Journal of Cellular Biochemistry 62:443–453 (1996)

Interleukin 4 Inhibits Hepatocyte Growth Factor-Induced Invasion and Migration of Colon Carcinomas

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Abstract Hepatocyte growth factor (HGF) is known to have a number of biological properties including promoting tumor progression of human carcinomas. Metastasis involves a number of events that are attributed to induction by paracrine factors such as HGF. Identification of natural inhibitors of these events would allow better control of tumor progression. Recently we demonstrated that interleukin 4 (IL-4) can regulate proliferation of various human carcinoma cell lines. In the present study, we used established human colon carcinoma cell lines and primary colon carcinoma cell cultures to determine if IL-4 could regulate HGF-induced cell proliferation and other events of tumor progression such as MMP (matrix metalloproteinases)-1, -2, and -9 production, cell migration and cell-matrix invasive activity. All colon carcinoma cell lines expressed HGF and IL-4 receptors. IL-4 significantly inhibited HGF-induced proliferation of one cell line. Cell-matrix invasion was significantly enhanced by HGF (0.1–10 ng/ml); IL-4 (1–10 U/ml) significantly inhibited HGF-induced invasion in a dose-dependent manner. IL-4 also inhibited HGF-induced cell-matrix invasion of metastatic colon carcinoma cells and HGF-induced cell migration. HGF enhanced MMP-1, -2, and -9 production by cell lines. This effect could be inhibited by IL-4. These findings indicate that IL-4 is a potent inhibitor of HGF-induced invasion and metastasis-related functions of human colon carcinoma cells.

Key words: cancer, collagenase, *Met*, cytokine, metastasis, motility

Hepatocyte growth factor was first identified and cloned as a potent stimulator of DNA synthesis in cultured hepatocytes [Nakamura et al., 1989; Gohda et al., 1988]. HGF's effects are elicited through HGFR, also known as the c-*Met* protooncogene product, a member of the tyrosine kinase growth factor receptor family [Bottaro et al., 1991; Naldini et al., 1991]. HGF is secreted by mesenchymal stromal cells, macrophages, hepatic sinusoidal endothelial cells, Kupffer cells, fibroblasts, and skin fibroblasts [Noji et al., 1990; Konishi et al., 1991; Matsumoto et al., 1992]. Recently it has been shown that scatter factor, which induces motogenesis of tumor cells, is identical to HGF [Matsumoto and Nakamura, 1993]. HGF also induces mitogenic, motogenic, and morphogenic effects on normal and malignant cells [Stoker et al., 1987; Tajima et al., 1992; Matsumoto et al., 1991; Bussolino et al., 1992; Montesano et al., 1991; Tsarfaty et al., 1992]. It has been implicated that HGF may play a significant role in tumor invasion and metastasis [Tajima et al., 1992; Weidner et al., 1990]. HGFR is often overexpressed in carcinomas of the gastrointestinal tract [Di Renzo et al., 1992; Konishi et al., 1991]. HGF is produced during tissue destruction, inflammation, and in various other disease states of the liver [Jiang et al., 1993; Kaneko et al., 1992]. Gastrointestinal tumors that invade and establish metastases in the liver can cause

Abbreviations used: bp, base pair; cDNA, complementary DNA; IL-4, interleukin-4; IL-4R, interleukin-4 receptor; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor (*Met*); MMP-1, -2, -9, matrix metalloproteinases; PBS, phosphate buffered saline; RT-PCR, reverse-transcriptase polymerase chain reaction.

Received October 10, 1995; accepted February 16, 1996.

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major tissue destruction and inflammation. The activation of HGF during tissue destruction could potentially facilitate tumor progression of HGFR positive tumor cells in this microenvironment.

HGF is a paracrine factor produced predominantly by mesenchymal derived cells on induction by carcinoma cells [Matsumoto and Nakamura, 1993]. Seslar et al. [1993], have recently demonstrated that HGF secretion by human fibroblasts is regulated in a paracrine fashion by human breast tumor cell lines. Clinical investigations have shown that HGF level is increased in malignant pleural effusion, in sera of cancer patients [Kenworthy et al., 1992], and in the serum of patients receiving therapeutic treatment to the liver [Kaneko et al., 1992]. Identification of host factors that regulate HGF's effect on carcinoma cells would play an important role in controlling tumor metastasis and invasion.

Functional IL-4R is expressed in a wide range of human cancer cells such as melanoma [Hoon et al., 1991a], renal cell [Hoon et al., 1991b; Obiri et al., 1993], gastric [Morisaki et al., 1992], lung [Tungekar et al., 1991], breast and colon carcinomas [Toi et al., 1992; Kaklamanis et al., 1992]. IL-4 significantly inhibits the proliferation of human carcinomas and induce differentiation of most cells [Hoon et al., 1991b; Morisaki et al., 1992]. IL-4, an immune cytokine predominantly produced by CD4+ T cells, mast cells, and basophil cells [Paul and Ohara, 1987], has an interesting role in regulating non-hemopoietic tumor growth [Hoon et al., 1996].

A multiple number of successful biological events are required in order for tumor cells to establish distant metastatic colonies [Nicolson, 1982; Liotta and Stetler-Stevenson, 1991]. These events include tumor cell proliferation, motogenesis, invasion of tissue, and extracellular matrix degradation. Invasive tumor cells release endopeptidases referred as matrix-metalloproteinases (MMP) that have a broad spectrum of proteolytic activity for several types of extracellular matrix [Martrisian, 1990]. Some of the major MMPs secreted by highly invasive neoplastic cells include: MMP-1, an interstitial collagenase that degrades collagen types I, II, and III; MMP-2 (72 kD) and MMP-9 (92 kD) are collagenases that degrade denatured collagen (gelatin), and collagen types IV, and V [DeClerck et al., 1992; Fridman et al., 1995]. Collectively, these enzymes participate in the complex of events involved in invasion and metastasis. Recent studies indicate that there are a number of host factors which can regulate these events [Martrisian, 1990]. In this study we provide evidence that IL-4 can significantly inhibit HGF-induced cell proliferation, cell migration, cell-matrix invasion, and MMP-1, -2, and -9 production by colon carcinoma cells.

