# Production of Interleukin 15 by Human Colon Cancer Cells Is Associated with Induction of Mucosal Hyperplasia, Angiogenesis, and Metastasis

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## ABSTRACT

*Purpose:* The purpose of this study was to identify a mediator produced by human colon cancer cells that is responsible for the induction of hyperplasia in the adjacent mucosa.

*Experimental Design:* Seventy human colon cancer surgical specimens were immunostained to determine the presence of cytokines that can induce hyperplasia in the adjacent mucosal. Human colon cancer cells with low and high metastatic potential were implanted into the cecal wall of nude mice. The resulting lesions were studied by immunohistochemistry to detect possible mediators of mucosal hyperplasia.

*Results:* Immunostaining of 70 colon cancer specimens from 70 patients suggested that mucosal hyperplasia and distant metastasis were associated with the expression of interleukin (IL)-15 and, to a lesser extent, transforming growth factor α. The production of IL-15 by colon cancer cells was not associated with the infiltration of natural killer cells into the tumors. Cecal tumors produced in nude mice by human colon cancer cells with low and high metastatic potential (KM12C and KM12SM cells, respectively) ex-

pressed similar levels of transforming growth factor α, and expression of IL-15 was detected only in the metastatic KM12SM cells and was associated with hyperplasia of the surrounding mucosa. The expression of the IL-15 receptor in rat intestinal epithelial cells (IEC6 cells) was confirmed by immunoblotting with antibodies against IL-15 receptor α and IL-2 receptor β and γ subunits and by a binding assay using $^{125}$I-labeled IL-15 ($K_d$ = 0.011 nM). IL-15 stimulated proliferation of the IEC6 cells, even under serum starvation. Treatment of IEC6 cells with IL-15 decreased doxorubicin-mediated cytotoxicity. In IEC6 cells treated with either IL-15- or KM12SM-conditioned medium, immunoblotting revealed a decrease in the production of p21Waf1, Bax, and Bak and an increase in the production of cyclin E, proliferating cell nuclear antigen, the phosphorylated active form of AKT, basic fibroblast growth factor, and vascular endothelial growth factor, changes associated with cell growth, survival, and induction of angiogenesis.

*Conclusions:* These data indicate that IL-15 produced by metastatic colon carcinoma cells can induce hyperplasia in the mucosa adjacent to colon cancer, thus contributing to angiogenesis and progression of the disease.

## INTRODUCTION

Hyperplastic changes in the mucosa adjacent to human colon cancer are associated with disease progression (1). These changes, which include high Ki-67 labeling indices, increased production of proangiogenic molecules, and intense angiogenesis, have the potential to promote the growth of colon cancers and produce metastasis (2). These initial findings in specimens of human colon cancers were confirmed in a nude mouse model implanted orthotopically with colon cancer cells. Specifically, high levels of PCNA[2] and increased cell proliferation on bromodeoxyuridine labeling in highly metastatic KM12SM cells were associated with hyperplastic morphology in the adjacent mucosa, whereas in KM12C (the parental cell line of KM12SM that has low metastatic potential) cells, PCNA and proliferation levels were lower, and the adjacent mucosa showed little or no hyperplasia (2). Increased levels of VEGF and basic fibroblast growth factor and reduced levels of mouse IFN-β in the mucosa adjacent to highly metastatic KM12SM tumors were associated with dense vascularization at the mucosa-tumor boundary (2).

However, the factors that induce hyperplasia in the mucosa

[2] The abbreviations used are: PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; IL, interleukin; DXR, doxorubicin; RT-PCR, reverse transcription-PCR; IL-15R, IL-15 receptor; NK, natural killer; ROS, reactive oxygen species; FBS, fetal bovine serum; Ab, antibody; pAKT, phospho-AKT.

adjacent to colon cancer are unknown. Colon cancers produce various growth factors and cytokines, such as EGF and TGF-$\alpha$, that can induce epithelial cell proliferation (3, 4). IL-15, an autocrine/paracrine factor produced by colon cancer cells (5), has been shown to promote the proliferation, motility, and invasiveness of colon cancer cells as well as increase their tolerance resistance to apoptosis (5). IL-15 has also been shown to play a role in the repair of colon mucosal epithelium and to cooperate with growth factors and cytokines, such as TGF-$\alpha$, TGF-$\beta$, IL-1, IL-2, and IL-4, that are involved in inflammatory bowel disease (6). Whether the expression of IL-15 by human colon cancer cells is also associated with the induction of mucosal hyperplasia and angiogenesis was the subject of our present study.

## MATERIALS AND METHODS

**Surgical Specimens.** Seventy formalin-fixed, paraffin-embedded archival surgical specimens from 70 patients with primary colon adenocarcinomas that had invaded the subserosal layer were randomly selected from the Hiroshima University Hospital and the JR Hiroshima General Hospital of West Japan Railway Company. In 33 of the 70 cases, lymph node metastases were detected (Dukes' C). The other 37 cases had no evidence of lymph node metastasis (Dukes' B). None of the patients had distant metastases at the time of resection of the primary neoplasms.

