Colon adenocarcinoma cells inhibit anti-CD3-activated killer cell induction

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Abstract. Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. Recently, in vivo administration of anti-CD3 antibody has been shown to induce mouse T lymphocytes to mediate major-histocompatibility-complex (MHC)-unrestricted tumoricidal activity which is distinct from natural-killer-cell-derived LAK activity. It has therefore been suggested that anti-CD3 therapy may find application in tumor immunotherapy in humans. However, the effectiveness of anti-CD3-activated killer cell induction within the environment found in the vicinity of colon adenocarcinoma cells has not been evaluated. The present report demonstrates that colon cancer cells of human (HT-29) and mouse (MCA-38) origin markedly inhibit the generation of activated killer cells in murine spleen cell cultures. DNA synthesis and interleukin-2 production by spleen cells following stimulation with anti-CD3 antibody are also profoundly depressed in the presence of MCA-38 and HT-29 adenocarcinoma cells. MCA-38- and HT-29-mediated inhibition of activated killer cell development is exerted through the production of a tumor-associated soluble factor that is distinct from transforming growth factor β or prostaglandins. Local immunosuppression associated with sites of tumor growth may therefore represent a major obstacle to successful anti-CD3 immunotherapy of certain colon adenocarcinomas.

Key words: Colon cancer – Immune suppression – Anti-CD3 antibody – MHC-unrestricted CTL

Introduction

Adenocarcinoma of the colon is a major health problem, in large part because fewer than one-half of all cases are curable by surgery [28]. Furthermore, advanced intestinal cancers have proven resistant to conventional chemotherapy with 5-fluorouracil, which is currently the most effective antineoplastic agent used in the treatment of metastatic disease [28]. Attempts have therefore been made to treat colon cancer with adoptive immunotherapy, which employs interleukin (IL)-2 to induce the patients’ own lymphocytes to mediate tumoricidal activity [20]. Unfortunately, in clinical trials, colon adenocarcinomas have responded poorly to adoptive immunotherapy with lymphokine-activated killer (LAK) cells [32].

One possible explanation for the failure of LAK cell therapy as a treatment for colon cancer may lie in the ability of certain tumors to secrete immunosuppressive molecules and thereby evade immunological killing [23]. Transforming growth factor β (TGFβ), which is released from many neoplastic tissues [27], is able to block both cytotoxic T lymphocyte (CTL) and LAK activity [14, 22]. A related molecule, glioblastoma-derived T cell suppressor factor, has been shown to mediate similar inhibitory effects [16]. Immunosuppression is also exerted by tumor-elaborated molecules that are distinct from TGFβ. For example, human oral squamous carcinoma cell lines produce a soluble factor that inhibits mitogen- and alloantigen-stimulated lymphocyte proliferation [26], while human colon cancer cells maintained in culture secrete a novel suppressor factor which blocks the induction of LAK cells by IL-2 [7].

LAK cells are derived predominantly from the relatively few natural killer cells found in peripheral blood [24]. However, T cells are much more abundant in the circulation and constitute the vast majority of tumor-infiltrating lymphocytes [2]. It may therefore be preferable to employ LAK-like CTL in cancer immunotherapy. In this regard, mouse spleen cell cultures stimulated with monoclonal antibody (mAb) against CD3 of the T cell receptor complex develop potent major-histocompatibility-complex (MHC)-
unrestricted tumoricidal activity [17, 29]. Moreover, in mouse models of cancer, low-dose therapy with anti-CD3 mAb has been shown to prevent malignant progresor tumor growth [8], as well as mediate the regression of experimental hepatic and pulmonary metastases more effectively than immunotherapy with IL-2 and/or LAK cells [10, 12]. Activation of T cells and subsequent antitumor activity induced by anti-CD3 therapy may therefore represent an alternative to conventional LAK therapy for treatment of colon cancer.

The present study was undertaken to determine whether anti-CD3 mAb can effectively induce MHC-unrestricted cytolytic activity in mouse spleen cell populations in the presence of colon cancer cell lines of mouse (MCA-38) or human (HT-29) origin. The results indicate that both tumor cell lines produce immunosuppressive factor(s), which can be distinguished from TGFβ and prostaglandins, that interfere with the generation of anti-CD3-activated killer (AK) cells.

**Materials and methods**

*Mice.* Female C57BL/6 mice, 6 weeks old, were purchased from Charles River Canada (Laval, Que.). Prior to use in experiments, mice were maintained on standard laboratory chow and water supplied ad libitum in our animal care facilities.

*Medium and reagents.* RPMI-1640 medium (ICN Flow, Mississauga, Ont.) was supplemented with 10 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all ICN Flow), and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (Gibco BRL, Burlington, Ont.). Hereafter, this will be referred to as complete RPMI-1640 medium. Recombinant murine IL-4 was purchased from Genzyme (Cambridge, Mass.) while recombinant human TGFβ1 was kindly provided by Nacalai Tesque Inc., Kyoto, Japan. Turkey anti-human TGFβ polyclonal antibody (IgG2), which crossreacts with murine TGFβ, was obtained from Collaborative Research Inc., Bedford, Mass. The hybridoma-producing hamster anti-mouse CD3 mAb (clone 145-2C11) [17] was generously provided by Dr. J. Bluestone (University of Chicago, Chicago, Ill.). Indomethacin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol to produce a 0.004 M stock solution. This stock was diluted in complete RPMI-1640 medium to obtain the final indomethacin concentrations used in this study.

