H. Sasaki, H. Kasumi, M. Yoshimoto, T. Iwanaga, et al. 2001. Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. *Cancer Res. 61:445.*
15. Orosz, P., A. Kruger, M. Hubbe, J. Ruschoff, P. V. Hoegen, and D. M. Mannel. 1995. Promotion of experimental liver metastasis by tumor necrosis factor. *Int. J. Cancer 60:867.*
16. Onozaki, K., K. Matsushima, B. B. Aggarwal, and J. J. Oppenheim. 1985. Human interleukin 1 is a cytocidal factor for several tumor cell lines. *J. Immunol. 135: 3962.*
17. Takikawa, O., T. Oku, H. Yasui, and R. Yoshida. 1993. Synergism between IFN-γ and IL-1αβ in growth inhibition of an allografted tumor. *J. Immunol. 151:2070.*
18. Mckenzie, R. C., E. S. Park, W. R. Brown, G. S. Shivji, and D. N. Sauder. 1994. Effect of ultraviolet-inducible cytokines on melanoma growth in vivo: stimulation of melanoma growth by interleukin-1 and -6. *Photodermatol. Photoimmunol. Photomed. 10:74.*
19. Bani, M. R., A. Garofalo, E. Scanziani, and R. Giavazzi. 1991. Effect of interleukin-1β on metastasis formation in different tumor systems. *J. Natl. Cancer Inst. 83:119.*
20. Pecceu, F., P. Dousset, D. Shire, E. Cavrois, E. Marchese, P. Ferrara, M. Kaghad, X. Dumont, and J. Lupker. 1991. Human interleukin 1β fused to the human growth hormone signal peptide is *N*-glycosylated and secreted by Chinese hamster ovary cells. *Gene 97:253.*
21. Tanaka, M., K. Narumi, M. Isemura, M. Abe, Y. Sat, T. Abe, Y. Saijo, T. Nukiwa, and K. Satoh. 2000. Expression of the 37-kDa laminin binding protein in murine lung tumor cell correlates with tumor angiogenesis. *Cancer Lett. 153:161.*
22. Yoshida, T., Y. Kaneko, A. Tsukamoto, K. Han, M. Ichinose, and S. Kimura. 1998. Suppression of hepatoma growth and angiogenesis by a fumagillin derivative TNP470: possible involvement of nitric oxide synthase. *Cancer Res. 58: 3751.*

23. Sonoda, Y., N. Mukaida, J. Wang, M. Shimada-Hiratsuka, M. Naito, T. Kasahara, A. Harada, M. Inoue, and K. Matsushima. 1998. Physiologic regulation of postovulatory neutrophil migration into vagina in mice by a C-X-C chemokine(s). *J. Immunol. 160:6159.*
24. Kilian, P. L., K. L. Kaffka, A. S. Stern, D. Woehle, W. R. Benjamin, T. M. Dechiara, U. Gubler, J. J. Farrar, S. B. Mizel, and P. T. Lomedico. 1988. IL-1α and IL-1β bind to the same receptor on T cells. *J. Immunol. 136:4509.*
25. Liotta, L. A., and E. C. Kohn. 2001. The microenvironment of the tumour-host interface. *Nature 411:375.*
26. Grunstein, J., G. Roberts, O. Mathieu-Costello, D. Hanahan, and R. S. Johnson. 1999. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. *Cancer Res. 59:1592.*
27. Inoue, K., J. W. Slaton, B. Y. Eve, S. J. Kim, P. Perrotte, D. Balbay, S. Yano, M. Bar-Eli, R. Radinsky, C. A. Pettaway, and C. P. N. Dinney. 2000. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin. Cancer Res. 6:2104.*
28. Xin, X., S. Yang, G. Ingle, C. Zlot, L. Rangell, J. Kowalski, R. Schwall, N. Ferrara, and M. E. Gerritsen. 2001. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. *Am. J. Pathol. 158:1111.*