**Immunohistochemistry.** Consecutive 4-$\mu$m sections were stained immunohistochemically using the immunoperoxidase technique described previously (2). Anti-IL-15 polyclonal Ab (clone L-20; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TGF-$\alpha$ monoclonal Ab (Oncogene Research Products, Cambridge, MA) were used at a concentration of 1 $\mu$g/ml. The association between the IL-15 and TGF-$\alpha$ expression and hyperplastic changes in the mucosa adjacent to the tumors was determined. Mucosal hyperplasia was defined as the presence of cryptic columns in the mucosa within 2 mm from the edge of the tumors that were 1.5 times as tall as columns in the mucosa 10 cm away from the tumor (Fig. 1*A*). For detection of NK cells, we used the anti-CD56 Ab (DAKO Corp., Carpinteria, CA) and anti-IL-15R$\alpha$ polyclonal Ab (clone H-107; Santa Cruz Biotechnology) at the concentration of 1 $\mu$g/ml. Specimens were color-developed with diamine benzidine hydrochloride (DAKO Corp.). Meyer's hematoxylin (Sigma Chemical Co., St. Louis, MO) was used for counterstaining. The number of NK cells was determined by counting cells in 100 high-power fields, which were equivalent to a 20-mm$^2$ area.

**Cell Cultures.** IEC6 rat intestinal epithelium cells were purchased from Dainihon Pharmaceutical Co. (Tokyo, Japan). The human colon cancer cell line KM12C was established in our laboratory from surgically resected primary colon cancer (7, 8). KM12SM cells were established in culture from liver metastases produced by KM12C cells growing in the cecal wall of nude mice (7, 8). The two cell lines were routinely maintained in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% FBS (Whittaker M. A. Bioproducts, Inc., Walkersville, MD) at 37°C in 5% $CO_2$ and 95% air.

**Animal Model.** Specific pathogen-free male athymic BALB/c mice were purchased from the Animal Production Area
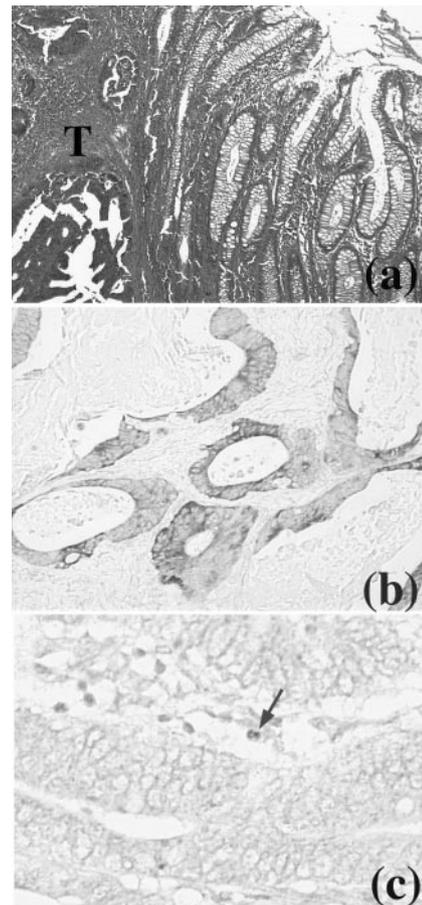


*Fig. 1* Immunostains of colon cancer (Dukes' classification C). *A,* hyperplastic changes in mucosa adjacent to the cancer (*T*; H&E stain). *B,* IL-15-positive staining in cancer cells. *C,* infiltration of tumor by a few NK cells (*arrow*) was detected with anti-CD56 Ab. *Bar,* 100 $\mu$m.

of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Animals were maintained and used according to institutional guidelines in facilities approved by the American Association of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and the NIH. The mice were used when they were 8–10 weeks of age. To produce cecal tumors, we implanted 1 $\times$ 10$^6$ viable KM12SM or KM12C cells into the cecal wall of nude mice, as described in detail previously (9).

**RT-PCR.** The expression of IL-15 and TGF-$\alpha$ mRNA was assessed with RT-PCR using 0.5 $\mu$g of total RNA extracted with a RNeasy kit (Qiagen, Hilden, Germany). The primer sets were as follows: (*a*) human IL-15, 5'-CAA-AGC-ACC-TAA-CCT-A-3' (forward) and 5'-ATA-ACT-TGT-AAC-TCC-A-3' [reverse (referred to GenBank AF031167)]; (*b*) rat IL-15, 5'-CAA-AGA-GGA-GGC-GTT-C-3' (forward) and 5'-CTT-TCA-ATT-TTC-TCC-A-3' [reverse (referred to GenBank NM013129)]; and (*c*) human TGF-$\alpha$, 5'-GAG-TGC-AGA-CCC-GCC-CGT-GGC-3' (forward) and 5'-CCA-GGA-GGT-CCG-CAT-GCT-CAC-3' [reverse (referred to GenBank M31172)]. $\beta$-Actin was also amplified for internal control. PCR products were electro-

phoresed in 2% agarose gel and stained with ethidium bromide. The experiment was performed three times.

**In Vitro Cell Growth.** IEC6 cells seeded at a density of 2000 cells/well in 96-well plates were cultured for 12 h in regular medium. The cells were washed twice with PBS and treated under conditions detailed below in this section. Cell growth was monitored after 12, 24, 36, 48, 72, and 96 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (5). Absorbance at a 595 nm wavelength was measured. Each experiment was performed three times.

IEC6 cells were cultured in medium containing IL-15 and under serum-free conditions with or without 10 ng/ml IL-15 (Peprotec Ltd., London, UK). To neutralize the effects of IL-15, we used polyclonal anti-IL-15 goat Ab (clone L-20; Santa Cruz Biotechnology) or polyclonal anti-IL-15Rα goat Ab (clone H-107; Santa Cruz Biotechnology) at concentrations of 2 μg/ml (5). Goat IgG Ab (2 μg/ml) was used as a control. At this concentration, the Abs were not toxic (data not shown). For serum starvation, cells seeded onto 96-well tissue culture plates were washed twice with PBS and treated with serum-free RPMI 1640 after 12 h of culture in regular medium. Each experiment was performed three times.

