Several investigators have demonstrated that human colon cancers were extensively infiltrated by lymphocytes and that this infiltration is associated with improved survival (Murray et al., 1975; Carlon et al., 1985; DiGiorgio et al., 1992). However, tumour infiltrating lymphocytes (TILs) isolated from colon cancer tissues were expanded after culture in the presence of interleukin (IL)-2, but have failed to lyse autologous tumours in cytotoxicity assays (Yoo et al., 1990; Home et al., 1993). Anti-tumour effects of colon cancer-derived TILs may be mediated by cytokine secretion. Little is known about cytokine production from colon cancer-derived TILs and its functional role.

The human colon cancer cell line colo205 established from ascitic fluid of patients with poorly differentiated colorectal carcinoma has a unique morphological character that it grows non-adherently like haematopoietic cell lines (Semple et al., 1978). colo205 cells gradually show the cobblestone-like flat morphology and adhere closely to plastic after confluent condition, indicating that those cells differentiate into well-differentiated stage. We have reported that interferon-γ (IFN-γ) induced cell–cell adhesion of colo205 before the confluent stage (Kanai et al., 1993). This IFN-γ-induced aggregation was completely inhibited by anti-carcinoembryonic antigen (CEA) monoclonal antibody (mAb). In contrast to Th1 cytokine IFN-γ, the effect of Th2 cytokines such as IL-4 in the cell–cell adhesion of colo205 remains unclear. IL-13 is a cytokine that shares structural characteristics with IL-4, and has been found to mirror the effects of IL-4 in a wide variety of cells (Zurawski and deVries, 1994). Obiri et al reported that IL-4 receptor (IL-4R) and IL-13 receptor (IL-13R) are expressed on human colon cancer cells and these receptors may have a functional role (Obiri et al., 1993, 1996).

To assess the role of IL-4 and IL-13 in colon cancer cell adhesion, we investigated the effect of both cytokines in human colon cancer cell line, colo205 cell–cell adhesion. IL-4 receptor was expressed on the cell surface of colo205, and recombinant IL-4 inhibited colo205 cell–cell adhesion in a dose-dependent fashion without inhibiting cell proliferation. Flow cytometric analysis revealed that monoclonal antibodies (mAbs) directed against E-cadherin and carcinoembryonic antigen (CEA) inhibited colo205 cell–cell adhesion and IL-4 significantly inhibited the expression of E-cadherin and CEA. IL-13 also inhibited colo205 cell–cell adhesion. These results indicated that IL-4 and IL-13 inhibited colon cancer cell–cell adhesion by down-regulation of E-cadherin and CEA molecules. We then investigated the expression of both cytokines from freshly isolated colon cancer tumour-infiltrating lymphocytes (TILs). With reverse transcription-polymerase chain reaction and flow cytometric analysis, we demonstrated that colon TILs expressed IL-4 and IL-13 mRNA and protein. These results suggest that Th 2 type cytokines IL-4 and IL-13 locally-produced from TILs may regulate colon cancer adhesion by down-regulation of adhesion molecules. © 2000 Cancer Research Campaign

Keywords: IL-4; IL-13; colon cancer; cell adhesion

MATERIALS AND METHODS

Cell lines
The human poorly differentiated colorectal carcinoma cell line (colo205) was obtained from ATCC (Rockville, MD, USA). The cell culture and all assays in this study were performed in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin–streptomycin and 5 x 10⁻⁵ M 2-ME.

Cytokines and reagents
Recombinant human IL-4 and IL-13 were purchased from Pharmingen (San Diego, CA, USA). Recombinant human IFN-γ was kindly provided by the Shionogi Pharmaceutical Co. (Osaka,
Japan). Phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), Ca ionophore and brefeldin A were obtained from Sigma (St Louis, MO, USA).

Monoclonal antibodies

The fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse IgG was purchased from TAGO (Burlingame, CA, USA). The mAb C3080 (anti-human CEA) was purchased from Immunotech (Marseille, France). The mAb HEDC-1 (anti-human E-cadherin) was purchased from Takara Biomedicals (Tokyo, Japan). The mAb BD4-8 (anti-human IL-4) and the mAb B69-2 (anti-human IL-13) were purchased from Pharmingen (San Diego, CA, USA). The mAb IT2.2 (anti-human B7-2) was kindly provided from Dr Miyuki Azuma (National Children’s Medical Research Center, Tokyo). Isomatched mouse IgGl and streptavidin–FITC were purchased from Sigma.

