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INITIAL DESCRIPTION OF A TUMOR ENHANCING ACTIVITY PRODUCED BY MURINE SPLENOCYTES

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Summary: We have shown that certain murine tumors grow more slowly and spread less readily in immune deficient animals. We have also demonstrated that immunologic factors explain certain aspects of this difference. In the work presented we demonstrate that a subpopulation of splenocytes produce a factor(s) that enhances tumor cell proliferation *in vitro*. We also describe an *in vitro* model to determine the level of tumor stimulatory activity. We found that the tumor cell growth-enhancing activity (TEA) is heat stable but sensitive to trypsin digestion, low pH and β-mercaptoethanol. TEA production is found to be insensitive to mitogen stimulation such as concanavalin A, lipopolysaccharide, and phytohemagglutinin. Among the known growth factors and interleukins we have tested (interleukin 1-7, basic FGF, EGF, TGF-β PDGF, GM-CSF, and MCSF), none appear to account for TEA activity.

In clinical medicine it has been commonly observed that cancers, although more frequent in occurrence (1), are characterized by slower rate of growth and less frequent metastases in older patients (2-4). In murine models, we have examined a wide range of host factors that might account for this age effect (5-10). We believe that age-associated changes in immune function explain the observed changes in tumor growth. Lymphocytes and monocytes produce a wide array of factors that are important for wound healing, angiogenesis, lymphocyte activation, etc., and these factors may provide a growth stimulus for tumor cells. An age-associated reduction in the production of such factors might thereby account for reduced tumor cell proliferation.

In the work reported herein, we present evidence that splenic cells produce a factor that stimulates murine B16 melanoma cells to proliferate. Preliminary characteristics of this factor indicate that it is a protein produced by adherent cells and is most likely distinct from previously described monokines. Results of our characterization of this factor are the subject of this report.

MATERIALS AND METHODS

Experimental animals

Young (2-4 months) male C57 Bl/6 (B6) mice were purchased from Harlan Sprague Dawley at six weeks of age and maintained in the AAALAC-approved animal care facility at the Medical Sciences Center, University of Wisconsin. These mice (used in the experiments in which spleen cell supernatants were prepared) were maintained for at least two weeks prior to experimentation under isothermal condition (22°C), 12 hour photoperiods and *ad libitum* feeding of Purina laboratory chow.

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Maintenance of cloned cell lines

B16 melanoma (F10) was originally supplied by the National Cancer Institute's tumor repository, and has been maintained in our laboratory in culture or frozen for more than ten years. B16 melanoma (F10) was grown as a monolayer in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, L-glutamine, Hepes buffer and antibiotics. When confluent (usually three days), the cells were detached from the flask by incubating with trypsin-EDTA and reseeded in fresh media.

The cytotoxic T-cell line (CTLL-2) was grown in suspension in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, Hepes, antibiotics and recombinant IL-2. These cells were passed every three days by transferring to fresh media. The helper T-cell line (HT2) was maintained in a similar fashion.

The macrophage cell line (Raw 264.7) was grown as a monolayer in supplemented RPMI media as described above. The cells were transferred every two days using a rubber cell scraper to detach cells from the bottom of the flask. An additional macrophage cell line (αChyJ2), known to constitutively secrete IL-6 (11) was also used in certain experiments. The cells were passaged using trypsin-EDTA and cultured in RPMI 1640 containing 10% fetal calf serum as above.

Spleen cell cultures

Spleens were removed from healthy, immune competent young (2-4 months) mice and single-cell suspensions were prepared. Cells were teased from the spleen using syringe and needle into a petri-dish containing media. The cells were washed twice and viability was determined by trypan-blue exclusion. Cells were > 95% viable by this technique. Cells ($3x10^6$/ml) were incubated in serum-free RPMI 1640 at 37°C, 5% CO_2 for 24 hours and the culture supernatant (SCS) was aspirated, aliquoted and frozen at -70°C for later analysis.