DXR (Sigma Chemical Co.) was used to induce cytostatic and cytolytic effects. IEC6 cells were seeded onto 12-well cell culture plates with a cell density of 50,000 cells/well. The cells were treated for 24 h with various concentrations of DXR (0.003–0.3 μg/ml), with or without 10 ng/ml IL-15. Detached cells collected from the medium and PBS washings were stained with trypan blue. Attached cells were trypsinized briefly and stained with trypan blue. For each treatment group, live cells (unstained) and dead cells (stained) were counted separately using a Nesbeuer-type hemocytometer. Detached cells were also stained by Giemsa to confirm apoptosis.

**Preparation of Extracts.** Subconfluent cultures of cells were washed twice with PBS, harvested in PBS with a cell scraper, and centrifuged. The cell pellet was resuspended in a suspension buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin], and SDS was added to make up a 1% final concentration for cell lysis. The lysate was then boiled for 10 min and passed 20 times through a 21-gauge needle. The supernatant of the centrifuged ($10,000 \times g$, 5 min) lysate was used as total cell lysate for analyses.

**Cultured Cell Medium.** KM12SM or KM12C cells were cultured at a density of $1 \times 10^6$ cells/10-cm$^2$ tissue culture plate in serum-free RPMI 1640. After 48 h, the medium was collected, filtered (pore size = 0.22 μm; Becton Dickinson Labware, Bedford, MA), concentrated with a Centricon filter device (Millipore Corp., Bedford, MA), and mixed with an equal volume of fresh RPMI 1640 (20% FBS). KM12SM cultured medium was also treated overnight at 4°C with 1 μg/ml anti-IL-15 Ab (clone L-20; Santa Cruz Biotechnology) and then mixed with the same volume of RPMI 1640 [20% FBS (KM12SM cultured medium plus IL-15 Ab)]. IEC6 cells treated with KM12SM cultured medium or KM12SM cultured medium plus IL-15 Ab were harvested after 48 h, and the extracted cell lysates were subjected to immunoblotting.

**ELISA for IL-15.** An ELISA system was purchased from Genzyme-Techne Co. (Minneapolis, MN), and the assay

*Table 1* Association between hyperplasia in mucosa adjacent to colon carcinomas and production of metastases in 70 clinical specimens

| Mucosal hyperplasia[a] | No. of cases | Nodal metastasis | |
| --- | --- | --- | --- |
| | | Positive cases | Negative cases |
| Positive | 44 | 30 (68%)[b] | 14 |
| Negative | 26 | 3 (12%) | 23[b] |

[a] Hyperplastic change of mucosa was defined morphologically as cryptic columns 1.5 times taller than control mucosa 10 cm from cancer lesion.

[b] $P < 0.001$ (two-tailed Fisher's exact test).

was performed according to the company's instructions. For the assay, $1 \times 10^6$ cells were seeded into 6-well tissue culture plates and cultured for 12 h in regular medium. The cells were then washed twice with PBS and treated for 24 h with 2 ml of serum-free RPMI 1640. At that time, the cultured medium was collected and centrifuged. To confirm the specificity of ELISA, IL-15 (100 pg/dl) solubilized with PBS served as a positive control.

**Immunoblotting.** Total cell lysates were prepared as described above. Immunoblot analysis of 50-μg lysates was performed using 12.5% SDS-PAGE followed by electrotransfer onto nitrocellulose membranes. The membranes were first incubated with an appropriate primary Ab, and the filters were then incubated with peroxidase-conjugated anti-IgG Ab (Medical and Biological Laboratories, Ltd., Nagoya, Japan). The immune complex was visualized using the enhanced chemiluminescence Western blot detection system (ECL; Amersham Biosciences, United Kingdom Limited, Buckinghamshire, United Kingdom). The primary Abs used and their manufacturers were as follows: (*a*) anti-p21Waf1 and anti-cyclin E (PharMingen, San Diego, CA); (*b*) anti-PCNA (DAKO Corp.); (*c*) anti-VEGF, anti-AKT, anti-IL-2 receptor subunit β (clone C-20), anti-IL-2 receptor subunit γ (clone M-20), and anti-IL-15R subunit α [clone H-107 (all from Santa Cruz Biotechnology)]; (*d*) anti-pAKT (New England Biolabs, Inc., Beverly, MA); and (*e*) anti-tubulin (Zymed Laboratories, Inc., South San Francisco, CA). Specific signals on the developed films were stored in a computer and scanned using NIH Image software (NIH, Bethesda, MD).

**125I-Labeled IL-15 Binding Assay.** Recombinant human IL-15 (Peprotec, Ltd.) was labeled with Na$^{125}$I (Amersham) using iodination reagent (Pierce Biotechnology, Inc., Rockford, IL) to a specific activity range of 28,000–30,000 cpm/fmol. The binding assay was carried out by modifying a method described previously (5, 10). Cells were seeded in 6-well tissue culture plates at $1 \times 10^6$ cells/well. After 24 h, the cells were washed with binding medium (RPMI 1640 containing 1% BSA) and treated with diluted concentrations (100 nM) of $^{125}$I-labeled IL-15 (nonspecific binding) or unlabeled IL-15 (specific binding) in 0.5 ml of binding medium. The binding reaction was carried out for 4 h at 4°C. The cells were then lysed using 0.1 N NaOH and 0.025% SDS and counted in a liquid scintillation counter. Data were obtained from duplicate experiments.