Cell–cell adhesion by phase-contrast microscopy

Cell–cell adhesion was assessed by the formation of cell aggregates determined by at least two investigators using a phase-contrast microscopy. Initial numbers of plated cells were 1 \times 10^5. Percentage of aggregated cells was calculated according to the formula: (number of single cells)/(total number of cells) \times 100.

To investigate the role of adhesion molecules during morphological changes of colo205 cells, we assessed the effect of anti-CEA and anti-E-cadherin mAbs on the colo205 cell–cell adhesion. Briefly, 3 \times 10^5 colo205 cells per well (96-well flat bottom microplates) were cultured with anti-CEA mAb mAb (2 \mu g ml^{-1}) or anti-E-cadherin (2 \mu g ml^{-1}) alone, or in combination of both mAbs for 24–48 h.

Effect of IL-4 on cell proliferation

Effect of IL-4 on cell growth was also determined by counting viable cells on day 0 to day 6 after culturing in 24-well culture plates. Initial cell numbers on day 0 were 1 \times 10^5. Cultures in the absence or presence of rhIL-4 were set up in triplicate in 2 ml culture medium for each time point. At each point, cells from individual wells were harvested and counted under a microscope with a haemocytometer using a trypsin blue viability assay.

IL-4 and IL-13 mRNA expression by reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from 1.0 \times 10^6 colo205 cells, 1.2 \times 10^6 TILs and 1.0 \times 10^6 PHA (10 \mu g ml^{-1})-stimulated peripheral blood mononuclear cells (PBMC) using RNAzol (Cinna/Biotech Lab, Houston, TX, USA). RNA was also isolated from biopsied specimens of normal colonic mucosa and colon cancer tissues. One hundred nanograms each of total RNA was then reverse transcribed in the presence of RAV 2 reverse transcriptase (BRL, Bethesda, MD, USA). For cDNA synthesis, 4 ng ml^{-1} of total cellular RNA solution was heated at 65°C for 5 min and cooled rapidly. After adding 1 \mu l of 10 \mu M polymerase chain reaction (PCR) buffer (500 mM potassium chloride (KCI), 100 mM Tris–HCl buffer, pH 8.4, 15 mM magnesium chloride (MgCl), 0.01% gelatin), 1 \mu l of 25 mM aNTP (Takara), 1 \mu l of 10 \times hexanucleotide mixture (Boehringer Mannheim, Mannheim, Germany), 1 \mu l of 100 mM dithiothreitol (Boehringer Mannheim), 20 U of ribonuclease inhibitor (Takara), and 3 U of RNA-2 reverse transcriptase, the mixture was incubated at 42°C for 60 min, heated at 94°C for 5 min, and quick-chilled on ice. PCR was performed to amplify the cDNA of IL-4, IL-13, IL-4 receptor \alpha (IL-4R\alpha), common \gamma chain (\gamma) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Equal amounts of cDNA were used in each PCR. The PCR reaction mixture contained 10 \mu l of cDNA, 5 \mu l of 10 PCR buffer, 8 \mu l of 1.25 mM aNTP, 207 \mu l of DEPC-water, 5 \mu l each of 20 pM 5' and 3' primers and 1.5 U of Taq polymerase (Perkin-Elmer Cetus, Norfork, CT, USA). To amplify the cDNA of IL-4, IL-4R\alpha, \gamma, and GAPDH, the amplification was conducted at 94°C for 1 min, at 60°C for 2 min, at 72°C for 3 min for 40 cycles for IL-4, IL-13, IL-4R\alpha, \gamma and GAPDH. The forward and reverse human IL-4 primers were 5’ primer, CGG CAA CTT TCA CCA CGG ACA CAA GTG CGA TA and 3’ primer, ACG TAC TCT GGT TGG CCT CCT TCA CAG BGA CAG. The forward and reverse human IL-13 primers were 5’ primer, TGC CTC CCT CTA CAG CCC TCA and 3’ primer, CAG TTG AAC CGT CCT CCC TCG CG. The forward and reverse human IL-4R\alpha primers were 5’ primer, TGA AGT CTG GTA TTT ACA GAG C and 3’ primer, TCA AAC AAC TCA ACC CAT CGC ACC CAG C. The forward and reverse human \gamma primers were 5’ primer, GAA GAC ACC ACA GCT GAT TTC and 3’ primer, ACT CTC AGC CAG CTT AGA. The forward and reverse GAPDH primers were 5’ primer, TGA AGG TCG TTC GGA GAT AGT TG and 3’ primer, CAT GTA GGC CAT GAG TTC GC and 3’ primer, CAT GTA GGC CAT GAG TTC GC.

