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Growth factors for human tumor clonogenic cells elaborated by macrophages isolated from human malignant effusions

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Summary. We previously demonstrated that macrophages isolated from human malignant effusions support colony formation of autologous tumor cells in soft agar. We now demonstrate that macrophages (derived from effusions of patients with ovarian, breast, colon, or lung adenocarcinomas) secrete a soluble factor(s) that enhances the ability of a human epithelial tumor cell line (SW-13) to clone in soft agar. Macrophages increased colony growth 5 to 10-fold in a concentration dependent manner, although inhibition of cell growth was observed in the presence of high concentrations of macrophages. We attempted to increase production of tumor colony stimulating factor by exposing macrophages to lipopolysaccharide, concanavalin A, or phytohemagglutinin. Exposure of macrophages to these agents failed to increase their ability to secrete stimulatory factors. Macrophages were cultured for 1 day to 6 weeks in the presence of GCT-CM, a source of granulocyte-macrophage colony stimulating factor and the ability of these cultured macrophages to support colony growth assessed. The ability of macrophages to support colony growth declined gradually with time in culture reaching 50% of control values at 14 days, but remained at this level until 5 weeks of culture. The results of this study indicate the SW-13 cells may provide a quantitative assay for studying monocyte-derived tumor colony stimulating factors.

Introduction

A clonogenic assay for cells derived directly from human tumors is currently being used both to predict chemosensitivities of cells from individual patients and to study the biological control of anchorage-independent growth. Despite an extensive literature describing the clinical utility of this assay [11], the factors controlling the ability of primary human tumor cells to form colonies in agar are poorly understood.

We previously demonstrated that depletion of phagocytic macrophages (MO) from effusions derived from patients with ovarian cancer resulted in a decrease of tumor colonies [7]. In subsequent studies, we demonstrated HLA-DR negative MO enhanced growth of tumor cells in soft agar and that HLA-DR positive cells limited growth [6]. We further found irradiation of adherent accessory cells increased their ability to support tumor colony formation [8]. The effect of irradiation was mediated by T lymphocytes that controlled the ability of MO to produce diffusible tumor colony stimulating factors (TCSFs).

These observations were confirmed by Buick et al. [2] using human malignant effusions derived from patients with adenocarcinomas. Similarly, Welander et al. [15] found that cloning efficiencies of primary human ovarian cells were enhanced by xenogenic macrophages. The growth of many animal tumors is similarly potentiated by MO both in vitro and in vivo [3]. The concept of helper MO is complementary to and consistent with the concept of immunostimulation of neoplastic cell growth.

To further define the ability of MO isolated from human malignant effusions to produce diffusible TCSFs, we examined the ability of MO to stimulate the growth of a tumor cell line SW-13, derived from an adenocarcinoma of the adrenal cortex, that clones poorly in soft agar. This cell line forms colonies in soft agar upon stimulation with growth factors derived from epithelial tissue [4]. The results of the present study indicate MO significantly increased colony growth. SW-13 cells may be useful in studying factors derived from monocytes-macrophages that enhance anchorage-independent growth of human tumor cells.

Materials and methods

Preparation of tumor cell suspensions. Pleural or ascitic fluids (200–4,000 ml) were obtained aseptically in heparinized (10 units/ml) vacuum bottles from patients with histologically proven epithelial neoplasms. The presence of tumor cells in the fluid was verified by a clinical pathologist. Appropriate informed consent was obtained in all cases.

Fluids were passed through sterile gauze, centrifuged at 600 *g* for 10 min, and the cell pellet resuspended in McCoy's 5A medium containing 10% fetal bovine serum (FBS). Cells were washed twice in this medium and counted in a hemocytometer. Viable nucleated cell counts (determined using trypan blue) were routinely more than 90%. Differential counts were performed on slides prepared using a cytocentrifuge and stained by the Papanicolaou and Wright Giemsa methods and for nonspecific esterase reactivity [16].