*Table 2* Expression of IL-15 and TGF-α in 70 clinical specimens of human colorectal cancers is associated with induction of hyperplasia in mucosa adjacent to the lesions

| Mucosal hyperplasia[a] | No. of cases | No. of positive cases[b] | | |
|---|---|---|---|---|
| | | IL-15 | TGF-α | Both |
| Positive | 44 | 10 | 11 | 23 |
| Negative | 26 | 1 | 8 | 3 |
| P[c] | | 0.0444 | 1.0000 | 0.0007 |

[a] Hyperplastic change of mucosa was defined morphologically as cryptic columns 1.5 times taller than control mucosa 10 cm from cancer lesion.
[b] Positivity was determined by immunostaining.
[c] Calculated using the two-tailed Fisher's exact test.

*Table 3* Number of infiltrating NK cells in surgical specimens of human colon cancer

| Dukes' classification | IL-15 expression | No. of cases | No. of NK cells (per mm²)[a] | P[b] |
|---|---|---|---|---|
| B | (−) | 33 | 4.18 ± 2.37 | |
| B | (+) | 4 | 4.13 ± 1.72 | 0.7954 |
| C | (−) | 11 | 0.85 ± 0.25 | |
| C | (+) | 22 | 1.07 ± 0.44 | 0.1217 |

[a] The number of NK cells was determined by counting cells in 100 high-power fields equivalent to 20 mm².
[b] Calculated by unpaired Mann-Whitney $U$ test.

**DNA Fragmentation.** To examine DNA fragmentation, cells were lysed with hypotonic lysis buffer [0.2% Triton X-100, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0)] on ice for 20 min. After centrifugation, supernatant of the lysate was treated with proteinase K and RNase A at 65°C for 30 min and precipitated with 100% ethanol and 0.1 M sodium acetate. Precipitated DNA was resolved into small amount of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The whole amount of the purified DNA samples was separated on a 2% agarose gel. DNA fragmentation was assessed twice.

**Statistical Analysis.** Statistical significance was examined using the two-tailed Fisher's exact test or the unpaired Mann-Whitney $U$ test. $P$s < 0.05 were considered significant.

## RESULTS

**Mucosal Hyperplasia and Expression of IL-15 and TGF-α in Surgical Specimens of Human Colon Cancers.** Mucosal hyperplasia was observed in 44 of the 70 surgical specimens of human colon cancers (63%; Fig. 1*A*; Table 1) and found more frequently in metastatic (30 of 33) than in nonmetastatic (14 of 37) colon cancers ($P$ < 0.001). Among the 44 cases with mucosal hyperplasia, IL-15 expression in colon cancer cells was detected in 33 specimens (75%), and TGF-α expression was detected in 34 specimens (77%; Fig. 1*B*; Table 2). Of these 44 cases, 23 specimens (52%) showed expression of both IL-15 and TGF-α. TGF-α expression alone was detected in 8 of 26 cancers with normal adjacent mucosa (31%) and thus was not specific to mucosal hyperplasia ($P$ = 1.0000). In contrast, the expression of IL-15 or IL-15 combined with TGF-α was higher in cases with mucosal hyperplasia than in cases without mucosal hyperplasia ($P$ = 0.0444 and $P$ = 0.0007, respectively). Thus, IL-15 expression alone or IL-15 expression combined with TGF-α was frequently found in tumor cells of cases with mucosal hyperplasia. The adjacent mucosa did not express IL-15. NK cell infiltration was detected by immunostaining using anti-CD56 Ab in IL-15-producing and non-IL-15-producing tumors (Fig. 1*C*; Table 3). The number of NK cells infiltrating the tumors did not vary significantly between the IL-15-producing and the non-IL-15-producing tumors (Dukes' B or C cases). In contrast, the number of infiltrating NK cells was higher in Dukes' B cases (4.18 ± 2.29 cells/mm²) than in Dukes' C cases (1.00 ± 0.40 cells/mm²), regardless of IL-15 production ($P$ < 0.001).

**Induction of Hyperplasia in Colonic Mucosa by Human Colon Cancer Cells in Nude Mice Is Associated with Expression of IL-15.** The expression of IL-15 mRNA in KM12C and KM12SM human colorectal carcinoma cells was examined by RT-PCR (Fig. 2*A*) and by immunohistochemical analysis of the tumors growing in the cecum of nude mice (Fig. 2*B*). In agreement with our previous report (2), the mucosa adjacent to KM12SM tumor cells in nude mice cecum was thickened with elongated crypts and increased areas of PCNA-positive nuclei, whereas the mucosa adjacent to KM12C tumors had fewer hyperplastic changes (Fig. 2*B*). IL-15 mRNA was detected in KM12SM cells, and IL-15 protein was detected in the KM12SM tumors. The KM12C cells and tumors did not express IL-15. In contrast, TGF-α mRNA was detected in both KM12SM and KM12C cells. The expression of IL-15 and TGF-α mRNA in noncancerous rat IEC6 intestinal epithelial cells (examined by RT-PCR) was weak (data not shown). The concentration of IL-15 in the culture medium of KM12SM cells (determined by ELISA) was 420 ± 76 pg/dl (mean ± SD; $n$ = 3), whereas the concentrations of IL-15 in the culture media of KM12C and IEC6 cells were below the limit of detection. NK cell infiltration of the cecal tumors was detected by immunostaining using anti-IL-15Rα Ab (Fig. 2*B*). The number of infiltrating NK cells was 0.3 cell/mm² in IL-15-producing KM12SM tumors and 4.3 cells/mm² in the IL-15-negative KM12C tumors.