MATERIALS AND METHODS

Cell Lines

Human colon carcinoma cell lines CoSp, CoPe, and CoDo were derived from biopsy specimens of a serosal metastasis, a primary tumor, and a lung metastasis, respectively. All specimens were from patients who had elective surgery at Saint John's Hospital, John Wayne Cancer Clinic. These permanently established cell lines were characterized as colon carcinomas. The colon carcinoma cell line HT29 (ATCC, Rockville, MD) and erythroleukemia cell line K562 (ATCC) were used as controls. Cell lines used in the study had undergone more than 15 cell passages. The lines were cultured in culture flasks with RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bioproducts Inc., Calabasas, CA), antibiotics, and incubated in a humidified incubator with 7% CO_2 at 37°C.

Primary Cell Cultures

Primary cultures were established from biopsy specimens of an omental metastasis (CoST) and a liver metastasis (CoZH). Fresh tissues were minced into small pieces (~ 10 mm^3), digested by type I collagenase for 2 h at 37°C, washed twice with RPMI 1640, and resuspended in RPMI culture medium containing 10% heat-inactivated fetal bovine serum. The cells were seeded in multiple 24-well culture plates (Costar, Cambridge, MA) and cultured in a humidified incubator with 7% CO_2 at 37°C. The medium was replaced each week. Primary cultures were used only after histological identification as carcinomas. After 4 weeks of culture, epithelial colonies were harvested and used for invasion assays.

Reagents

Human recombinant HGF was purified from culture media of CHO (Chinese hamster ovary) cells transfected with plasmid containing human HGF cDNA [Nakamura et al., 1989]. HGF purity was > 98% as determined by SDS/PAGE,

and activity was expressed as ng/ml. Recombinant IL-4 (1.8×10^7 U/mg) was kindly supplied by Schering-Plough (Kennilsworth, NJ). A polyclonal rabbit anti-human HGFR (*Met* product) antibody which recognizes the C-terminal amino acid sequence of the β-chain [Tempest et al., 1988] was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A mouse monoclonal anti-human IL-4R antibody (M57) was kindly supplied by Immunex (Seattle, WA). Antibodies were used for Western blotting and flow cytometry analysis [Morisaki et al., 1992].

Western Immunoblot Analysis

HGFR expression in CoSp, CoPe, and CoDo cells was assessed by Western blot analysis. Subconfluent cells in T75 cm^2 culture flasks (Corning, NY) were washed twice with cold phosphate buffered saline (pH 7.2) and lysed in 1 ml of lysis buffer containing 200 mg/ml of phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Cell lysates were adjusted to 2 mg/ml protein and assessed using the ECL Western blotting analysis system (Amersham, IL). Briefly, 30 μg of each protein sample from colon carcinoma cell lines was gel-fractionated and electroblotted to a nitrocellulose membrane (Bio-Rad, Richmond, CA). The immunoblots were incubated with anti-HGFR antibody. Antibody binding was detected by incubating blots with peroxidase-labeled anti-rabbit antibody followed by ECL detection reagents. The blots were then exposed to autoradiography film (Hyperfilm-ECL).

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA of colon carcinoma cells was isolated using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH), following the manufacturers instructions. 1 μg of total RNA was used in the RT-PCR assay to detect IL-4R and HGFR mRNA. RNA was added to a 20 μl reaction mixture containing 5× RT buffer, 10 mM dinucleotide triphosphate mixture (dNTPs), RNasin, oligo dT_{15} primer, and avian myeloblastosis virus reverse-transcriptase. Reagents for the reverse-transcriptase reaction were obtained from Promega (Madison, WI). The mixture was incubated for 2 h at 37°C, heated to 99°C for 5 min, and then chilled on ice. Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute, UCLA core facilities. The sequences for IL-4R primers were derived from the GenBank: 3′ primer, 3′-GGGCTTGAAGGAGCCCTTCCA-5′; and 5′ primer, 5′-ATGGGGTGGCTTTGCTCTGGG-3′. The IL-4R PCR cDNA product was 345 bp. The primer sequences for HGFR were: 5′ primer, 5′-TGGATGGCTTTGGAAAGTCTG; and 3′ primer, 3′-TTTTACGTTCACATAAGTAGC-5′. The HGFR PCR cDNA product was 336 bp. The primer sequences for β-actin were: 5′ primer, 5′-CCTTCCTGGGCATGG AGTCCTG-3′; and 3′ primer, 3′-GGAGCAATGATCTTGATCTTC-5′. The β-actin PCR product was 201 bp. The RT-PCR mixture consisted of 10 μl of 10X RT-PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin) (Perkin Elmer Cetus, Norwalk, CT), 8 μl of 10 mM dNTPs mixture, 1 μl of each oligonucleotide primer (100 pmol/μl), 0.5 μl of AmpliTaq DNA polymerase (2.5 units/μl) (Promega), and 20 μl of RT mixture [Morisaki et al., 1993]. Sterile double-distilled water was added to bring the volume up to 100 μl. 100 μl of mineral oil was added to prevent evaporation. The RT-PCR reaction for β-actin and HGFR was performed in an Omni-Gene temperature cycler (Hybaid, Middlesex, England): 1 cycle at 95°C for 3 min; 95°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles; and 72°C for 10 min. IL-4R RT-PCR was run at 1 cycle for 95°C for 3 min; 95°C for 1 min, 63°C for 1 min, 72°C for 1 min for 35 cycles; and 72°C for 10 min. The RT-PCR cDNA products were assessed in a 2% agarose gel containing ethidium bromide. A 100 base pair (bp) DNA ladder (GIBCO BRL Life Technologies Inc., Gaithersburg, MD) was used as a bp reference marker.