Isolation of macrophages from effusions. Tumor cell suspensions were incubated overnight at 37 °C in a humidi-

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fied atmosphere of 5% CO_2 in air in 100-mm plastic tissue culture dishes (Falcon 3003) at a concentration of 2×10^6 cells/ml in McCoy's 5A medium containing 10% autologous effusion fluid. Nonadherent cells were removed and the adherent cell layer washed twice with 5 ml of 0.002% EDTA-saline. The adherent cells were then removed using EDTA-trypsin. Viability of the adherent cells was 85% of that observed before overnight culture. In cases where adherent cells were used as a feeder layer, a known number of adherent cells were suspended in the bottom layer of enriched McCoy's 5A media in 0.5% agar.

In the experiments indicated, adherent cells were incubated for an additional 24 h in the presence of 30 μg/ml lipopolysaccharide (LPS, W.S. Typhosa 0901, Difco Detroit, Mich.) 30 μg/ml concanavalin A (Con A, 3× recrystallized, Sigma, St. Louis, Mo.), 1% phytohemagglutinin (PHA, GIBCO, Grand Island Biological, Grand Island, NY), or RPMI 1640 media alone. Cells were washed three times at the end of the incubation period, harvested as described, and replated in the 0.5% agar underlayers.

Long-term cultivation of macrophages. In some cases, adherent cells were incubated for longer times as specified in RPMI 1640 medium containing 10% FBS (Sterile Systems, Logan Utah) and 10% GCT conditioned media (GCT-CM) (GIBCO) as a source of macrophage colony stimulating factor [9]. The medium was changed twice weekly and cells subcultured using EDTA-trypsin when indicated.

Cell culture. The SW-13 cell line (CCL 105), derived from an adenocarcinoma of the human adrenal cortex, was obtained from the American Type Culture Collection (Rockville, Md.). The cells were grown at 37 °C in L-15 media (GIBCO) supplemented with 10% FBS (Sterile Systems). All cell lines were used in the logarithmic growth phase for experiments within 10–15 passages of the original frozen stock.

Soft agar colony assay. Base layers of 0.3 ml of 0.5% agar (Difco) containing enriched McCoy's 5A media [5] were prepared in individual wells of 24-well cluster dishes (Costar Cambridge, Mass.). Macrophages were incorporated into the base layers at the concentrations indicated in the text. An 0.3 ml overlayer of 0.3% agar containing SW-13 cells (5×10^3) was applied. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and colonies of more than 40 cells were counted between 10 and 12 days after plating in a Zeiss inverted phase microscope.

Statistical analysis. The Student's *t*-test was used on paired samples to compare control to experimental groups. Data are expressed as mean ±SE (4 points). Significance was established at $P \leq 0.05$.

Results

Enhancement of SW-13 colony formation in soft agar by tumor-associated MO

Adherent cells from 10 patients with either adenocarcinoma of the ovary (4), colon (2), breast (3) or lung (1) were isolated as described. The tumor type and differential count of adherent cells for each patient sample are presented in Table 1. Both the total cell count and relative proportions of cell types varied among patients. However, in 9 out of 10 cases, inflammatory cells accounted for more than 50% of total nucleated cells. In addition, MO accounted for more than 95% of adherent cells isolated at 16 h. To directly test if MO produced factors that stimulate colony growth of SW-13 cells, MO, in increasing numbers, were added to the 0.5% agar underlayer. SW-13 cells were added at a low density (5×10^3 cells/well) in the 0.3% agar overlayer. SW-13 cells clone poorly in soft agar at this density [4]. The results (Fig. 1) of five experiments using cells from 5 different patients (# 1–5, Table 1) indicated that increasing numbers of tumor-associated MO stimulated, in a dose-dependent manner, the growth of SW-13 cells. However, SW-13 colony growth decreased when greater than 1×10^5 MO/well were plated.

