Analysis of Human Ascites Effect on Clonogenic Growth of Human Tumor Cell Lines and NRK-49F Cells in Soft Agar

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Abstract. We studied the factors that control the capacity of tumor cells to grow in soft agar. And, we analyzed the effect of cell-free ascites (CFA) obtained from ovarian cancer patients in combination with various media on the cloning efficiency of H-134 and OVCAR-3^{nu} ovarian carcinoma cell lines and the WiDr colon carcinoma cell line.

Seven batches of CFA consistently enhanced the soft agar growth of tumor cells more efficiently than tested sera. The addition of charcoal-treated bovine serum albumin (BSA) lowered the amount of CFA required for optimal tumor cell growth. As little as 1.25 ng of epidermal growth factor (EGF)/ml further improved OVCAR-3^{nu} soft agar growth in combination with all of the amounts of CFA tested. Thus, neither BSA nor EGF seems to account for the colony-stimulating effect of ascites on tumor cells.

Four batches of CFA were tested for stimulating soft agar growth of normal rat kidney (NRK-49F) fibroblasts; all induced colonies of different morphologies. This effect was potentiated by EGF, which suggests the presence of several transforming growth factor-like activities in CFA.

The results show that differences in cloning efficiency of tumor cells of one or two orders of magnitude can be found between standard (anchorage-dependent growth-supporting) media and media optimized for soft agar growth, such as CFA in the presence of EGF. This paper will discuss the similarity in effects of CFA on various tumor cells and NRK cells, and possible implications of the stimulatory effects of CFA.

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Introduction

The ability of tumor cells to form progressively growing colonies while suspended in semisolid soft agar or methylcellulose-containing medium is generally considered to be related to the transformed state of the cells [1]. Although this correlation may not be steadfast, cell growth in agar has been widely used as an indicator of neoplastic transformation in selective culture of human tumor stem cells from effusions and tumor biopsies from patients [2, 3].

Success in the culture of human primary tumor cells in agar has remained modest regarding the percentage of tumor specimens that grow and cloning efficiency (CE, generally below 0.1%). Thus, the routine prospective use of agar cultures for in vitro testing for drug sensitivity has not been realized [4, 5]. Low CE may either reflect the low percentage of truly clonogenic cells in human tumors or inadequate culture methods and conditions [6]. In recent years, attempts have been made to determine the physical [7, 8], nutritional and hormonal factors [9-11] that influence the growth of tumor cells in semisolid medium. Also, it has been shown that soluble substances from adherent phagocytic cells present in malignant effusions [12] or cell-free ascites (CFA) [13] improve the CE of primary ovarian carcinoma cells.

Parallel to developments in culture techniques for primary human tumor cells, there has been an intensive search for growth factors which may be responsible for autocrine growth regulation of tumor cells [14]. Important classes of polypeptide growth factors, operationally defined as transforming growth factors (TGFs) on the basis of their capacity to induce the loss of density-dependent growth inhibition in monolayers and to induce soft agar colony growth of immortalized indicator cells, such as normal rat kidney (NRK) cells, have been isolated from a variety of non-neoplastic and neoplastic sources [15]. The presence of TGFs in conditioned medium (CM) from chemically or virally transformed rodent cells, various mouse, rat and human tumor cell lines [16] and human tumors [17] may suggest that these polypeptides enhance the ability of tumor cells to grow in soft agar. However, correlations between TGF production and the CE of human tumor cells have not been observed until now [18, 19].

Furthermore, distinct polypeptides, one stimulatory for NRK soft agar growth (TGF) and one inhibitory for the soft agar growth of human colon carcinoma cells (TIF), have been isolated from rat malignant ascites [20]. The presence of relatively large amounts of TGF in rat ascites [20] and our finding that human ascites improves the CE of human tumor cells [13] warrants further work in this field.

To expand our knowledge of determinants of and requirements for human tumor cell growth in soft agar, we performed experiments on the modulatory effect

of human CFA, different serum batches, albumin and EGF on the CE of two ovarian carcinoma cell lines and one colon carcinoma cell line with different growth characteristics in soft agar.

Materials and Methods

Chemicals and Media

Cell culture media and reagents, including Dulbecco's modified Eagle's medium, minimal essential medium (MEM), McCoys 5A and CMRL 1066 medium, fetal calf serum (FCS), newborn calf serum (NBCS), horse serum, Hank's balanced salt solution (HBSS), tryptic soy broth, L-ascorbic acid, Na-pyruvate, L-serine, asparagine, glutamine and insulin, agar, penicillin, streptomycin and trypsin were from GIBCO Biocult Ltd., Paisley, Scotland. EGF, culture grade, was purchased from Collaborative Research Inc., Waltham, MA. Bovine serum albumin (charcoal-treated/fatty acid-free, BSA-ch/FAF) was purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were of analytical grade.