Cell surface expression of adhesion molecules and cytokine receptors by flow cytometry analysis

Examination of IL-4R, IL-13R, and IFN-\gamma receptor (IFN-\gammaR) expression on cell surface by FACScan (Becton-Dickinson, Mountain View, CA, USA) was carried out using biotin-labelled IL-4, IL-13, and IFN-\gamma as previously described (Fanslow et al., 1993). Briefly, biotin-N-hydroxysuccinimide (Calbiochem, La Jolla, CA, USA) and purified human recombinant IL-4, IL-13, and IFN-\gamma were mixed at a molar ratio of 12:1. One hundred microlitres each of IL-4 (1 mg ml^{-1}), IL-13 (0.1 mg ml^{-1}) and IFN-\gamma (0.1 mg ml^{-1}) in 0.1M NaHCO3, pH 8.5 were mixed with 10 ml biotin-N-hydroxysuccinimide (1 mg ml^{-1} in dimethyl sulphoxide) for 30 min at room temperature, vortexing every 10 min. At the end of the incubation period, the reaction mixture was thoroughly dialysed in PBS and adjusted to 10 mg ml^{-1} in PBS plus 0.01% NaN3. For IL-4R, IL-13R and IFN-\gammaR determination, 5 \times 10^5 colo205 cells were incubated with 50 ng biotinylated IL-4, IL-13 or IFN-\gamma in a volume of 100 \mu l PBS + 0.02% NaN3, 2% heat-inactivated FCS for 30 min at 4°C. After washing three times, cells were further incubated in 50 \mu l of diluted streptavidin–FITC for 30 min at 4°C. Cells were washed three times and analysed by the FACScan (Becton-Dickinson, Mountain View, CA, USA).

To estimate the quantity the CEA and E-cadherin expression on colo205 cells, flow cytometric analysis was done. The cells were harvested using 0.25% trypsin, washed with PBS three times, and then incubated with anti-E-cadherin mAb, anti-CEA mAb or...
isomatched mouse IgG1 mAb. After washing and incubating with FITC-conjugated goat anti-mouse IgG, the cells were suspended at \(1 \times 10^6\) cells ml\(^{-1}\) and the fluorescence intensity on the surface of the cells was analysed with a FACSscan.

### Isolation of TILs

Tissue samples were obtained with informed consent, from two patients with colorectal cancer. Mucosal samples included macroscopically and microscopically cancerous areas and unaffected normal areas of the colon. Tissues were minced with scissors, and then digested with 0.4% collagenase (Sigma) solution in RPMI-1640 supplemented with 50 \(\mu\)g ml\(^{-1}\) gentamicin (GIBCO). After washing the tumour/lymphoid single cell suspension, tumour cells were separated from TILs by discontinuous Ficool-Paque gradient of 40% and 60% Percoll, and washed in RPMI-1640 medium. Viability and purity was assessed by trypan blue dye exclusion.

### Flow cytometry analysis of IL-4 and IL-13 expression in colon cancer TILs

To investigate IL-4 and IL-13 at protein level, freshly isolated TILs were cultured in 24-well plates \((2 \times 10^6\) ml\(^{-1}\)\) for 24 h at 37\(^\circ\)C and 5% carbon dioxide in RPMI-1640 supplemented with 2 mM glutamine, 100 IU ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin, and 10% FCS. Cells were stimulated with PMA \((10\) ng ml\(^{-1}\)) + Ca ionophore \((20\) ng ml\(^{-1}\)) in the presence of 10 \(\mu\)g ml\(^{-1}\) brefeldin A. Brefeldin A was added at the beginning of the culture for a maximum of 12 h to inhibit protein secretion.