**Expression of IL-15R by IEC6 Cells.** Next, we examined whether intestinal epithelial cells expressed the IL-15R. Production of the IL-15R-specific α subunit and β and γ subunits, which are common with IL-2, was detected in IEC6 cells (Fig. 3*A*). The IL-15Rα level was not affected by the treatment of cells with IL-15- or KM12SM-conditioned medium (Fig. 5). A binding assay with ¹²⁵I-labeled IL-15 revealed two types of binding kinetics to the IEC6 cells (Fig. 3*B*). Scatchard plot analysis showed both high-affinity binding sites ($K_d$ = 0.011 nM, $B_{max}$ = 0.012 fmol) and low-affinity binding sites ($K_d$ = 0.07 nM, $B_{max}$ = 0.016 fmol).

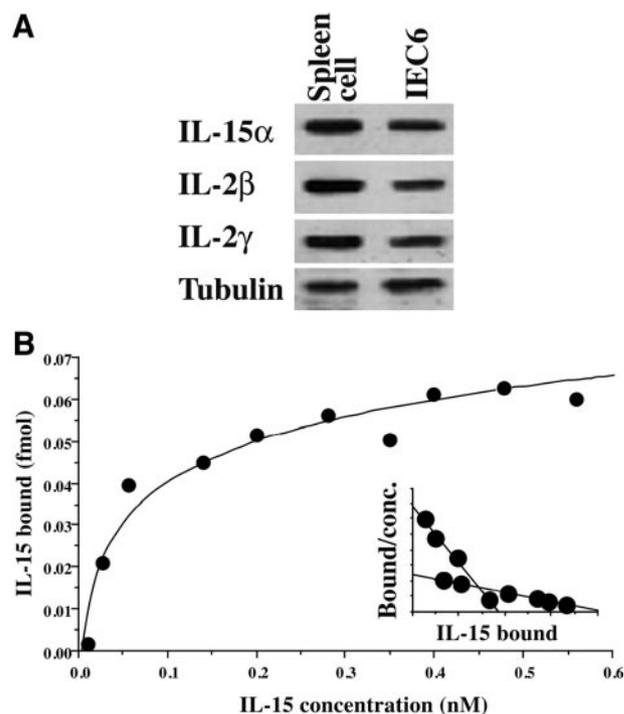**Effects of IL-15 on the Growth and Survival of IEC6 Intestinal Epithelial Cells.** Next, we examined the effect of IL-15 on growth of IEC6 cells (Fig. 4*A*). Culturing cells in medium containing IL-15 increased their proliferation by 24%. IEC6 cells were cultured under serum-free conditions with or without 10 ng/ml IL-15. Treatment of IEC6 cells with IL-15 increased their growth by 21% compared with serum-starved cells without IL-15. Treatment of IEC6 cells with anti-IL-15 Ab or anti-IL-15Rα Ab neutralized the IL-15-induced growth en-
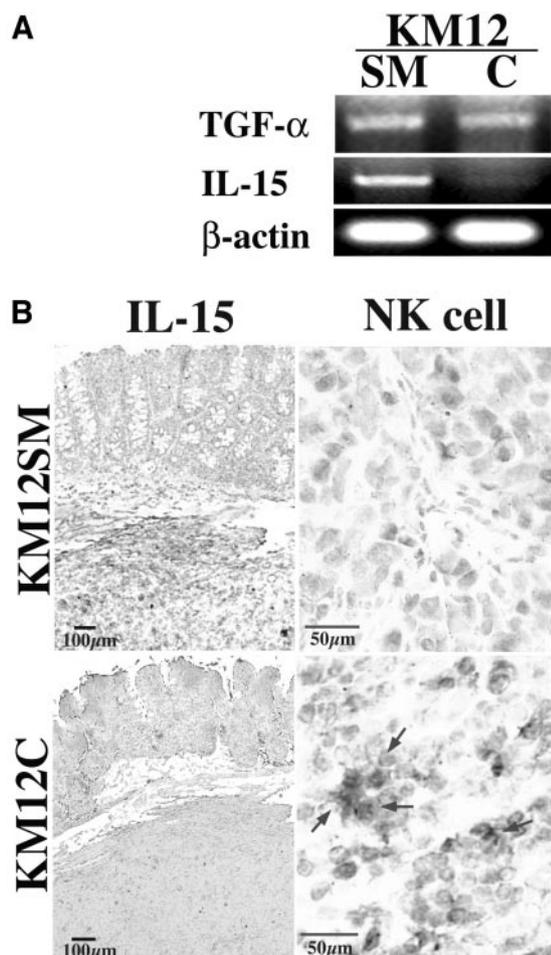
Fig. 2 A, expression of TGF-α and IL-15 mRNA by KM12SM (high metastatic potential) and KM12C (low metastatic potential) human colon cancer cells as determined by RT-PCR. β-Actin mRNA served as an internal control. B, immunostaining of human IL-15 and mouse NK cell marker (IL-15Rα) in KM12SM and KM12C tumors growing in the cecum of nude mice. Strong IL-15 immunoreactivity was found in KM12SM cells but not in KM12C cells. Few NK cells were found in the KM12SM tumor. The mucosa adjacent to the KM12SM tumor showed thickened hyperplastic changes. Arrow, IL-15Rα-positive NK cell.