29. Yoshida, S., M. Ono, T. Shono, H. Izumi, T. Ishibashi, H. Suzuki, and M. Kuwano. 1997. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor α-dependent angiogenesis. *Mol. Cell. Biol. 17:4015.*
30. Maier, J. A. M., M. Mariotti, P. M. Comoglio, and M. R. Soria. 1996. Interleukin 1 induces an autocrine loop hepatocyte growth factor/c-Met in murine Kaposi-like spindle cells. *Oncogene 13:1009.*
31. Chen, S. Y., J. Viallet, R. H. Schwall, T. Nakamura, and M. S. Tsao. 1998. Paracrine effects of hepatocyte growth factor/scatter factor on non-small-cell lung carcinoma cell lines. *Br. J. Cancer 77:2162.*
32. Liss, C., M. J. Fekete, R. Hasina, C. D. Lam, and M. W. Lingen. 2001. Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages. *Int. J. Cancer 93:781.*
33. Yamada, T., S. Fujieda, S. Yanagai, H. Yamamura, R. Inatome, H. Yamamoto, H. Igawa, and H. Saito. 2001. IL-1 induced chemokine production through the association of Syk with TNF receptor-associated factor-6 in nasal fibroblast lines. *J. Immunol. 167:283.*
34. Li, J., M. A. Perrella, J.-C. Tsai, S.-F. Yet, C.-M. Hsieh, M. Yoshizumi, C. Patterson, W. O. Endege, F. Zhou, and M.-E. Lee. 1995. Induction of vascular endothelial growth factor gene expression by interleukin-1β in rat aortic smooth muscle cells. *J. Biol. Chem. 270:308.*
35. Akagi, Y., W. Liu, K. Xie, B. Zebrowski, R. M. Shaheen, and L. M. Ellis. 2001. Regulation of vascular endothelial growth factor expression in human colon cancer by interleukin-1β. *Br. J. Cancer 80:1506.*
36. Ristimaki, A., K. Narko, B. Enholm, V. Joukov, and K. Alitalo. 1998. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J. Biol. Chem. 273:8413.*
37. Dong, G., Z. Chen, Z.-Y. Li, N. T. Yeh, C. C. Bancroft, and C. V. Waes. 2001. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. *Cancer Res. 61:5911.*
38. Arenberg, D. A., S. L. Kunkel, P. J. Polverini, M. Glass, M. D. Burdick, and R. M. Strieter. 1996. Inhibition of interleukin-8 reduces tumorigenesis of human non-small-cell lung cancer in SCID mice. *J. Clin. Invest. 97:2792.*
39. Yoshiji, H., S. Kuriyama, D. K. Ways, J. Yoshiji, Y. Miyamoto, M. Kawata, Y. Ikenaka, H. Tsujinoue, T. Nakatani, M. Shibuya, and H. Fukui. 1999. Protein kinase C lies on the signaling pathway for vascular endothelial growth factor-mediated tumor development and angiogenesis. *Cancer Res. 59:4413.*
40. Pilewski, J. M., T. S. Bumbalo, A. G. Davis, and J. M. Siegfried. 2001. Hepatocyte growth factor promotes tumor growth in a novel in vivo model of human lung cancer. *Am. J. Respir. Cell Mol. Biol. 24:556.*
41. Uchida, D., H. Kawamata, F. Omotehara, K. Nakashiro, T. Kimura-Yanagawa, S. Hino, N. M. Begum, M. O. Hoque, H. Yoshida, M. Sato, and T. Fujimori. 2001. Role of HGF/c-Met system in invasion and metastasis of oral squamous cells carcinoma cells in vitro and its clinical significance. *Int. J. Cancer 93:489.*
42. Bell, A., Q. Chen, M. C. DeFrances, G. K. Michalopoulos, and R. Zarnegar. 1999. The five amino acid-deleted isoform of hepatocyte growth factor promotes carcinogenesis in transgenic mice. *Oncogene 18:887.*
43. Di Renzo, M. F., R. P. Narsimhan, M. Olivero, S. Bretti, S. Giordano, E. Medico, P. Gaglia, P. Zara, and P. M. Comoglio. 1991. Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene 6:1997.*
