Secretion of Bioactive Granulocyte-Macrophage Colony-stimulating Factor by Human Colorectal Carcinoma Cells

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Abstract

Secretion of several cytokines by colorectal carcinoma cells has been substantiated. These do not include granulocyte-macrophage colony-stimulating factor (GM-CSF) until now. We show that the supernatant of two human colorectal carcinoma cell lines, LS1034 and SW480, stimulates proliferation of GM-CSF-dependent M07e cells. The activity was constitutively secreted by LS1034 cells and could be induced by serum-free culture conditions in SW480 cells. Addition of a neutralizing anti-GM-CSF antibody completely inhibited this activity. Preabsorption with anti-GM-CSF antibody removed all M07e growth-stimulating activity from LS1034 and SW480 supernatant. Western blot analysis revealed the presence of GM-CSF in LS1034 supernatant. Our results indicate that human colorectal carcinoma cells secrete indeed biologically active GM-CSF.

Introduction

Although production of several growth factors has been demonstrated for colorectal carcinoma cells (1-3), the list of colorectal carcinoma-derived cytokines does not include GM-CSF to date. Among the colony-stimulating factors which regulate differentiation and maturation of hematopoietic precursor cells, GM-CSF supports growth of several myeloid lineages (4, 5). Therefore, GM-CSF is used in clinical protocols to reduce the myelosuppressive effects in cancer patients after chemotherapy (6-9). Secretion of GM-CSF has been reported for tumor cells of different origin (10, 11). In some instances, GM-CSF promotes tumor growth in an autocrine fashion (12, 13). Our present study now describes the secretion of GM-CSF by human colorectal carcinoma cells which is active in a biological assay.

Materials and Methods

Cytokines and Antibodies. rhu GM-CSF and rhu IL-3 were purchased from Gibco (Basel, Switzerland). Neutralizing rabbit anti-hu IL-3 and mouse monoclonal anti-hu GM-CSF antibody were obtained from Genzyme (Cambridge, MA). [methyl-3H] thymidine (3H TdR) with a specific activity of 925 GBq/mmol was obtained from Amersham (Aylesbury, United Kingdom).

Cell Lines and Culture Conditions. The human colorectal carcinoma cell lines HT-29, SW480 (both obtained from the American Type Culture Collection, Rockville, MD), and LS1034 (14) were maintained in Dulbecco’s modified Eagle medium:Ham’s F-12 nutrient mixture (1:1; Gibco) supplemented with 2 mM l-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazinedisulfonic acids and 5% FCS. M07e cells, a generous gift of Dr. P. G. Natali (Regina Elena Cancer Institute, Rome, Italy), were cultured in RPMI 1640 (GIBCO).

Received 4/14/94; accepted 6/2/94.

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1 This work was supported by the Swiss National Foundation (Grant 31-28590-90) and Grant FCR 293 from the Ligue Suisse contre le Cancer to H. L.

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3 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; rhu, recombinant human; SCF, stem cell factor; SN, supernatant; hu, human; FCS, fetal calf serum; PBS, phosphate-buffered saline.

Preparation of SN from Colorectal Carcinoma Cell Lines. Cells were seeded at 5 X 10⁴ to 1 X 10⁵/ml in complete medium and SN were taken after 4 days. For preparation of serum-free SN, cells were grown to 90% confluence in complete culture medium. They were washed twice with PBS and SN was harvested after a 4-day culture period in serum-free medium. All SN were centrifuged at 600 x g for 10 min to remove cellular debris.

M07e Proliferation Assay. SN of colorectal carcinoma cell lines were tested for stimulation of factor-dependent M07e as described (15). Briefly, M07e cells were washed twice with RPMI 1640 with 10% FCS, resuspended in the same medium, and distributed at 1 X 10⁴ cells/well into 96-well flat-bottomed microtiter plates. They were incubated in the presence of colorectal carcinoma cell SN for 3 days at 37°C and 5% CO₂ in a final volume of 200 μl. In neutralization experiments, antibody and cytokines or SN were incubated together for 1 h at 37°C prior to the addition of cells. The cells were pulsed with 3H TdR during the last 4 h of culture (0.5 μCi/well). They were harvested on filter papers and dried. The incorporated radioactivity was determined using an Automatic Filter Counting System (Inotech, Dottikon, Switzerland). Results were confirmed in at least three independent experiments. All measured were in duplicate.

Preabsorption of Colorectal Carcinoma Cell SN with Anti-hu GM-CSF Antibody. Anti hu-GM-CSF antibody (1 μg/ml), diluted in coating buffer (0.1 M Na₂CO₃, pH 9.6), was coupled to sterile enzyme-linked immunosorbent assay plates (Falcon, Lincoln Park, NJ) by overnight incubation at 4°C. Plates were washed three times with PBS, incubated for 2 h at 37°C with PBS/2% bovine serum albumin to saturate free binding sites and washed again three times with PBS. Colorectal carcinoma cell SN or recombinant cytokines were added and incubated for 4 h at 37°C. Thereafter, the activity of the SN was tested on M07e as described above.