Assessment of Cell Proliferation

Colon carcinoma cells were assessed for cell proliferation in the presence of IL-4 and HGF. Cells were seeded in 12-well tissue culture plates in regular serum-supplemented culture medium, as described above, with specific concentrations of HGF. Cells were seeded at 5×10^4/well in triplicates. Control cultures consisted of cells with culture medium alone. Cell proliferation/viability was assessed using a microscope, by a standard trypan blue viability exclusion technique with a cell-counting hemocytometer. Counts were performed in duplicates for each well and expressed as the mean. Overall counts for individual experiments were expressed as mean of triplicate measurements. Analysis of IL-4 effect on HGF on colon carcinoma cells was carried out as described above with the following modifications: Cell cultures were seeded with

5×10^4 cells/well in triplicates for harvesting on days 3, 5, and 7. The cell cultures were treated with IL-4, HGF, IL-4 + HGF, or culture medium alone after seeding (day 0).

Migration and Cell-Matrix Invasion Assay

The invasive response of colon carcinoma cells to HGF was assessed by a standard chemoinvasion assay using microplate Transwell chambers, prepared as previously described [Uchiyama et al., 1992; Mareel et al., 1991]. The 9-mm diameter filters of 8-μm porous cell culture Transwell inserts (Falcon, Indianapolis, IN) were coated with 100 μg basement membrane matrix, Matrigel (Collaborative Research, Bedford, MA). The Transwells with filters were then placed in 24-well plates. 400 μl of culture medium containing an indicated amount of HGF was added to the lower wells, and 300 μl of culture medium containing 5×10^4 cells with or without IL-4 was added to the upper wells. After 72 h of incubation, noninvading cells left on the upper surface of the filter were completely removed and visually verified under a phase contrast microscope. The filters were fixed with methanol and stained with Giemsa solution. The cells on the lower surfaces of filters were counted using a 200× objective on a Nikon phase contrast microscope (Nikon, Melville, NY). Total number of cells were counted in five representative microscopic fields for each filter. The migration assay was performed under identical conditions as the cell-matrix invasion assay except, non-coated filters were used in the Transwells.

Analysis of MMP-1 Production

Colon carcinoma cells from individual cell lines were seeded in 12-well tissue culture microplates (10^6 cells/well) in 2 ml of RPMI 1640 containing HGF and/or IL-4 and cultured at 37°C in a tissue culture incubator for 24 h. Supernatant was harvested and concentrated 10× to a volume of 200 μl using Centricon 10 concentrators (Amicon Division, Beverly, MA). MMP-1 in the supernatant was measured using a human specific MMP-1 sandwich ELISA (Amersham International, Buckinghamshire, England), following the manufacturers instructions. Supernatant samples were tested in duplicate for each assay. A standard curve was performed in each assay to calculate MMP-1 levels. The ELISA reaction was read at 450 nm using a Vmax kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA).

Zymography

Reverse zymogram gel analysis was performed to assess gelatinase and collagenase IV activity [Liabakk et al., 1996] of colon carcinoma cell lines. Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis was performed with gelatin (0.1%) incorporated into the acrylamide mixture prior to polymerization following the manufacturers instructions (Novex, San Diego, CA). Cells (10^6) were seeded in 12-well culture plates in 2 ml of culture medium with or without cytokines. The culture medium was harvested after 24 h incubation at 37°C and then concentrated 10× using a Centricon 10 concentrator. Gels were stained with Coomassie blue R-250 (detection limit is less than 10 units of collagenase) and destained with 40% methanol and 10% acetic acid. A clear zone in the gel indicated the presence of protein with gelatinolytic activity. Gels were scanned using a computer photoanalyzer program and the image was digitally inverted. Each image was scanned several times for analysis. The images were then quantitated using a dual-wavelength flying-spot scanner (Shimadzu Corp., Kyoto, Japan). The greater the degree of enzyme activity the larger and brighter the band.

Statistical Analysis

Student's *t*-test was used for statistical analysis of data.

RESULTS

IL-4R and HGFR Expression by Colon Carcinoma Cell Lines

To verify that CoSp, CoPe, and CoDo express IL-4R and HGFR, RT-PCR analysis was performed. All three lines expressed IL-4R and HGFR mRNA (Fig. 1). The level of HGFR expression by the three colon carcinoma cell lines was also assessed by Western blotting (Fig. 2) and densitometric analysis. The HGFR, 140 kD protein, was expressed by all three colon carcinoma cell lines. The expression levels in relative densitometric units from highest to lowest was CoSp (216,000), CoDo (6330), and CoPe (3960), respectively. HT29 colon carcinoma cell line was a positive control for HGFR expression (112,000 densitometric units), and the K562 cell line (0 densitometric units) was used as a negative con-