Enriched McCoys (E-McCoys) medium consisted of McCoys 5A supplemented with Na-pyruvate (0.022%), L-serine (0.042 mg/ml) and penicillin/streptomycin (200 U/ml). Double enriched (DE)-McCoys contained, in addition to the above, the following components added immediately before the experiment: 10% of a mixture of horse serum/FCS (1:2), 20% tryptic soy broth, asparagine (0.1 mg/ml) and glutamine (1.4 mM).

DE-CMRL consisted of CMRL 1066 plus freshly added insulin (2 U/ml), L-ascorbic acid (0.3 mM), penicillin/streptomycin (200 U/ml), 10% horse serum, asparagine (0.1 mg/ml) and glutamine (3.25 mM). All sera were stored at -20°C and heat-inactivated before use (30 min, 56°C).

The combination DE-McCoys/DE-CMRL is referred to as Hamburger-Salmon medium.

Ascites Collection

Ascites fluids were collected in sterile plastic containers (containing 10 U/ml heparin) from untreated patients with ovarian malignancies. Cells were removed as quickly as possible by centrifugation for 20 min at 400 g (4°C) and CFA was stored at -20°C . Batch 463, containing 0.03% blood contamination based on red blood cell count before centrifugation, was partly centrifuged a second time for 15 min at 4,000 g (4°C) to remove potential residual blood cells. No differences in experimental results were seen between the two treatments. In some experiments, heat-inactivated CFA (30 min, 56°C) was used, which gave essentially the same stimulating activity as untreated CFA. In preliminary experiments we established that heparin had no growth stimulatory or inhibitory effects by using a batch of CFA prepared without heparin and adding heparin to culture dishes. Protein and albumin concentrations of the CFA batches used were 43.8 ± 3.5 mg/ml and 25.5 ± 1.4 mg/ml respectively (mean $\pm$ SE, $N = 4$).

Cells and Culture Techniques

H-134 cell line was established from malignant ascites collected in heparin (10 U/ml ascites) from a 62-year-old patient with serous papillary adenocarcinoma of the ovary. The

cell line is tumorigenic, forming undifferentiated tumors when cells are injected s.c. into nude mice. Karyotyping was performed after 45 passages by R.M. Slater and A.W.M. Nieuwint (Dept. of Human Genetics, University of Amsterdam). The analysis of thirty banded metaphases revealed a hypotriploid chromosome number (modal number was 64), with 48% of chromosomes showing abnormalities of which 13% were not identifiable. HLA-typing of these cells by standard microlymphocytotoxicity assay (L.P. de Waal, Dept. of Immunohematology, Netherlands Red Cross Laboratory, Amsterdam) revealed the expression of A28, AW19, BW21 and B5 antigens. OVCAR-3^{nu} cells were obtained from Dr. R.F. Ozols (National Cancer Institute, Bethesda, MD). WiDr human colon carcinoma cells were from the American Type Culture Collection (ATCC CCL 218; Rockville, MD) and NRK fibroblasts clone 49F, originally established by Drs. J.E. DeLarco and G.J. Todaro, were obtained from Dr. E.E.J. van Zoelen (Hubrecht Laboratory, Utrecht, The Netherlands). Cell lines were free of mycoplasma at initiation of the experiments as tested with Hoechst stain 33258. Cells were cultured routinely in bicarbonate buffered Dulbecco's modified Eagle's medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and 10% FCS, except for NRK cells which were grown in medium containing 10% NBCS. Cells were subcultured using trypsin (0.025%, w/v) and EDTA (0.05%, w/v) in CA²⁺- and Mg²⁺-free HBSS.

Soft Agar Growth Assays

Three human tumor cell lines were used to define the colony-inducing activity of CFA obtained from ovarian cancer patients. The cell lines were selected based on their different CE in soft agar. WiDr has a CE of about 10% according to specifications from the ATCC, and OVCAR AR-3^{nu} was reported to have a low CE of <1% [21]. H-134 has a high CE of 50%.

Exponentially growing, freshly trypsinized single cells from tumor lines or NRK cells (viability $\geq 98\%$ by trypan blue exclusion) were suspended in experimental medium mixed with 0.1 volume of 3% (w/v) agar and seeded in 35 mm Petri dishes (Falcon Labware Division, Oxnard, CA) in a volume of 1 ml on freshly prepared 1 ml underlayers containing 0.5% agar.

Cells used for soft agar growth assays were from passage 50-70 for H-134, 45-62 for OVCAR-3^{nu}, 3-15 after reception for WiDr and 15-21 for NRK. Cells in agar cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cultures were counted unfixed and unstained under an inverted microscope. Colonies $\geq 62 \mu\text{m}$ were scored at indicated days by examining 10% of the total dish area or more when colony count was less than 100. All incubations were done in triplicate. All experiments were repeated separately at least twice. Data are expressed as numbers of colonies $\geq 62 \mu\text{m}$ and divided by the number of inoculated cells times 100% (CE). The CE of individual experiments may vary, even with rigorous control of experimental conditions, but effects of various additions were reproducible and similar for the three cell lines.