The inhibition of SW-13 colony formation by high concentrations of MO was similar to that observed when autologous effusion-derived MO are used to support the growth of autologous nonadherent tumor cells [2], or when MO are used to stimulate granulocyte-macrophage (GM) colony growth [12]. The inhibition of colony formation by high numbers of monocyte-macrophages has been suggested to be due to MO production of prostaglandin E (PGE_2). Indomethacin, an inhibitor of prostaglandin synthesis, abrogates the PGE_2-induced inhibition of colony growth.

Table 1. Cytological analysis of cells derived from effusions

Patient number	Tumor site	TC	Differential (%)[a]			Meso	% Mac at 16 h[b]
			Mac	Lycte	PMN		
1	Ovary	13	60	27	0	0	100
2	Ovary	44	50	3	0	3	98
3	Ovary	5	86	2	0	7	100
4	Ovary	10	80	9	1	0	100
5	Colon	24	12	60	4	0	100
6	Colon	31	36	31	2	0	100
7	Breast	46	22	15	17	0	95
8	Breast	30	55	10	0	5	95
9	Breast	25	71	6	0	0	100
10	Lung	59	34	7	0	0	95

[a] TC, tumor cells; Mac, macrophages, Lycte, lymphocytes, and Meso, mesothelial cells

[b] Differential counts of both the starting cell suspension and the adherent cell population at 16 h after the start of cultures were performed as described in *Materials and methods* (500 cells/slide; two slides per specimen)

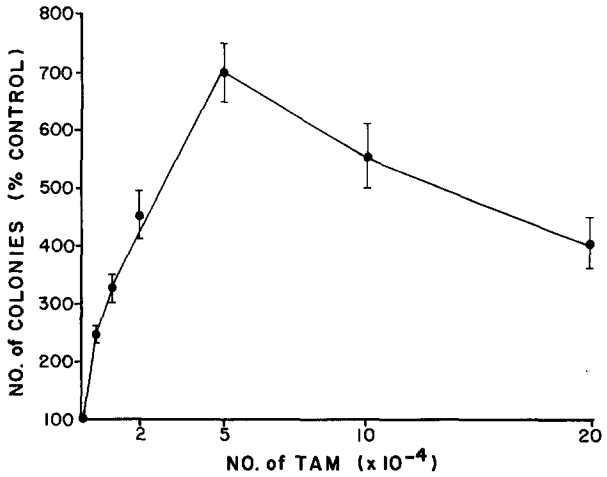

Fig. 1. Stimulation of colony formation of SW-13 cells by macrophages. Human MO (TAM), isolated as described from patients 1–5, Table 1 were plated in increasing concentrations in 0.5% agar underlayer. SW-13 cells (5×10^3) were plated in the upper layer and colonies enumerated at 10 days, 4 wells per point. The results for each patient at every cell density, were normalized to percent of control observed in the presence of SW-13 cells alone. The average increase in growth was calculated for each cell density. Points represents mean ±SE for 5 patients. Control number of colonies was 44±15

Therefore, indomethacin (1.4×10^{-7} M) was added to the 0.5% agar underlayer that contained graded numbers of MO (0, 1, 5, 10, 20, and 50×10^4). The results of three experiments, using cells from 3 patients with ovarian cancer, demonstrated that indomethacin did not reverse the inhibition of colony growth observed in the presence of high concentrations of MO ($P \leq 0.05$). The ability of MO to support colony growth was unaffected at any cell concentration tested by treatment with indomethacin (≤ 0.05) (data not shown).

Effect of mitogens on the ability of MO to stimulate SW-13 colony formation

Although MO produced colony stimulating factors for SW-13 cells constitutively, we attempted to increase production of TCSFs by several activating agents. MO were isolated as described and exposed to either LPS (30 μg/ml), Con A (30 μg/ml), PHA (1% v/v) or RPMI 1640 media and 10% FBS alone for 24 h. At the end of the incubation, cells were washed, harvested in EDTA-trypsin, and added to the 0.5% agar underlayers. The results of seven experiments (using cells from patients 1–5, 7, 8, Table 1) indicated that none of the activating agents tested significantly enhanced the ability of MO to support SW-13 colony formation (Table 2). PHA, LPS, or Con A-containing media alone failed to stimulate SW-13 colony formation (data not shown).