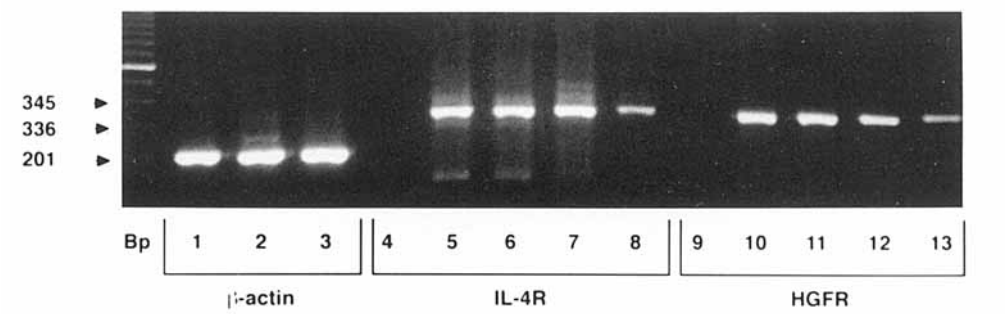

Fig. 1. Expression of IL-4R and HGFR mRNA of colon carcinoma cell lines. Bp, base pair ladder. *Lanes 1–3:* β-Actin (201 Bp); *lanes 4–8:* IL-4R (345 Bp); and *lanes 9–13:* HGFR (336 Bp). Lanes 4 and 9 are representative of RT-PCR control (no RNA) with IL-4R and HGFR primers, respectively. Lanes 1, 5, and 10 correspond to CoSp. Lanes 2, 6, and 11 correspond to CoPe. Lanes 3, 7, and 12 correspond to CoDo. Lanes 8 and 13 correspond to HT29 (positive control for IL-4R and HGFR, respectively). These results are representative of several experiments on cell lines performed at different cell passages.

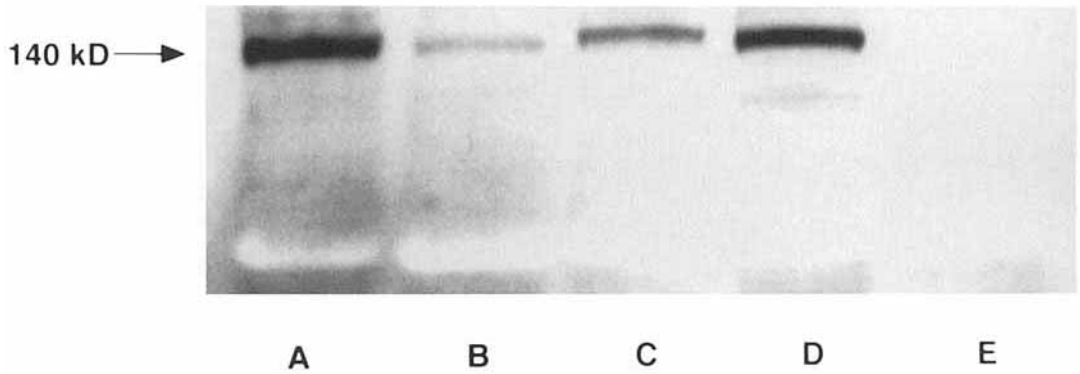

Fig. 2. Expression of HGFR (*Met*) in colon carcinoma cell lines. Solubilized proteins from CoSp, CoPe, and CoDo cell lines (*lanes A, B, C,* respectively) were assessed by Western blot with specific anti-*Met* antibody. HT29 cell line (*lane D*) and K562 cell line (*lane E*) were used as positive and negative controls, respectively. This is a representative Western blot of several different protein extractions from cell lines of different passages.

trol. Cell surface expression of IL-4R was verified by flow cytometry using a specific anti-IL-4R antibody. The expression of IL-4R was the highest on CoSp cells (50%) whereas, IL-4R on CoPe and CoDo cell lines was only 15 and 12%, respectively.

IL-4 Effect on HGF-Induced Proliferation of Colon Carcinomas

Only the CoSp line showed significant ($P < 0.01$) proliferation in response to 5, 10, and 20 ng/ml of HGF (Fig. 3). CoPe and CoDo cell proliferation was not significantly enhanced by HGF. These representative experiments demonstrated that all colon carcinoma cell lines expressing HGFR are not stimulated to proliferate in presence of HGF. The effect was not due to the proliferation rates of the individual cell lines. We therefore focused our attention on the HGF responding cell line CoSp. To determine if HGF-induced proliferation of CoSp cells could be inhibited by IL-4, 100 U/ml IL-4 was added to CoSp cell cultures with 5 ng/ml of HGF, and cell proliferation was examined after 3, 5, and 7 days. CoSp cell proliferation in the presence of HGF plus IL-4 was significantly ($P < 0.01$) inhibited compared to HGF alone on days 5 and 7 (Fig. 4). On day 7, cell cultures were examined, and by the trypan blue exclusion assay there was no evidence of cell death. There was no significant difference in cell number between treatment with medium alone and that treatment with IL-4 (100 U/ml) alone. These results demonstrated that IL-4 alone had no effect on CoSp cell proliferation.

IL-4 Effect on HGF-Activated Cell-Matrix Invasion

The invasive response of colon carcinoma cell lines to HGF was assessed by a chemoinvasion assay using the microplate Transwell chamber system (Table I). When cell lines were treated with HGF (1 and 10 ng/ml), cell-matrix invasion

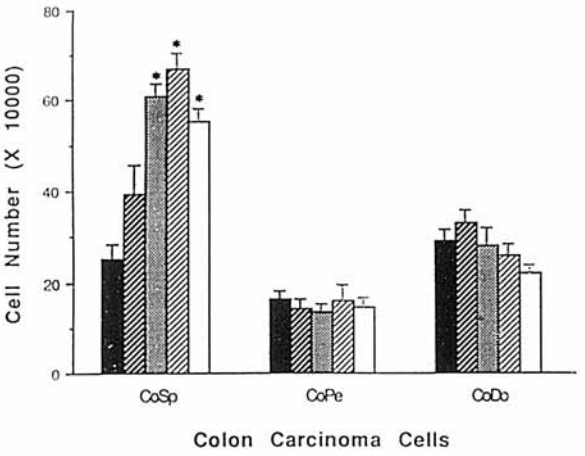

Fig. 3. Representative experiments of the effect of HGF on proliferation of human colon carcinoma cells. CoSp, CoPe, and CoDo cells were seeded at a density of 5 × 10^4 cells/well in 12-well plates. Cells were treated with culture medium containing 0 (■), 1 (▨), 5 (▤), 10 (▨), and 20 (□) ng/ml of HGF. The medium was changed at day 3, and at day 6 viable cells were counted using a hemocytometer. The mean ± SD of triplicate experiments are shown. *$P < 0.01$.