In a series of experiments Hamburger-Salmon medium was compared with MEM as the basal medium. In these experiments three different batches of horse serum all proved to be inferior to three different batches of FCS. It was noticed that the use of E-McCoys underlayer medium resulted in slightly higher CE than MEM + 10% FCS only for WiDr (data not shown). Therefore, in most subsequent experiments MEM was used as the basal medium.

Results

Effects of CFA on Soft Agar Growth of Human Tumor Cells

H-134 cells showed a cell concentration-dependent CE in Hamburger-Salmon medium or in MEM + 10% FCS (Fig. 1). However, no large colonies were seen at this timepoint (day 8). In preliminary experiments it was established that the addition of 50% CFA of all batches to the agar upperlayer was optimal for all four CFA batches. With the addition of 50% CFA to the upperlayer, CE was 50% and colony size was about 100 μm at all cell concentrations, indicating that maximal colony size was reached earlier with CFA than with Hamburger-Salmon medium [13].

The effects of increasing concentrations of four batches of CFA on OVCAR-3^{nu} and WiDr colony formation were compared with three batches of FCS. Figure 2 illustrates a representative experiment showing the superior effect of CFA compared with FCS. FCS effects were similar to the effects of pooled human AB serum, which was superior to the effects of NBCS (not shown). The colony size was larger with CFA than with FCS (while colony density was the same), indicating a quicker cell proliferation. The addition of EGF further improved the CE of the tumor cell lines. This is shown for OVCAR-3^{nu} cells (Fig. 2), which had the most obvious response to EGF. Dose-response experiments showed that 2.5 ng EGF/dish had a maximal effect at all CFA concentrations. EGF caused the growth of much larger colonies of H-134 and WiDr of a looser morphology, which nevertheless consisted of more cells than colonies grown without additional EGF.

Effects of BSA on Soft Agar Growth of Human Tumor Cells

Since we used batches of malignant CFA which contained a considerable amount of serum albumin and since beneficial effects of serum albumin on colony size and the CE of tumor cells in suspension culture have been described [22], the effect of a BSA supplement (3, 6, 15 or 30 mg/ml agar upperlayer) to the cultures was investigated. Two different batches of BSA fraction V had no effect on the CE of H-134; one of these batches slightly stimulated WiDr and OVCAR-3^{nu} growth (not shown). Two batches of BSA which were charcoal-treated and dialyzed (BSA-ch) had a large colony-stimulating effect on the tumor cell lines. The effect of 15 mg BSA-ch, which was optimal, is shown in Table I for WiDr and OVCAR-3^{nu}. One batch of BSA-FAF had effects similar to BSA-ch (Table I).

Because of reports that BSA may serve as a radical scavenger in cell culture [7], we performed some experiments to establish the effect of catalase (up to 40

Fig. 1. Soft agar growth of H-134 cells. Single cells were plated in 35 mm dishes in basal medium (○) and basal medium + 50% CFA (□). Underlayer medium was MEM + 10% FCS. Colonies $\geq 62 \mu\text{m}$ were counted at day 8. Results are mean $\pm$ SE of three separate experiments.

Fig. 2. Soft agar growth of OVCAR-3^{nu} cells. Single cells (30,000) were plated in 35 mm dishes in MEM containing different percentages of FCS (○, ●) or CFA (△, ▲) with (▲, ●) or without (△, ○) 10 ng EGF/dish. Underlayer medium was MEM + 10% FCS. Colonies $\geq 62 \mu\text{m}$ were counted at day 14. Results are mean $\pm$ SE of two (FCS) or four (CFA) separate experiments.

Table I. Effect of CFA and BSA on soft agar growth of WiDr and OVCAR-3^{nu} cells

Upperlayer medium		CE (%)	
		WiDr	OVCAR-3 ^{nu}
MEM + 10% FCS	—	0.7 ± 0.3 ^a	—
"	+ 15 mg BSA-ch	6.7 ± 3.2	—
"	+ 15 mg BSA-FAF	4.0 ± 2.1	—
MEM + 10% CFA	—	4.8 ± 3.0	—
"	+ 15 mg BSA-ch	18.1 ± 4.9	—
"	+ 15 mg BSA-FAF	14.3 ± 5.5	—
MEM + 25% CFA	—	11.3 ± 4.3	2.8 ± 0.2
"	+ 15 mg BSA-ch	21.9 ± 4.4	4.9 ± 0.6
"	+ 15 mg BSA-FAF	21.3 ± 2.6	—

Underlayer medium was E-McCoys + 10% FCS (WiDr) or MEM + 10% FCS (OVCAR-3^{nu}).