### RESULTS

#### IL-4 inhibits colo205 cell–cell adhesion

mRNA expression of IL-4R, that is comprised of two chains, IL-4R\(\alpha\) chain and common \(\gamma\) (\(\gamma_c\)) chain, in colo205 cells was analysed by RT-PCR analysis. IL-4R\(\alpha\) chain mRNA was detected in colo205 cells (Figure 1A). \(\gamma_c\) chain mRNA was also detected in colo205 cells. To confirm the expression of IL-4R in colonic epithelial cells, we analysed expression in colo205 cells by flow cytometry. As shown in Figure 1B, colo205 cells clearly expressed IL-4R on the cell surface. IFN-\(\gamma\) receptor was also expressed on colo205 cells as previously reported (Kanai et al, 1993).

colo205 cells changed their morphological shapes from floating and round cells to adherent and cobblestone-like cells after confluent stage (Figure 2A). Most cells in medium with IL-4 did not change shape, and kept floating and were round in morphology (Figure 2C, D). In contrast, IFN-\(\gamma\) induced strong cell–cell aggregation (Figure 2B). To assess the formation of cell adhesion, percentage of single cells were then determined under phase-contrast microscopy. As shown in Figure 3, IL-4 inhibited colo205 cell–cell adhesion in a dose-dependent fashion. Moreover, IFN-\(\gamma\) induced cell–cell adhesion was significantly \((P < 0.05)\) decreased by exogenous IL-4 (Figure 3).

To assess the effect of IL-4 in the proliferation of colo205 cells, viable cells were counted at various time points. The cell counts in colo205 cultures with various concentrations of IL-4 were almost the same as that in the culture of untreated colo205 cells at given point of time (Figure 4). We also examined the effect of IL-4 on the incorporation of thymidine into the DNA of colo205 cells and could not detect any significant difference between IL-4-treated and untreated cells (data not shown).

#### IL-4 induced down-regulation of the expression of CEA and E-cadherin molecules in colo205 cells

To elucidate the role of adhesion molecules during morphological changes of colo205 cells, we assessed the effect of anti-CEA and anti-E-cadherin mAbs on the colo205 cell–cell adhesion. As shown in Figure 5, anti-CEA \((2\) \(\mu\)g ml\(^{-1}\)) and anti-E-cadherin \((2\) \(\mu\)g ml\(^{-1}\)) mAbs but not control anti-B7-2 \((2\) \(\mu\)g ml\(^{-1}\)) mAb inhibited colo205 cell–cell adhesion at 24 h (Figure 5A) and 48 h (Figure 5B). As shown in Figure 5C, combination of anti-CEA and
anti-E-cadherin mAbs significantly inhibited colo205 cell–cell adhesion, as compared with anti-CEA mAb or anti-E-cadherin mAb alone did. This result indicated that E-cadherin and CEA are important molecules for colo205 cell–cell adhesion. We then analysed the expression of E-cadherin and CEA in colo205 cells by flow cytometry after cytokine treatment. As shown in Figure 6, E-cadherin and CEA molecules were down-regulated by IL-4 treatment. In contrast, E-cadherin and CEA molecules were constitutively expressed in untreated colo205 cells, and these molecules were up-regulated by IFN-γ treatment. Non-specific cross-reacting antigen (NCA) that is a CEA family was also down-regulated by IL-4 treatment (data not shown). These results indicated that IL-4 inhibited colon cancer cell–cell adhesion by down-regulation of E-cadherin and CEA molecules.
Effect of IL-4 and IL-13 on colon cancer cell adhesion

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Figure 6  Expression of E-cadherin and CEA molecules in colo205 cells. Cells were treated with IL-4 (100 U ml–1) or IFN-γ (100 U ml–1) for 72 h. Single-cell suspensions were stained with anti-E-cadherin or anti-CEA mAb followed by FITC-labelled goat anti-mouse IgG antibody. Dotted lines show the profiles of cells stained with control mouse IgG antibody and second antibody. Representative of three experiments was shown. Data are presented as histograms of relative number of cells (y-axis) versus fluorescence intensity (x-axis, log scale).