Preparation of spleen cell subpopulations

Prepared spleen cells were incubated with rat anti-mouse monoclonal antibodies (anti-L3T4, anti-Thyi.2 and anti-Lyt2, [Becton Dickinson]) at 1 mg/ml, for 30 minutes at 4°C with gentle agitation. The cells were centrifuged at 200g for 10 minutes and resuspended in phosphate buffered saline (PBS) containing 1% fetal calf serum. Antibody coated Dynabeads M-450 (7.5 ml/10^6 cells) were incubated at 4°C for 5 minutes. The target cells rosetted with specific antibody were isolated by applying a magnet to the outer wall of the test tube for 2-3 minutes (12).

Tumor cell proliferation assay

B16 melanoma cells were grown to confluency, briefly exposed to trypsin solution, washed twice and suspended at a density of $1x10^5$ cells/ml. The cells were then seeded into 96-well tissue culture plates at 2500 cells per well (25 µl) in the presence of dithiotriotol-reduced FBS (SH-FBS). Undiluted SCS, media or other sample were added to the B16 so that the final volume was 75 µl. The plates were incubated at 37°C, 5% CO_2 for 72 hours. For B16 melanoma standard, 0 to 50,000 cells were plated at 69 hours and allowed to equilibrate for three hours. Cell number in experimental and standard wells was determined by the MTT assay (13,14). Briefly, 10 µl of stock MTT (3-(4,5-dimethylthiozol-2yl)-2,5-diphenyl tetrazolium bromide) solution at 5mg/ml was added per 100 µl medium and plates were incubate at 37°C, 5% CO_2 for an additional four hours. Media was carefully aspirated and 100 µl of dimethyl sulfoxide (DMSO) was added per well. Mitochondrial dehydrogenases from viable cells reduce tetrazolium salt to purple formazan crystals. The formazan crystals were dissolved in DMSO and the color intensity was measured on a Dynatech ELISA plate reader using a wavelength of 570 nm, a reference wavelength of 630 nm and calibration of 1.99.

RESULTS

Preliminary characterization of a spleen cell factor that enhances B16 melanoma growth *in vitro*

Spleen cells from healthy, immune competent, young mice were cultured in medium for 24 hours without added serum or other mitogens. The spleen cell supernatant (SCS) was found to stimulate B16 melanoma cell growth in culture. Generally, B16 melanoma cells do not proliferate

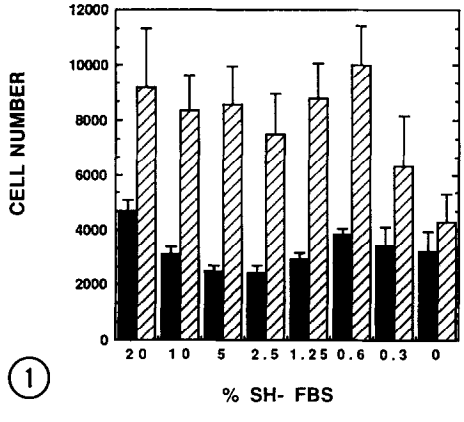
in the absence of FBS or reduced FBS (SH-FBS) in which the protein growth factors have been inactivated (15). However, the addition of SCS to the cultures enhanced tumor cell proliferation (Figure 1) in a dose-dependent manner (Figure 2) and the activity declined after 24 hours in culture (Figure 3). In additional experiments we found that spleen cell supernatants from rats and from rhesus monkeys, prepared in a manner analagous to that described for the murine spleen cells, produced comparable amounts of TEA (data not shown).

Cellular source of growth promoting activity

Spleen cell populations were partially purified using adherence to plastic and magnetic bead-conjugated monoclonal antibody separation techniques. Serum free supernatants from these partially purified populations were tested for B16 growth-promoting activity. The data indicate that the most likely cell source for TEA are those of the monocyte/macrophage lineage (Table 1). However, it is likely that more than one cell subset influences TEA production. This is perhaps reflected by the modest TEA activity from purified murine macrophage, T and B-cell clones as depicted in Figure 4.