Fig. 3 A, production of IL-15R-specific α subunit and β and γ subunits common to IL-2 in IEC6 cells. Rat spleen cells served as positive controls. Fifty μg of total cell lysate were subjected to 12.5% SDS-PAGE and electroblotted onto nitrocellulose membranes. Tubulin served as an internal control. B, specific binding of IL-15 to IEC6 cells was detected using ^{125}I-labeled IL-15. Scatchard plot analysis of ^{125}I-labeled IL-15 binding generated two classes of binding sites, high-affinity sites and low-affinity sites (inset).

hancement of IEC6 cells under serum starvation conditions (Fig. 4B).

To examine the effects of IL-15 on cell survival, IEC6 cells were incubated for 24 h in media containing different concentrations of DXR. The numbers of viable and dead cells were determined using trypan blue dye exclusion staining. As shown in Fig. 4, C and D, the presence of IL-15 significantly increased cell survival with a maximal effect at 0.003 μg/ml DXR. The number of viable IEC6 cells treated with IL-15 and 0.003 μg/ml DXR was 1.2 times higher than that of cells treated with DXR alone. For each concentration of DXR, IL-15 treatment decreased the number of dead cells to half the number in the untreated cells. Treatment of IEC6 cells with anti-IL-15 Ab or anti-IL-15Rα Ab prevented the anticytolytic effect of IL-15 (Fig. 4E), thus allowing apoptosis to occur in the IEC6 cells (Fig. 4F). We confirm the antiapoptotic effect of IL-15 on IEC6 cells treated with serum withdrawal (data not shown).

**Alteration of Expression of Cellular Proteins in IEC6 Cells by IL-15- and KM12SM-Conditioned Medium.** In the last set of studies, we examined the production by IEC6 cells of proteins associated with the cell cycle, cell survival, and the production of angiogenic molecules (Fig. 5). After 48 h of treatment with IL-15, the levels of cyclin-dependent kinase inhibitor p21Waf1, cyclin E, and PCNA in IEC6 cells were 21%, 192%, and 138%, respectively, the levels measured in untreated control cells, and the production of VEGF increased to 139%, respectively. The level of pAKT, an active form of the antiapoptotic serine/threonine kinase AKT (11), increased to 183% of the control level, whereas the total AKT protein level was unaltered by IL-15 treatment (Fig. 5). In cells exposed to IL-15, the levels of the proapoptotic factors Bax and Bak were reduced to 11% and 7%, respectively, as compared with the levels in control cells (data not shown).

The IEC6 cells also were cultured in preconditioned medium from KM12SM cells that contained 630 pg/ml IL-15 (as measured by ELISA). Treatment of IEC6 cells with KM12SM-preconditioned medium produced changes in the levels of expression of all of the above proteins, similar to the changes produced by IL-15 (Fig. 5). The effects of KM12SM-preconditioned medium on IEC6 cells were negated by pretreatment with anti-IL-15 Ab.