Ability of long-term cultures of MO to support colony formation

MO from 3 patients with adenocarcinoma of the ovary were cultured in the presence of GCT-CM for periods of 1 day to 6 weeks. We have previously demonstrated that MO increase 4 to 6-fold when incubated under these conditions [9] and comprise more than 95% of total cells. The cells divide for 2 weeks after initiation of the cultures, but maintain macrophage morphology, function, and immunological markers for up to 10 weeks. MO were harvested at various times and their ability to support SW-13 colony formation compared to that of freshly isolated MO. Results are presented in Fig. 2. MO from these 3 patients stimulated colony growth 10.2, 8.8, and 7.8-fold respectively with the numbers of colonies observed in the absence of MO 28±15. The ability of MO to support SW-13 colony formation declined steadily during the initial phase of culture, falling to 42% of control values at 14 days. The ability

Table 2. Effect of activating agents on the ability of MO to enhance colony growth

Activating agent	Number of colonies (% control)
None	800± 75[a]
PHA	875± 50
LPS	813±100
Con A	900± 50

MO from 7 patients (1–5, 7, 8 Table 1) were isolated as described and exposed to either media alone, PHA, Con A, or LPS-containing media for 18 h at the concentrations indicated in the text. Controls represent cells incubated without MO. The cells were then harvested and suspended in the bottom layer of 0.5% agar at 1×10^5 cells/well. The SW-13 cells (5×10^3 cells/well) were placed in the 0.3% agar overlayer (4 wells, group). Results were normalized to percent control SW-13 growth in the absence of MO and the means calculated for all patients for each activating agent. The number of colonies observed in the absence of MO was 28±12

[a] Mean±SEM

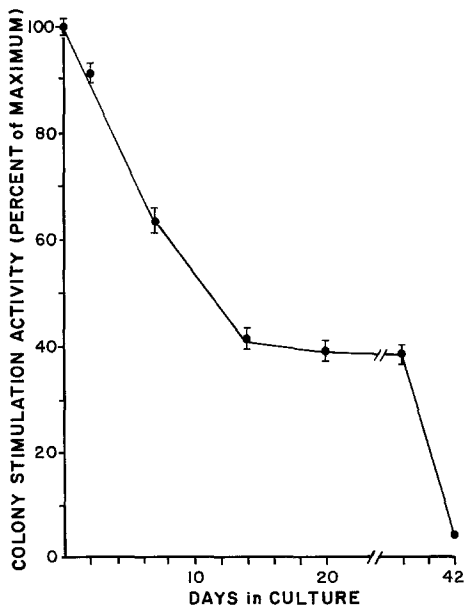

Fig. 2. Effect of cultured MO on growth of SW-13 cells. MO, isolated as described from 3 patients (3–5, Table 1) with ovarian cancer, were cultured in the presence of GCT-CM for 1 to 42 days. The ability of cells harvested at different times to support colony growth of SW-13 cells was assessed. The maximum number of colonies induced by MO harvested at different time points was assessed for each patients' culture. This was designated as 100% activity for that patient. The number of colonies induced by MO harvested at different times was then calculated as percent of maximum activity. Activities of MO harvested a different times, from the 3 patients were then calculated

to support colony formation remained at this lowered level until 35 days of culture, when it abruptly declined. No TCSF was observed 42 days after the initiation of culture. GCT-CM alone (10% v/v) did not affect SW-13 colony formation (data not shown).

Discussion

The results of this study confirm and extend previous findings indicating MO derived from human malignant effusions secrete diffusible factors that stimulate anchorage-independent growth of human tumor cells. In this case, allogeneic MO were able to stimulate the growth of a human epithelial tumor cell line, SW-13, indicating stimulation of colony growth was not HLA-restricted. This cell line may serve as an easily available, readily standardized bioassay for purification of MO-produced TCSF.