Figure 7  (A) Phase-contrast microscopic pictures of untreated, IL-4-treated (100 U ml–1), IL-13 (10 ng ml–1)-treated, or IFN-γ (100 U ml–1)-treated colo205 cells at 2 and 6 days after treatment. Similar results were obtained in each of five independent experiments. (B) IL-13 inhibited cell–cell adhesion of colo205 cells. Subconfluent cells were incubated with IL-13 (0.1–10 ng ml–1), or IFN-γ (10 U ml–1)+IL-13 (0.1–10 ng ml–1) for 24 h. After incubation, numbers of single cells and total cells were measured by phase-contrast microscopy. The percentage of single cells (number of single cells/number of total cells) is represented. The figure shows the results of average range in triplicate. (C) IL-13 receptor expression on the cell surface of colo205 cells by flow cytometric analysis.
TILs using intracellular staining method by flow cytometric analysis respectively. (from colon cancer tissues by RT-PCR analysis. mRNA from PHA-stimulated normal colonic mucosa and colon cancer tissues by RT-PCR analysis. Figure 8A) IL-4 and IL-13 mRNA expression in biopsied specimens of colon cancer tissues as well as those of normal colonic tissues (Figure 8A). Expression of both IL-4 and IL-13 mRNA in colon cancer tissues was not increased as compared with that in normal colonic tissues. We then assessed IL-4 and IL-13 mRNA expression in freshly-isolated TILs from colon cancer. Both cytokine mRNAs were demonstrated by RT-PCR analysis (Figure 8B). To confirm the expression of IL-4 and IL-13 in colon TILs, we analysed intracellular expression of IL-4 and IL-13 in cells stimulated with PMA+Ca ionophore in the presence of 10 µg ml⁻¹ brefeldin A by flow cytometry. As shown in Figure 8C, colon TILs expressed both IL-4 and IL-13 protein in the cytoplasm. Expression of both IL-4 and IL-13 protein on isolated TILs from colon cancer tissues was not increased as compared with that in lamina propria T-cells from normal tissues (data not shown).

DISCUSSION

Disrupted cell–cell adhesion is thought to be responsible for the invasiveness and metastasis potential of colon cancer cell. In poorly differentiated tumours, there are numerous small groups of non-adhesive cells as well as isolated cancer cells. Little is known about the role of cytokines in cellular properties that confer disordered cohesiveness. We demonstrated in a previous study that Th1 cytokine IFN-γ induces cell–cell adhesion of colo205 with up-regulation of CEA molecule (Kanai et al, 1993). In contrast to IFN-γ, the effect of Th2 cytokines such as IL-4 and IL-13 which shares structural and functional characteristics with IL-4 in the cell–cell adhesion of colon cancer cells remains unclear. Lahm et al has reported a morphological change in WiDr cells after exposure to IL-4 (Lahm et al, 1994). Cells after IL-4 treatment acquired a round morphology, while control cells grown in culture medium alone showed the epithelial-like flat morphology and closely attached to plastic. But they did not demonstrate the mechanism underlying the phenomena. It has been widely accepted that effector CD4+ T-cells are subdivided into two subsets Th1 and Th2 on the basis of their cytokine profiles. Th1 and Th2 subsets interact and regulate each other in functional activity. Disproportion between Th1 and Th2 subsets is thought to be associated with susceptibility to various phenomena. Therefore, it is possible that Th2 cytokines as well as Th1 cytokine IFN-γ may regulate colon cancer cell–cell adhesion.

In the present study, we found that Th2 cytokines IL-4 and IL-13 inhibited colon cancer cell–cell adhesion. Several investigators reported an inhibitory effect of IL-4 on in vitro proliferation of human cancer cell (Hoon et al, 1991; Morisaki et al, 1992; Obiri et al, 1993; Lahm et al, 1994), while colorectal carcinoma cell lines Co-115 and SW1116 showed no significant alteration of the proliferation in the presence of IL-4 (Jung et al, 1993). We also could not show any significant difference in cell proliferation between IL-4-treated and untreated colo205 cells. These results suggest that inhibitory effect of IL-4 and IL-13 on colon cancer cell–cell adhesion is not due to inhibiting cell growth, and that IL-4 and IL-13 may serve as negative regulators of colon cancer cell–cell adhesion.

It is well known that E-cadherin mediates cell–cell adhesion, and changes of E-cadherin expression have been reported in human carcinomas (Boyer et al, 1992). Examination of cancer tissues demonstrated a correlation in the expression of E-cadherin relative to the state of differentiation and dedifferentiation (Tamm et al, 1990; 1992).

IL-13 inhibits colo205 cell–cell adhesion

Most cells in the medium with IL-13 did not change the shapes and kept floating and round (Figure 7A). As shown in Figure 7B, IL-13 inhibited colo205 cell–cell adhesion in a dose-dependent fashion and partially inhibited IFN-γ-induced cell–cell adhesion. We also analysed IL-13 receptor expression on colo205 cells by flow cytometry. As shown in Figure 7C, colo205 cells expressed IL-13 receptor on the cell surface. These results indicated that IL-13 also inhibited colon cancer cell–cell adhesion in a similar manner to IL-4.

IL-4 and IL-13 expression in freshly-isolated colon cancer TILs

RT-PCR analysis demonstrated that IL-4 and IL-13 mRNA was expressed in biopsied specimens of colon cancer tissues as well as
Effect of IL-4 and IL-13 on colon cancer cell adhesion

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