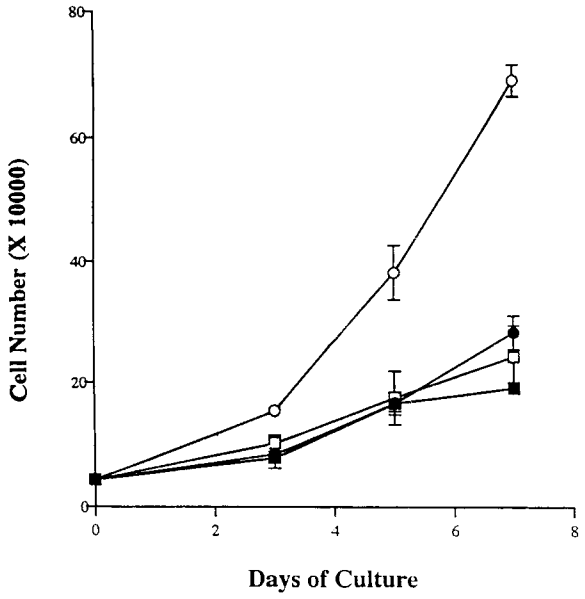

Fig. 4. Proliferation of CoSp cells in the presence of HGF and/or IL-4. The CoSp cells were plated in 12-well plates at a density of 5 × 10^4 cells/well. Cells were cultured in medium alone (●), 5 ng/ml HGF (○), 100 U/ml IL-4 (□), and 5 ng/ml HGF plus 100 U/ml IL-4 (■). The mean cell numbers ± SD of triplicate experiments are shown of a representative experiment.

by all cell lines was significantly ($P < 0.01$) enhanced in a dose-dependent manner. The cell invasion/migration induced by HGF was the highest for CoSp cells, followed by CoPe and then CoDo cells. The most significant enhancement by HGF (10 ng/ml) above controls was 35, 14, 7 fold by CoDo, CoPe, and CoSp, respectively. IL-4 (100 U/ml) alone did not have any major effects on invasion/migration when compared to non-treated cells.

To determine whether HGF-activated cell-matrix invasion/migration by colon carcinoma cells could be modulated by IL-4, various concentrations of IL-4 (0.1, 1, 10, and 100 U/ml) were added to the cultures in the presence of 10 ng/ml HGF. In the presence of 1 and 10 U/ml IL-4, cell-matrix invasion/migration by CoSp, CoPe, and CoDo cells was significantly reduced. Only CoSp cells were inhibited at 0.1 U/ml IL-4. In the presence of 10 U/ml IL-4 CoDo cells showed the greatest inhibition of invasion/migration. This suggested that the effect of IL-4 was not related to IL-4R expression since IL-4R expression was the lowest in CoDo cells. The inhibition of cell-matrix invasion/migration flattened out at around 10 U/ml of IL-4. The highest levels of inhibition by IL-4 were 69%, 86%, and 86% for CoSp, CoPe, and CoDo, respectively. Inhibition at 100 U/ml IL-4 was similar to that at 10 U/ml IL-4. IL-4 alone had no major inhibiting effect on cells.

To evaluate cell migration without Matrigel invasion the assay was performed in Transwells with noncoated filters. Cell migration was observed by all the colon cell lines in presence of HGF (10 ng/ml): 476 ± 25, 134 ± 7, 34 ± 1 (mean of triplicates ± SEM) total number of invading cells in 5 microscopic fields per filter (as in Table I legend) for CoSp, CoPe, and CoDo, respectively. IL-4 (10 U/ml) inhibited HGF-induced migration: 221 ± 33, 94 ± 7, 20 ± 1 (mean of triplicates ± SEM) total number of invading cells in 5 microscopic fields per filter for CoSp, CoPe, and CoDo, respectively. IL-4 inhibition of HGF-induced migration was 54%, 30%, 41% for CoSp, CoPe, and CoDo, respectively. Significant ($P < 0.05$) inhibition of migration was observed by CoSp and CoDo cell lines. HGF did not induce migration of control K562 cell line. In general IL-4 inhibition of HGF-induced migration for all cell lines was less in noncoated filters compared to the invasion/migration of Matrigel coated filters.

IL-4 Effect on HGF-Activated Cell-Matrix Invasion by Primary Colon Cells

To determine if the effects of HGF and IL-4 on cell-matrix invasion were an artifact of an in

TABLE I. Effect of IL-4 on HGF-Activated Cell-Matrix Invasion by Colon Carcinomas

Test	IL-4 (U/ml)	HGF (ng/ml)	Total number of invading cells in 5 microscopic fields		
			CoSp	CoPe	CoDo
A	0	0	72 ± 4	3 ± 1	0 ± 0
B	0	0.1	85 ± 10	1 ± 1	5 ± 1**
C	0	1	232 ± 28**	7 ± 1*	14 ± 1**
D	0	10	480 ± 46**	42 ± 3**	35 ± 2**
E	0.1	10	302 ± 14* (37)	41 ± 4 (3)	36 ± 1 (0)
F	1	10	229 ± 32* (52)	20 ± 3* (52)	14 ± 2** (60)
G	10	10	148 ± 10** (69)	6 ± 1** (86)	5 ± 1** (86)