Preliminary biochemical evaluation (Table 2)

We have also begun to characterize the biochemical nature of TEA. We found that the activity persists despite incubation at 80°C for 30 minutes. Activity, however, was lost when incubated at pH 2 for two hours or in the presence of β-mercaptoethanol. TEA production was not influenced by mitogen stimulation (ConA, LPS and PHA).



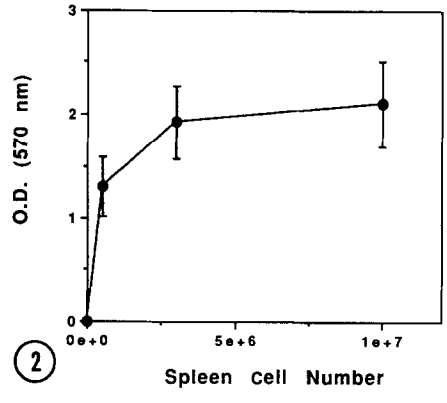


Figure 1.
Tumor enhancement activity.
Spleen cell supernatants (SCS) were prepared as described in Methods. Unconcentrated SCS, when added to media with varying amounts of reduced fetal bovine serum (SH-FBS), resulted in enhanced tumor cell growth as detected in the MTT assay.

Figure 2.
Dose-response in TEA production.
Spleen cells were plated at different densities and the serum free conditioned medium was collected and tested for TEA activity in the MTT assay. As can be seen, TEA activity increases with increasing number of cultured spleen cells.

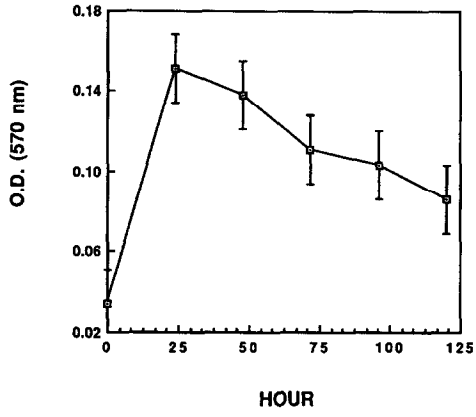


Figure 3.
TEA production in spleen cultures of varying time.
Spleen cells were plated at $3x10^6$ cell/ml in serum free medium and the supernatants were collected and tested for TEA activity. The data demonstrate that optimal TEA production is at 24 hours of culture.

Test of other known growth factors for TEA activity upon B16 melanoma

In order to rule out the possibility that TEA is a single already characterized growth factor, we attempted to match the stimulatory effect of SCS upon B16 melanoma with other known growth factors or neutralize the tumor growth promoting activity by antibodies against them. The growth factors (basic fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, transforming growth factor-β, granulocyte/macrophage colony stimulating factor, macrophage colony stimulating factor and Interleukins 1 to 7) were added in a dose-response manner to B16 melanoma cell cultures and cell proliferation

TABLE 1

BIOCHEMICAL CHARACTERISTICS

Variable	Tumor Enhancing Activity
Heat	Activity persists despite heating SCS to 80°C for 30 minutes.
pH	Loss of activity if pH of SCS temporarily (2 hrs.) reduced to 2.5.
Trypsin	Results in loss of the majority of activity.
Reduction	SCS reduced by β-mercapto-ethanol results in near complete loss of activity.
Size	Greater than 50 Kd.
Mitogen stimulation	TEA production is insensitive to mitogen stimulation, such as Con A, LPS and PHA

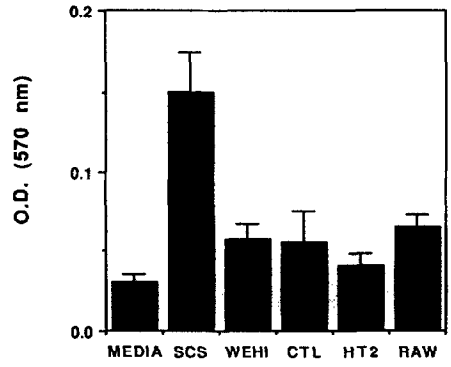


Figure 4.
Serum free conditioned medium prepared from several cloned cell lines was tested for tumor enhancing activity. Under conditions analagous to primary spleen cell culture, supernatants did not produce comparable tumor cell stimulatory effect in vitro.

was measured by MTT assay. Doses of the various cytokines were chosen based upon the published optimal range for each factor. In separate experiments, available monoclonal antibodies were incubated with SCS and then added to B16 cell cultures to assess for neutralization of TEA activity. We were not able to identify similar activity among the cytokines we have tested, and the antibodies did not neutralize the tumor stimulatory activity of SCS. The results of these experiments are listed in Table 3.