Western Blot Analysis. Serum-free SN of LS1034 and HT-29 cells were concentrated by Amicon centrifugation (membrane cutoff, 10 kDa). Fifty μl of 100-fold concentrated SN were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. rhu GM-CSF was detected by Western blot analysis using a monoclonal anti-hu GM-CSF antibody (5 μg/ml) using the enhanced chemiluminescence detection system (Amersham).

Results and Discussion

The proliferation of M07e, a human megakaryoblastic leukemia cell line, strictly depends on the presence of hematopoietic growth factors such as GM-CSF and IL-3 (15). LS1034 colon carcinoma cells, cultured in medium with 5% FCS or under serum-free conditions, released a factor(s) into the SN which strongly enhanced proliferation of M07e (Fig. 1). Serum-free culture of SW480 induced secretion of a similar activity. In this cell line, serum appears to suppress factor production since SN of SW480 cells cultured in medium with 5% FCS did not support proliferation of M07e (Fig. 1). In contrast, SN of HT-29 cells did not contain detectable M07e growth-promoting activity (Fig. 1).

A number of cytokines have been reported to enhance M07e growth-promoting activity (Fig. 1).
GM-CSF secretion by human colon carcinomas

GM-tSF secretion by human colon carcinomas in 100-fold concentrated LS1034 SN. In contrast, no GM-CSF was detectable in SN from HT-29 cells (Fig. 4, Lane 3). LS1034 cells secrete GM-CSF constitutively. A cluster on chromosome 5q23–31 contains the genes for GM-CSF, IL-3, IL-4, and IL-5 (19). Alterations in the APC gene, which maps to 5q21–22 occur frequently in colorectal carcinomas (20). Cytogenetic analysis revealed no normal chromosomes 5 in LS1034 (14). Moreover, LS1034 showed loss of heterozygosity for several markers on chromosome 5q but not 5p (21). Thus, one might speculate that constitutive expression of GM-CSF in LS1034 is due to genetic alterations.

Dilution of SN (1/n)

Fig. 1. Supernatants from colorectal carcinoma cell lines stimulate proliferation of M07e. M07e cells were cultured in the presence of SN from LS1034 (●), SW480 (□), LS1034 (○) or HT-29 (●, ○) cells, cultured in medium with 5% FCS (□, △, ▽) or under serum-free conditions (●, ▽, ○). Proliferation of M07e in the presence of medium alone was 115 cpm.

It was possible that M07e cells were induced to secrete GM-CSF by a factor(s) present in the colorectal carcinoma cell SN, thereby promoting their own growth. SCF has been reported to support proliferation of M07e by inducing endogenous GM-CSF (17). However, it appeared unlikely that colorectal carcinoma-derived SCF was responsible for the response since anti-SCF antibody did not block the proliferation of M07e induced by rhu SCF (data not shown). To definitely exclude the possibility that a second cytokine present in colorectal carcinoma cell SN triggered GM-CSF secretion in M07e, LS1034 and SW480 SN were preabsorbed with anti-GM-CSF antibody (Fig. 3, top). Preabsorption with anti-GM-CSF antibody completely abolished the capacity of LS1034 and SW480 SN to stimulate proliferation of M07e (Fig. 3, bottom). Thus, GM-CSF secreted by colorectal carcinoma cells was responsible for the proliferation of M07e, thereby identifying this cytokine as another factor being produced by colorectal carcinoma cells. These results were confirmed by Western blot analysis. LS1034-derived GM-CSF appeared as a strong band of 22 to 26 kDa (Fig. 4, Lane 2), probably due to a different extent of glycosylation. Densitometric analysis revealed about 2 ng of GM-CSF present in 100-fold concentrated LS1034 SN. In contrast, no GM-CSF was detectable in SN from HT-29 cells (Fig. 4, Lane 3).

Fig. 2. Anti-GM-CSF antibody neutralizes the activity in colorectal carcinoma cell SN. rhu GM-CSF (2 ng/ml), rhu IL-3 (2 ng/ml), and colorectal carcinoma cell SN were incubated in the presence of anti-IL-3 (2.5 μg/ml) (□) or anti-GM-CSF antibody (2.5 μg/ml) (●). An activity of 100% is defined as the proliferation of M07e in the absence of antibody. Results represent the mean of two experiments.

Fig. 3. Preabsorption of colorectal carcinoma cell SN with anti-GM-CSF antibody. M07e-induced proliferation by rhu GM-CSF (1 ng/ml), rhu IL-3 (10 ng/ml), and colorectal carcinoma cell SN either untreated (○) or preabsorbed with anti-GM-CSF antibody (1 μg/ml) (●).
Acknowledgments

We gratefully acknowledge the excellent assistance of M. Allegrini and P. Dubied in preparing the photographic artwork.

References


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