Cell-matrix invasion was assayed in microplate Transwells with Matrigel coated filters. IL-4 was added in the upper well (Matrigel side) with 5×10^4 tumor cells; HGF was added in the lower well. Data shown are the total number of cells (mean of triplicate experiments ± SEM) migrating from the upper well to the lower surface of the filter; total number of cells refers to the assessment of five representative microscopic fields for each filter. Microscopic fields were observed with a 200 × objective. Tests B, C, and D were compared to test A; tests E, F, and G were compared to test D: $^*P < 0.05$, $^{**}P < 0.01$. These are representative of several experiments. Bracket numbers refer to percent inhibition when 10 ng/ml HGF alone was compared to IL-4 plus *HGF* of individual cell lines. IL-4 10 U/ml alone did not have any major effect on the individual cell lines invasion/migration compared to the cells treated with culture medium alone.

vitro established cell line, primary cultures of colon carcinomas isolated from an omental metastasis (CoST) and a liver metastasis (CoZH) were studied. Tumor biopsy specimens were carefully dissected to remove normal tissue, minced, resuspended in culture medium, and then seeded in 24-well culture plates. After several weeks, the epithelial tumor cell clusters adhered and grew, forming epithelial cell colonies. The epithelial cell colonies in wells were identified by phase-contrast microscope, harvested and then used for the experiments. Primary cultures of both colon carcinoma cultures showed significantly ($P < 0.01$) enhanced cell-matrix invasion in the presence of HGF (Fig. 5). Addition of IL-4 significantly ($P < 0.01$) inhibited HGF-induced invasion (Fig. 5). There was no significant effect on cell-matrix invasion with IL-4 alone. The results of these studies confirmed those on established colon carcinoma cell lines.

IL-4 Effect on HGF-Activated MMP-1 Production

Experiments were performed to examine if modulation of cell invasiveness could be related to production of MMP-1. MMP-1 degrades interstitial and fibrilliar collagens. In the presence of 10 ng/ml HGF, MMP-1 production by each of the colon carcinoma cells was significantly ($P < 0.05$) enhanced compared to culture medium alone (Fig. 6). The enhanced MMP-1 secretion by HGF was inhibited by IL-4 (500 and 250 U/ml). At 500 U/ml IL-4 MMP-1 secretion returned to control levels. IL-4 alone did not enhance MMP-1 production above background levels.

IL-4 Effect on HGF-Activated MMP-2 and -9 Production

Zymgram gel analysis was used to examine the production of MMP-2 and -9 by colon cell lines. Figure 7 shows a representative example of several experiments of zymograms of CoSp and CoPe. Zymogram gelatin degradation bands were assessed using relative densitometric units obtained from a photo image scanner. HGF (10 ng/ml) treatment of cell lines enhanced MMP-2 production above non-treated cells, 9.1, 2.4, and 1.1 fold from CoSp, CoPe, and CoDo cell lines, respectively. However, HGF (10 ng/ml) treatment of cell lines did not significantly enhance MMP-9 production of CoSp, CoPe, and CoDo (1.5, 1.1, 1 fold, respectively) above respective untreated cell lines. IL-4 (500 U/ml) significantly inhibited HGF induced MMP-2 levels (64%) and MMP-9 levels (48%) of the CoSp line.

A collagen degradation assay was performed using ^{3}H-labeled collagen type IV [Nakajima et al., 1987]. MMP-2 and -9 collagenases degrade type IV collagens [Tetlow et al., 1993]. HGF enhanced collagen degradation by CoSp but not other cell lines (data not shown). IL-4 abrogated the HGF-induced collagenase IV activity.

Expression of HGFR on Cells After IL-4 Treatment

HGFR expression was assessed by flow cytometry to determine if it could be modified by HGF, IL-4 or HGF plus IL-4 treatment. Colon carcinoma cells were treated with HGF (10 ng/ml), IL-4 (100 U/ml) or HGF (10 ng/ml) plus IL-4