Finally, the IEC6 cells were cultured in preconditioned
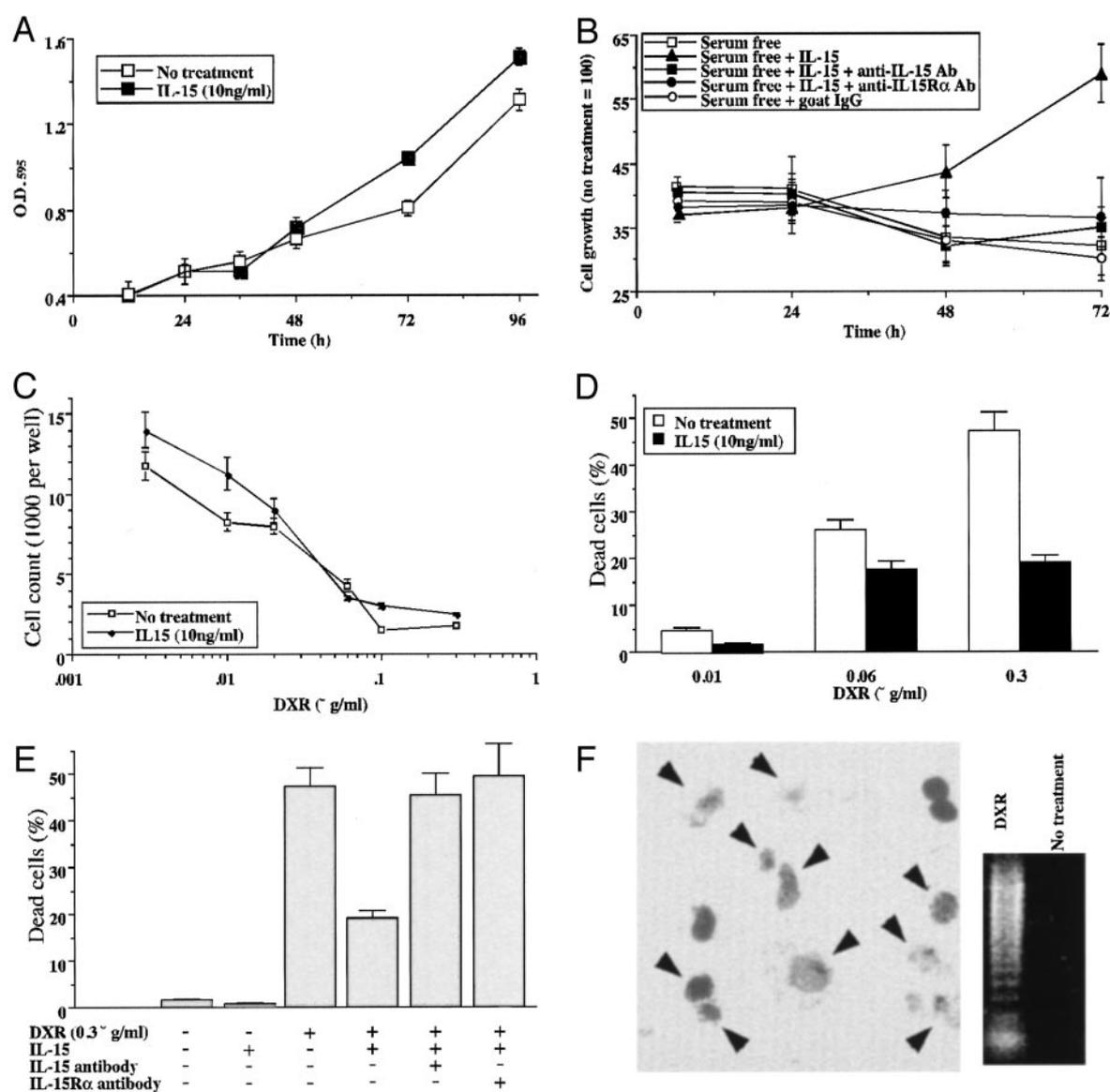
*Fig. 4* *A*, the effect of IL-15 on growth of IEC6 cells. Cells seeded in 96-well plates at a density of 2000 cells/well were treated with medium with or without 10 ng/ml IL-15. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 12–96 h later. Results represent the mean ± SD. *B*, neutralizing effect of anti-IL-15 or anti-IL-15Rα Abs on IL-15-induced cell growth of serum-starved cells. IEC6 cells cultured in serum-free medium were treated with IL-15 (10 ng/ml) alone or with anti-IL-15 Ab (2 μg/ml) or anti-IL-15Rα Ab (2 μg/ml). Cell growth is represented as a relative value compared with that of IEC6 cells growing in regular medium. *C* and *D*, anticytostatic and anticytolytic effects of IL-15. IEC6 cells were treated with different concentrations of DXR (0.003–0.3 μg/ml) for 24 h. The number of viable (*C*) and dead (*D*) cells was determined by trypan blue staining. *E*, neutralizing effect of anti-IL-15 or anti-IL-15Rα Abs on DXR-induced cytolysis. IEC6 cells treated with various combinations of DXR (0.3 μg/ml), IL-15 (10 ng/ml), anti-IL-15 Ab (2 μg/ml), or anti-IL-15Rα Ab (2 μg/ml) for 24 h. The number of dead cells was determined by trypan blue staining. *F*, Giemsa staining of IEC6 cells treated with DXR (0.3 μg/ml; *left panel*). *Arrowheads* point to apoptotic figures. DNA purified from DXR-treated IEC6 cells showed fragmentation (*right panel*).

medium from IL-15-negative KM12C cells (Fig. 5). The expression level of each of the above proteins produced by IEC6 cells treated with KM12C-preconditioned medium changed little or not at all from the levels found in untreated IEC6 cells.

## DISCUSSION

The expression of IL-15 has been shown to play a role in colon cancer progression by influencing cell growth, invasion,

and apoptosis (10). In the present study, expression of IL-15 was associated with the induction of mucosal hyperplasia by metastatic colon cancer cells, suggesting that IL-15 expression is closely related to the production of metastasis. Data from experimental systems support the trophic effect of IL-15. Implantation of human colon cancer cells into the cecal wall of nude mice leads to the establishment of a growing tumor (9). In this orthotopic mouse colon cancer model, highly metastatic
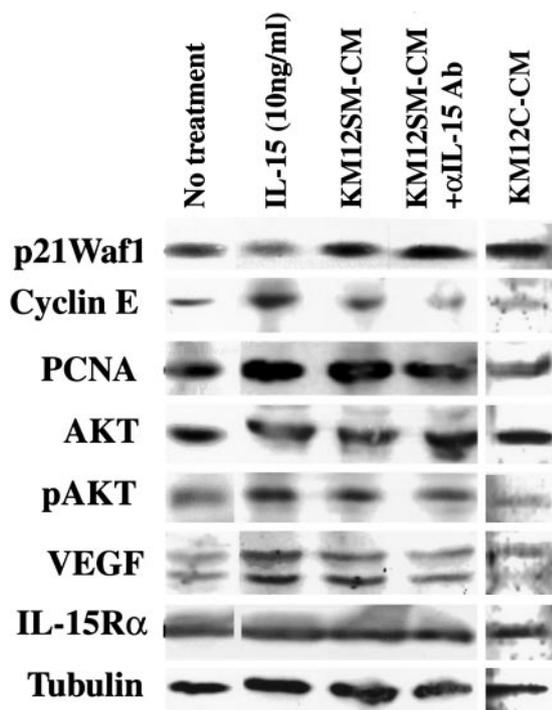
*Fig. 5* Effects of IL-15-, KM12SM-, and KM12C-preconditioned media on protein expression in IEC6 cells. The production of p21Waf1, cyclin E, PCNA, total AKT, pAKT, VEGF, and IL-15R α subunit was examined by immunoblotting. Tubulin served as an internal control. Cells were treated with 10 ng/ml human IL-15, concentrated KM12SM-conditioned medium (*KM12SM-CM*), concentrated KM12SM-conditioned medium pretreated with anti-IL-15 Ab (*KM12SM-CM+αIL-15Ab*), or concentrated KM12C-conditioned medium (*KM12C-CM*) for 48 h. Fifty μg of total cell lysate were subjected to 12.5% SDS-PAGE and electroblotted onto nitrocellulose membranes.