*Cell lines.* YAC-1 murine lymphoma, P815 murine mastocytoma, and HT-29 human colon adenocarcinoma cell lines were obtained from American Type Culture Collection, Rockville, Md. The murine MCA-38-LD (liver-derived) colon adenocarcinoma was kindly provided by Dr. S. Gallinger (Samuel Lunenfeld Research Institute, Toronto, Ont.). The CTLL-2 and HT-2 T cell lines were a generous gift from Dr. T. Issekutz (IWK Hospital, Halifax, N. S.). All cell lines, with the exception of the CTLL-2 and HT-2 lines, were maintained by passage in complete RPMI-1640 medium. CTLL-2 and HT-2 cells were grown in complete RPMI-1640 medium supplemented with 15% concanavalin-A-stimulated rat spleen cell culture supernatants. All cell lines were maintained at 37°C in a 95% humidified atmosphere of 5% CO2 and were verified to be free of *Mycoplasma* contamination prior to use in this study.

*Lymphocyte/adenocarcinoma cocultures.* Individual wells (2 cm² area) of 24-well flat-bottom multiplates (ICN Flow) were seeded with 10⁵ MCA-38 or HT-29 tumor cells in 1 ml complete RPMI-1640 medium. Once the tumor cell monolayers had grown to confluence, fresh medium plus 5×10⁵ C57BL/6 spleen cells (minus erythrocytes which had been depleted by osmotic shock) were added to each well. Control wells that lacked tumor cell monolayers received only spleen cells. AK cells were generated by adding anti-CD3 hybridoma supernatants to the desired wells at a final dilution of 1/40. In some experiments, spleen cells were placed in culture well inserts (Millipore, Mississauga, Ont.), separated from the tumor cells by a cell-impermeable membrane (0.4 μm pore diameter). Lymphocyte/adenocarcinoma cocultures were then cultured for 48 h (or as otherwise noted) at 37°C in 95% humidified air and 5% CO2. At the end of this time, AK cells were harvested by gentle aspiration with a Pasteur pipette. Examination of the wells with an inverted microscope confirmed that tumor cell monolayers had remained intact throughout this process. AK cells were washed three times with phosphate-buffered saline (pH 7.2), resuspended in complete RPMI-1640 medium, counted and adjusted to the desired cell concentration prior to being assayed for cytolytic effectector function in a ⁵¹Cr-release assay. In some experiments DNA synthesis by AK cells was measured by transferring aliquots of spleen cells at 12-, 24-, 48- and 72-h intervals to 96-well microtiter plates and pulsing for 6 h with 0.5 μCi tritiated thymidine ([³H]TdR; ICN Flow). Cultures were harvested onto glass-fiber filter-paper using a Titertek cell harvester and [³H]TdR incorporation was determined by liquid scintillation counting. Data are expressed as mean radioactivities (cpm) of triplicate samples, plus or minus the standard deviation.

### Cytolytic ⁵¹Cr-release assay.

YAC-1 and P815 target cells were incubated for 1 h at 37°C with 100 μCi sodium [⁵¹Cr] chromate (ICN Flow), washed three times, and resuspended to a concentration yielding 5×10⁴ target cells/well. AK cells and target cells were added to wells of 96-well "V"-bottom microtiter plates (ICN Flow) at various effector-to-target (E:T) ratios in a 0.2-ml volume of complete RPMI-1640 medium. Plates were incubated for 4 h at 37°C in 95% humidified air and 5% CO2. Following centrifugation at 400 g for 5 min, supernatants (0.1 ml) were collected from microtiter wells and the ⁵¹Cr released from lysed target cells was measured in a Beckman Gamma 8000 sample counter. Percentage lysis was calculated according to the formula

\[
\text{lysis} (\%) = \left(1 - \frac{E}{M} \right) \times 100
\]

where *E* is the release from experimental samples, *S* is the spontaneous release, and *M* is the maximum release upon lysis with 10% sodium dodecyl sulfate. Data are presented as the mean percentage lysis of triplicate samples. The standard deviation was consistently less than 10% of the mean.

### IL-2 bioassay.

IL-2-dependent CTLL-2 cells were washed and their concentration adjusted to 10⁵ cells/ml in complete RPMI-1640 medium. CTLL-2 cells (0.1 ml) and culture supernatants (0.05 ml) to be assayed for IL-2 bioactivity were combined in 96-well flat-bottom microtiter plates (ICN Flow) and incubated for 24 h at 37°C and 5% CO2 in 95% humidified air. Negative controls consisted of CTLL-2 cells cultured with complete RPMI-1640 medium while positive controls consisted of CTLL-2 cells cultured with concanavalin-A-stimulated rat spleen cell culture supernatants. During the final 6 h of culture, DNA synthesis in CTLL-2 cells was measured by pulsing each well with 0.5 μCi [³H]TdR. Data are expressed as mean radioactivities (cpm) of triplicate samples, plus or minus the standard deviation.

### TGFβ bioassay.

This assay is based on the ability of TGFβ to inhibit IL-4-driven proliferation of HT-2 cells [30]. Briefly, HT-2 cells were washed and adjusted to a concentration of 2×10⁵ cells/ml in complete RPMI-1640 medium. HT-2 cells (0.05 ml) and recombinant IL-4 at a final concentration of 100 U/ml were added to 96-well flat-bottom microtiter plates, along with 0.05 ml culture supernatants to be assayed for TGFβ activity. Control wells received complete RPMI-1640 medium instead of culture supernatants. Recombinant human TGFβ1 (0.6 nM) was added to some wells as a positive control. Wells containing only HT-2 cells plus complete RPMI-1640 medium served as negative controls. Plates were incubated for 24 h at 37°C and 5% CO2 in 95% humidified air. DNA synthesis was measured by pulsing HT-2 cells with 0.5 μCi [³H]TdR during the final 6 h of culture. Data are expressed as mean radioactivities (cpm) of triplicate samples, plus or minus the standard deviation.