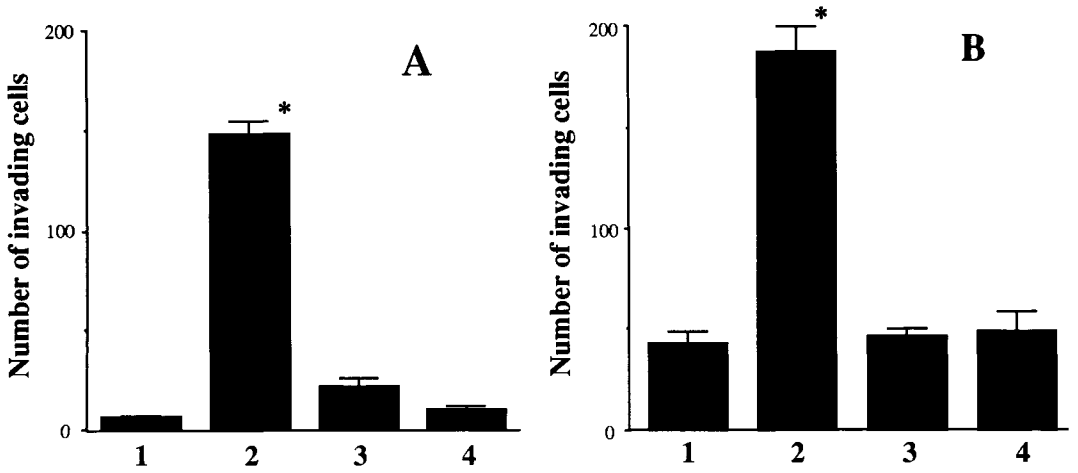

Fig. 5. Effect of IL-4 on HGF-induced cell-matrix invasion of primary cultures by colon carcinoma cells. Cell-matrix invasion was assayed in Transwells with 8-μm pore filter coated with Matrigel using primary culture cell lines: **A:** CoST and **(B)** CoZH. In the upperwells 5×10^4 tumor cells were added in all assays. Columns represent the following tests: (*1*) culture medium alone in upper well (Matrigel side) and lower well; (*2*) medium in upper well, HGF (20 ng/ml) in lower well; (*3*) IL-4 (100 U/ml) in upper well and HGF (20 ng/ml) in lower well; (*4*) IL-4 (100 U/ml) in upper well, culture medium in lower well. Data are the total number of cells (mean of triplicate experiments ± SEM) migrating from the upper well to the lower surface of the filter. The total number of cells was obtained by assessing five representative microscopic fields for each filter. Column 2 of Figures A and B was significantly greater compared to all other columns ($*P < 0.01$). These are representative experiments of several.

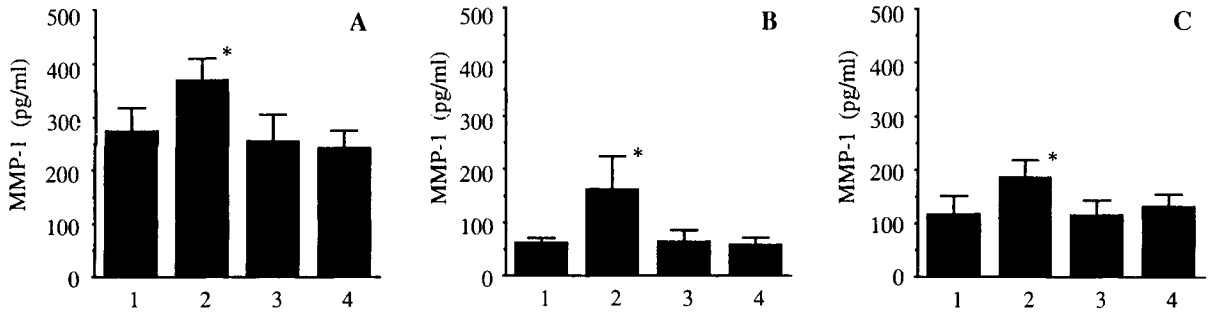

Fig. 6. Effect of HGF and IL-4 on MMP-1 production by colon carcinoma cell lines. Individual colon carcinoma cells (1×10^6/well) with or without HGF and/or IL-4 and cultured for 24 h at 37°C. MMP-1 production of culture supernatant was measured by an MMP-1 ELISA. Columns represent mean pg/ml ± SD of quadruplicate assays for response of individual cell lines to treatment: **A:** CoSp; **(B)** CoPe; and **(C)** CoDo. Columns represent the following: (*1*) control (culture medium only); (2) HGF (10 ng/ml); (*3*) IL-4 (500 U/ml); and (*4*) HGF (10 ng/ml) plus IL-4 (500 U/ml). All cell lines treated with HGF (column 2) were significantly greater ($*P < 0.05$) compared to other tests (columns 1, 3, and 4). These are representative results of two experiments.

(100 U/ml) for 3 days. HGFR expression was compared to that of non-treated cells. HGF alone, IL-4 alone or HGF plus IL-4 treatment did not affect HGFR expression.

DISCUSSION

HGF-induced cell matrix-invasion, cell migration, and MMP-1, -2, and -9 production by human colon carcinoma cells was significantly inhibited by IL-4. This is a novel and important effect of IL-4 on regulating tumor cell properties related to invasion and metastasis. HGF has been shown to have differential biological effects on cancer cells. In general, proliferation and invasion of cancer cells are not related, but together they play a critical role in tumor progression leading to successful metastasis. HGF enhanced cell-matrix invasion in all colon carcinoma cell lines but stimulated cell proliferation in only one cell line. HGFR was expressed on all the colon cell lines, particularly on CoSp cells. This suggests that HGF-induced cell proliferation may relate to higher levels of HGFR. The different biological events induced by HGF may

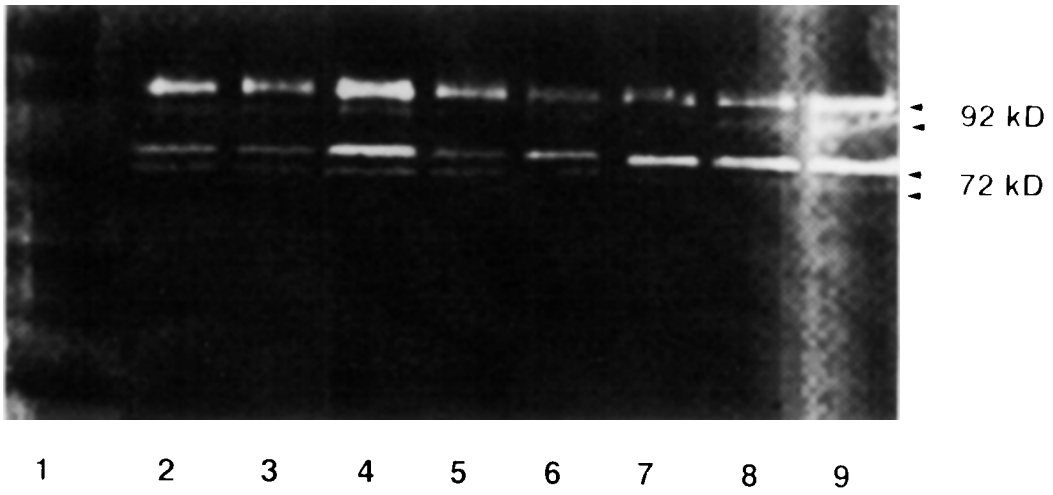

Fig. 7. Zymogram gel analysis of MMP-2 and MMP-9 production after treatment of colon cell lines. *Lane 1* represents pre-stained molecular weight standard (Bio-Rad). The following represent treatment of individual cell lines: *Lanes 2–5* are CoSp and *lanes 6–9* are CoPe; lanes 2 and 6, control cells with no treatment; lanes 3 and 7, 500 U/ml IL-4 treatment; lanes 4 and 8, 10 ng/ml HGF treatment; and lanes 5 and 9, 500 U/ml IL-4 + 10 ng/ml HGF treatment. *Arrows* indicate: MMP-9, 92 kD band and active form band underneath; and MMP-2, 72 kD band and active form band underneath. These experiments are representative of several zymography studies on the individual cell lines.