KM12SM human colon cancer cells expressed IL-15, whereas low-metastatic KM12C cells did not. Moreover, the present immunohistochemical analysis of autochthonous colon cancer specimens revealed that whereas metastatic colon cancer cells express IL-15, the adjacent mucosa does not. As we reported previously on colo320 cells (5), IL-15 shows direct effects on KM12SM colon cancer cells. IL-15 enhances cell motility and invasion *in vitro* on KM12SM cells, whereas it has less effect on KM12C cells (data not shown). KM12SM cells also express EGF receptor at high levels, which is closely associated with metastatic potential of the cells through autocrine stimulation by TGF-α (2, 3, 12). In contrast, KM12C cells express EGF receptor at lower levels than KM12SM cells (2, 13). The pair of KM12SM and KM12C cells is a suitable model for analyzing roles of IL-15 in colon cancer.

IL-15 can induce proliferation, invasion, and production of proangiogenic factors by colon cancer cells (5). Our present data indicate that IL-15 produced by colon cancer cells can induce cell growth and reduce DXR-induced apoptosis of intestinal epithelial cells. IL-15 also increases the production of angiogenic factors by intestinal epithelial cells, which suggests that IL-15 can contribute to both mucosal hyperplasia and angiogenesis and, hence, to tumor progression and

metastasis (1, 2). Colon cancer cells produce various growth factors, including TGF-α (3), and KM12SM cells produce IL-15 as well. In IEC6 cells treated with KM12SM-conditioned medium plus anti-IL-15 Ab, the up-regulated expression of pAKT and VEGF was partially neutralized by TGF-α produced by the KM12SM cells. Treatment of IEC6 cells with KM12SM-conditioned medium plus both anti-IL-15 and anti-TGF-α Abs neutralized up-regulation of pAKT and VEGF expression (data not shown).

In lymphoid cells, IL-2 receptor β/γ complex and IL-15Rα are required for signal transduction and high-affinity binding of IL-15, respectively (14). It was suggested recently that IL-15Rα is also involved in signal transduction in certain cell types (15); for example, synovial cells involved in rheumatoid arthritis express functional IL-15R complex (16). Our data demonstrate that IEC6 intestinal epithelial cells express IL-15Rα and IL-2 receptor β/γ subunits and have a high affinity for IL-15 (*i.e.,* the IL-15Rs are functional).

IL-15 is a chemoattractant to NK cells, and hence its expression is considered to be protective against the progression of colon cancers (17) and other neoplasms (18–20). In advanced cancers, the infiltration of NK cells into tumors and the activity of NK cells is reported to be suppressed (21–23). In the present study, we found that the number of NK cells infiltrating tumors was not associated with IL-15 production and that fewer NK cells infiltrated metastatic (Dukes' C) tumors than infiltrated nonmetastatic (Dukes' B) tumors. Also, the experimental KM12SM tumors (which have high metastatic potential) had fewer infiltrating NK cells than did the KM12C tumors (which have low metastatic potential). Collectively, these findings suggest that cancer cells may possess a mechanism to combat NK cells. NK cells express surface receptors that transduce intracellular signals through SHP-1 activation and inhibit cellular function (24–27). Activation of this inhibitory pathway in NK cells by products released from tumor cells has not been reported. ROS can inhibit NK cell activity (28), and tobacco carcinogens suppress NK cell activity through ROS produced by activated cyclooxygenase 2 and increased levels of prostaglandin $E_2$ (29). Because many types of cancer cells produce ROS, inhibition of NK cells by ROS from KM12C or KM12SM tumors may be relevant to our study findings. Various growth factors and cytokines are produced by colon cancer cells (3). Some, such as TGF-β (30), TGF-α (31), hepatocyte growth factor and insulin-like growth factor II (32), and GM3 (33), are reported to have inhibitory effects on NK cells. In the present study, we found that many IL-15-producing colon cancers concurrently produced TGF-α. These findings suggest that NK cell activation and the anticancer effects of IL-15 might be negated by ROS and growth factors concurrently produced by tumor cells. IL-15 has opposite biological effects in colon cancer cells than in the host immune system: the direct action of IL-15 on colon epithelial cells and cancer cells promotes the growth of cancer cells (5, 34, 35), whereas its activation of NK cells can have an antitumor effect (15–18). In the above scenario, the IL-15-producing colon cancer cells exploit the protumor effects of IL-15 in cancer-cancer and cancer-mucosa interactions to promote tumor progression and metastasis.

Tumor cells that stimulate proliferation of adjacent normal tissues can benefit from the induction of angiogenesis necessary to support the hyperplasia. Moreover, hyperplastic tissues produce a variety of growth factors that act as autocrine factors on normal cells and as paracrine factors on tumor cells. For example, scirrhous-type gastric cancers that produce TGF-β or platelet-derived growth factor can induce production of hepatocyte growth factor by fibroblasts (36, 37), and pancreatic adenocarcinomas that produce basic fibroblast growth factor or EGF can induce desmoplastic stromal changes or hyperplasia of duct epithelia associated with neovascularization (38). The present data from human colon cancers indicate that production of IL-15 can stimulate intestinal epithelial cells to proliferate and produce proangiogenic molecules. The angiogenesis supporting the induction and maintenance of the hyperplasia also contributes to the progressive growth of the colon cancer.

## ACKNOWLEDGMENTS

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