44. Kuba, K., K. Matsumoto, K. Date, H. Shimura, M. Tanaka, and T. Nakamura. 2000. HGF/NK4, a four kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. *Cancer Res. 60:6737.*
45. Maemondo, M., K. Narumi, Y. Saijo, K. Usui, M. Tahara, R. Tazawa, K. Hagiwara, K. Matsumoto, T. Nakamura, and T. Nukiwa. 2002. Targeting angiogenesis and HGF function using an adenoviral vector expressing the HGF antagonist NK4 for cancer therapy. *Mol. Ther. 5:177.*
46. Peplinski, G. R., K. Tsung, E. D. Whitman, J. B. Meko, and J. A. Norton. 1995. Construction and expression in tumor cells of a recombinant vaccinia virus encoding human interleukin-1β. *Ann. Surg. Oncol. 2:151.*
47. Peplinski, G. R., K. Tsung, J. B. Meko, and J. A. Norton. 1995. In vivo gene therapy of a murine pancreas tumor with recombinant vaccinia virus encoding human interleukin-1β. *Surgery 118:185.*
48. Bjorkdahl, O., A. G. Wingren, G. Hedlund, L. Ohlsson, and M. Dohlsten. 1997. Gene transfer of a hybrid interleukin-1β gene to B16 mouse melanoma recruits leukocyte subsets and reduces tumor growth in vivo. *Cancer Immunol. Immunother. 44:273.*
49. Itoh, S., T. Hattori, H. Hayashi, Y. Mizutani, M. Todo, T. Takii, D. Yang, J. C. Lee, S. Matsufuji, Y. Murakami, et al. 1999. Antiproliferative effect of IL-1 is mediated by p38 mitogen-activated protein kinase in human melanoma cell A375. *J. Immunol. 162:7434.*
50. Beaupre, D. M., M. Talpaz, F. C. Marini III, R. J. Cristiano, J. A. Roth, Z. Estrov, M. Albitar, M. H. Freedman, and R. Kurzrock. 1999. Autocrine interleukin-1β production in leukemia: evidence for the involvement of mutated RAS. *Cancer Res. 59:2971.*
51. Dankbar, B., T. Padro, R. Leo, B. Feldmann, M. Kropff, R. M. Mesters, H. Serve, W. E. Berdel, and J. Kienast. 2001. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interaction in multiple myeloma. *Blood 95:2630.*
52. Chen, Z., P. S. Malhotra, G. R. Thomas, F. G. Ondrey, D. C. Duffey, C. W. Smith, I. Enamorado, N. T. Yeh, G. S. Kroog, S. Rudy, et al. 1999. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. *Clin. Cancer Res. 5:1369.*
53. Huang, Y. T., T. S. Sheen, C. L. Cheng, C. J. Lu, Y. Chang, J. Y. Chen, and C. H. Tsai. 1999. Profile of cytokine expression in nasopharyngeal carcinomas: a distinct expression of interleukin 1 in tumor and $CD4^+$ T cells. *Cancer Res. 59:1599.*
54. Kostura, M. J., M. J. Tocci, G. Limjuco, J. Chin, P. Cameron, A. G. Hillman, N. A. Chartrain, and J. A. Schmidt. 1989. Identification of a monocyte specific pre-interleukin 1β convertase activity. *Proc. Natl. Acad. Sci. USA 86:5227.*
55. Takanami, I., K. Takeuchi, and S. Kodaira. 1999. Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology 57:138.*
56. Leek, R. D., C. E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, and A. L. Harris. 1996. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res. 56:4625.*
57. White, E. S., S. R. B. Strom, N. L. Wys, and D. A. Arenberg. 2001. Non-small cell lung cancer cells induce monocytes to increase expression of angiogenic activity. *J. Immunol. 166:7549.*