be dependent on other factors such as the physiological or activation state of the cell [Tajima et al., 1992; Giordano et al., 1993].

Our studies strongly suggest that HGF-activated invasion/migration may play a critical role in colon carcinoma metastasis. Colon carcinomas predominantly metastasize to the liver and this phenomenon may relate to the high level of expression of HGF in the inflamed or damaged organ [Noji et al., 1990; Kenworthy et al., 1992]. Although surgical resection is one of the best treatment for the cure of hepatic metastases of colon cancer, the tissue destruction caused by surgery may induce the production of HGF. In the presence of HGFR positive colon cells additional metastases may develop. Our results using metastatic colon carcinoma cells in primary cultures suggest that this effect is occurring in vivo. Other organs such as the lung and kidney [Konishi et al., 1991; Yanagita et al., 1992], both common sites of metastatic tumor development, also produce HGF. HGF's facilitation of proliferation and invasion strongly suggest that this factor is very instrumental in promoting aggressive tumor progression.

One of the novel findings of this study is cell-matrix invasion and cell migration enhanced by HGF is inhibited in the presence of IL-4. Inhibition of cell-matrix invasion was observed at a relatively low IL-4 dose for both highly-invasive colon carcinoma primary cultures and cell lines. IL-4's effect was not due to down-regulation of HGFR because IL-4, HGF, or IL-4 plus HGF did not modulate HGFR expression level. We examined the production of MMP-1, -2, and -9 since these enzymes are important in extracellular matrix degradation during invasion [Matrisian, 1990; Liabakk et al., 1996]. MMP-1 production was significantly enhanced by HGF, and this enhancement was significantly inhibited by IL-4. MMP-2 and -9 production was enhanced but to a lesser degree that varied among the cell lines. HGF induced collagenase activity and cell-matrix invasion together are important in the process of tumor metastasis. IL-4 inhibition of both these processes suggests that this cytokine has an important role in controlling metastatic spread of colon carcinomas. Correlations have been made with collagenase activity (MMP-1, -2, -9) and colorectal tumor progression [Van der Steppen et al., 1990; Hewitt et al., 1991; Liabakk et al., 1996]. Recently, a study has shown that metalloproteinase biosynthesis by human alveolar macrophages can be suppressed by IL-4 [Lacraz et al., 1992]. IL-4 may affect other cellular physiological events related to motogenesis and invasiveness, such as modulation of protein synthesis or cytoskeletal modulation [Rosen et al., 1990].

HGFR is located predominately in the lumen side of carcinomas, including colon carcinomas [Tsarfaty et al., 1992]. Similar distribution of IL-4R has also been reported in colon carcinoma

tissues [Kaklamanis et al., 1992]. The expression of HGFR and IL-4R may be related to the degree of differentiation of carcinoma cells. Recently, we have shown that gastric carcinoma cells are locked into a G_1/G_0 cell-cycle after IL-4 treatment. This effect depends on the level of IL-4R expression [Morisaki et al., 1992; 1994]. Previously, we have shown that IL-4's inhibitory effect on cell proliferation is related to the level of IL-4R expression [Morisaki et al., 1992]. However, other cellular functions induced by IL-4 are not directly related to receptor expression level [Hoon et al., 1991a,b].

$CD4^+$ T cells, mast cells, and basophils are the hosts natural source of IL-4 [Paul and Ohara, 1987]. Tumor-infiltrating lymphocytes (TILs) in colon carcinomas are primarily $CD4^+$ T cells [Balch et al., 1990]. Since IL-4 is secreted by $CD4^+$ T cells, colon carcinoma cell metastasis/invasion induced by HGF may be downregulated by activated tumor-infiltrating $CD4^+$ T cells. $CD4^+$ tumor-infiltrating lymphocytes may play an important role in regulating tumor cell progression at primary and secondary sites. Other cell types such as mast cells and basophils and are also present in inflamed gastrointestinal tissue. Immune cytokine downregulation of tumor proliferation and other activities is an important effective component of the host anti-tumor response [Hoon et al., 1996]. IL-4 is one of the most prominent member of the hemopoietic receptor superfamily that has anti-tumor immune effects.

In summary, we have shown that IL-4 downregulated HGF-induced cell-matrix invasion, cell migration, and MMP-1, -2 and -9 production in human colon carcinoma cells. Since these events play a major role in the metastasis of colon cancer IL-4 may be very appropriate as an adjuvant therapy for patients whose colon carcinomas are IL-4R positive. IL-4 has a dual effect of directly acting on various tumor cell events related to progression and activating immune responses [Hoon et al., 1993]. Currently, IL-4 therapy is being used to treat patients with advanced lung and colon carcinomas.

ACKNOWLEDGMENTS

The authors thank E. Famatiga and C. Kuo for their excellent technical assistance, and G. Berry for editorial assistance. Supported by the Rogovin-Davidow Foundation, The Joyce and Ben Eisenberg Foundation, and Career Development Award of the American Cancer Society (R.E.).

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