While MO enhanced colony growth, high concentrations of MO proved inhibitory. This is similar to the inhibition of GM and B lymphocyte [12] colony growth by high concentrations of monocytes. In those cases, the inhibition of colony growth was due to monocyte secretion of PGE_2. Indomethacin, an inhibitor of PGE_2 synthesis, did not enhance the ability of MO to support SW-13 colony formation. Thus it is likely that other factors, not related to PGE_2, inhibited SW-13 colony growth. Sredni et al. [14] have demonstrated MO produce a low mol. wt. factor, distinct from PGE_2, that inhibits T lymphocyte colony formation.

We found that MO produced TCSFs constitutively and that secretion could not be enhanced by exposure of MO to LPS, PHA, or Con A. This is in contrast to our previous work indicating secretion of TCSFs by peripheral blood monocytes was enhanced by exposure to LPS [10]. These data suggest the MO may have been activated in vivo. This is likely as MO were isolated from effusions with high numbers of inflammatory cells. In a somewhat analogous study, Bitterman et al. [1] showed alveolar macrophages from normal individuals did not release detectable amounts of a fibroblast growth factor unless activated. In contrast, alveolar macrophages from patients with interstitial lung disorders associated with inflammation of alveolar structures produced fibroblast growth factor without activation.

MO were expanded in culture and maintained for 6 weeks in the presence of GCT-CM. The activity of TCSFs secreted by these cells steadily declined with time in culture, but MO cultured for 5 weeks still enhanced colony formation 4-fold. At this point, secretion of TCSFs declined precipitously and no activity could be detected at 6 weeks. This limits the usefulness of long-term cultures of effusion-derived MO as a source of TCSF activity. This sudden decline is probably not due to lymphokine-induced differentiation as morphological and immunological characteristics of the cultured macrophages do not change after the 3rd week of culture [9]. Cessation of cell division alone cannot account for the loss of TCSF secretion as cells stop dividing after 2 weeks of culture, a time when colony stimulating factor levels remain high. Cell viability is also high (85%) at 5 weeks. Therefore, the sudden decrease in TCSF levels in older cultures remains unexplained.

Despite the differences in tumor histology, we observed MO stimulated SW-13 colony growth in a dose-dependent fashion in all cases tested. We attribute this similarity in MO behavior to the fact that these cells were derived from a highly selected group of patients. First, only effusions containing a large number of MO initially could be used in the isolation studies. Second, only those cultures in which more than 95% of adherent cells were MO were used. Third, the overall cellular composition of the effusion fluids was similar despite differences in tumor of origin, as the majority of cells were MO or lymphocytes. The use of a highly select group of patients' cells may have contributed to the uniformity of results obtained.

Studies seeking to define the effects of MO on anchorage-independent growth of human epithelial tumor cells have been hampered by the lack of appropriate, reproducible model systems. Cloning efficiencies of primary human tumor cells in soft agar are low, and growth rates highly variable [11]. We chose the SW-13 cell line as a quantitative assay for TCSFs as Halper and Moses [4] demonstrated epithelial derived polypeptides significantly stimulated cloning efficiencies of these cells in soft agar. Both acid-ethanol extracts and conditioned media from three epithelial tumor cell lines of different histologies stimulated cell growth. Extracts from the majority of freshly excised human carcinomas also stimulated cell growth. Conditioned media derived from confluent SW-13 monolayers enhanced cloning efficiencies of SW-13 cells plated at low densities. These authors, however, did not report if either peripheral blood monocytes, or MO derived from the human tumors examined affected SW-13 colony growth. The ability of SW-13 cells to clone reproducibly in soft agar and to respond to colony stimulating factors may prove useful in purification of TCSF and in clarifying its mechanism of action.

The existence of a MO-derived factor that stimulates the growth of human tumor epithelial cells adds to a growing body of evidence that indicates accessory cells secrete factors that may play a role in maintaining the transformed phenotype. Macrophage-derived growth factors may be of special importance as epithelial proliferation in the absence of inflammation does not promote tumor progression in the two-step model of carcinogenesis [13]. These findings suggest humoral factors secreted by MO may enhance tumor growth in vitro.

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