TABLE 2

CELL TYPES THAT PRODUCE TUMOR ENHANCING ACTIVITY

Source of supernatant	Results	Conclusion
Spleen adherent cells	Adherent cell supernatants stimulate B16 growth	Possible source of the tumor-enhancing activity is produced by adherent cells
Spleen non-adherent cells	Non-adherent cell supernatants stimulate B16 growth, but to a lesser extent	Non-adherent cells contribute to TEA production
Spleen T-cells (Thy1.2+)	No effect	Suggests T-cells alone do not produce tumor-enhancing activity
Spleen non-T-cells (Thy1.2-)	Stimulates B16 growth to a lesser degree	Supports macrophage as major source
Spleen Lyt 2+ cells	No effect	T cytotoxic/suppressor cells do not produce tumor-enhancing activity
Spleen L3T4+ cells	Activity present, but not as active as SCS	T helper cells may augment macrophage production of tumor-enhancing activity

TABLE 3

OTHER CYTOKINES AND TEA

Cytokines Tested	Experimental Variables	Results
IL-1	anti-IL-1(a and b) rmu IL-1	no effect
IL-2	rhu IL-2	no effect
IL-3	rmu IL-3	no effect
IL-4	anti-IL-4 rmu IL-4	no effect
IL-5	rmu IL-5	no effect
IL-6	anti-IL-6 rmu IL-6	no effect
IL-7	rhu IL-7	no effect
TNF	anti-TNF	no effect
EGF	purified porcine EGF/anti-EGF R	EGF stimulates B16 at 100ng/ml
FGF	anti-FGF	no effect
TGF-β	purified porcine TGF-β	no effect
PDGF	purified PDGF	no effect
PF-4	purified hu PF-4	no effect
GM-CSF	rmu GM-CSF	no effect
M-CSF	rmu M-CSF	no effect

DISCUSSION

One possible explanation for the observed reduced tumor growth in older animals and humans is that host tissues provide less support for tumor cell proliferation and growth with advanced age. That cells of the immune system are involved is supported by prior observations that many tumors grow less well in immune deficient animals (4,5). In the work which we have presented we provide evidence that splenic mononuclear cells provide a stimulus for tumor cell proliferation. This tumor enhancing activity appears not to result from the solitary activity of a number of previously characterized cytokines. Furthermore, the activity was heat stable, pH labile and sensitive to trypsin and β-mercaptoethanol. It was produced to a greater extent by adherent cells, and virtually not at all by T cells or T cell subsets. Interestingly, when T helper cells were cultured with adherent cells, maximal production was realized.

Other investigators have made similar observations from quite different systems. Sandru and colleagues (17) have reported a tumor stimulating factor produced by peripheral blood mononuclear cells from humans. In their system, a panel of responding tumor cell lines were tested and the stimulatory factor was active in some, but not all. Preliminary characterization has indicated that this factor, like the one which we have described, is the product of cells of the monocyte/macrophage lineage.

Hamburger and colleagues also have described a factor that is produced by macrophages that supports tumor cell growth in clonal cell assays (18,19). Like TEA and the factor studied by Sandru (17), this enhancing activity has been incompletely characterized but is sensitive to pH, heat and trypsin digestion.

It is apparent that normal cells can produce factors that may enhance tumor cell growth. These cytokines are probably involved in the complex interactions involved in wound healing or other host defenses. Perhaps the decline in the production of such factors with advancing age accounts for the observed defects in wound healing (21) as well as the reduced tumor growth rates in older cancer patients. Once we have characterized TEA more completely, we will develop strategies to address the latter hypothesis.

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