Upperlayer medium for OVCAR-3^{nu} contained 5 ng EGF. WiDr cells were seeded at 4,000 cells/dish and colonies ≥ 62 μm counted at day 7-10. OVCAR-3^{nu} cells were seeded at 30,000 cells/dish and colonies ≥ 62 μm counted at day 14.

^aMean ± SE of three separate experiments.

μg/ml) and superoxide dismutase (up to 300 μg/ml). Both enzymes showed minor effects on colony size and CE when used in excessive amounts, but could not induce colony growth in otherwise non-growing cultures (not shown).

Effects of CFA on Soft Agar Growth of NRK Cells

As a screen for the presence of transforming activity in CFA, four CFA batches were tested for soft agar growth-stimulating activity of NRK-49F fibroblasts. The stimulating effect of CFA on NRK colony growth is shown in Figure 3. All four batches had similar potency in stimulating NRK colony growth. In a typical experiment, NRK colony diameter was 80-150 μm with 50% CFA added (after seven days' growth) and up to 250-300 μm with additional EGF supplement. Colonies had a round, compact morphology and a varying percentage of loose, dispersed and sometimes branched morphology with individual cells clearly discernable (up to 50%). The latter colonies appeared in the absence, as well as the presence, of additional EGF. Figure 3 shows that 2.5 ng EGF/dish (= 1.25 ng/ml) had an optimal potentiating effect at all CFA concentrations.

The effect of ascites 463 was also compared with the effect of serum obtained from the same patient one h after ascites collection. The addition of 1% serum produced a 5.0% (3.0-7.0) CE (without EGF) and an 18.0% (17.9-18.1) CE when

Fig. 3. Soft agar growth of NRK cells. Single cells (2,000) were plated in 35 mm dishes in MEM + 10% NBCS containing different percentages of CFA without EGF (○) or with 2.5 ng (□) or 5 ng (△) EGF/dish. Underlayer medium was MEM + 10% NBCS. Colonies $\geq 62 \mu\text{m}$ were counted at day 7. Results are mean $\pm$ SE of two separate experiments.

5 ng EGF was included. The addition of 0.1% serum, which is still 10 times the amount of blood contamination present in 50% CFA 463, had no effect. Therefore, the stimulating activity present in CFA cannot be explained by the presence of growth factors in CFA from potential residual blood-borne platelets.

Discussion

During recent years there has been increased experimentation with the culture of human tumor cells in soft agar. These studies, however, were almost exclusively directed at in vitro testing of the sensitivity of tumor cells to drugs to improve cancer therapy. Only in recent reviews has the direction been changed toward enhancing our knowledge of the biology of plated tumor cells [6, 23].

We have reported the effect of malignant ascites from ovarian cancer patients on tumor cell growth [13]. This tissue fluid could be regarded as an in vivo medium conditioned by the presence of cancer cells and, therefore, as a potential source of polypeptide TGFs secreted by these cells. The present study was designed to compare the effects of a series of media, including CFA, and medium supplements, such as BSA and EGF, on the soft agar growth of a number of tumor cell lines

and on NRK cells as indicators for the presence of TGFs in human CFA [24]. Whether the results obtained for established tumor cell lines apply to the cloning of primary tumor cells has to be proven, but parallel effects of various medium constituents on primary tumor cells and tumor cell lines have been reported [25]. Moreover, the OVCAR-3^{nu} cell line was selected for its growth characteristics and CE which are similar to primary tumor cell features [21]. Theoretically, the use of immortalized cell lines in studies on the control of cell cycle entry by growth factors is considered to be a valid experimental model for the *in vivo* regulation of stem cell proliferation [23]. The CE of H-134 cells was clearly dependent on cell concentration, which may be an indication of the production of autocrine growth factors by the cells [24, 26]. H-134 cells produced and secreted some NRK colony-stimulating activity of the EGF-dependent type (unpublished observations) confirming reports for other cell lines [19, 24, 26]. The maximal CE of H-134 cells at low cell density could be obtained by providing a high FCS concentration or CFA to the cells. This suggests that higher concentrations of appropriate growth factors than in sera from diverse sources are contained in CFA [27]. Apart from the experiments described, three other batches of CFA were tested showing consistent stimulation of colony growth of tumor cell lines. Thus, in our experience, colony growth stimulation by CFA is a very general phenomenon, as was demonstrated before for stimulation of the CE of primary ovarian cancer cells [13]. Inhibition of colony formation by CFA has been reported by others [28]; however, without details of those experiments, the results cannot be adequately compared to those in the present study. Colony stimulation by CFA could be further enhanced by adding EGF to the cultures. Particularly in those experiments with OVCAR-3^{nu} cells, CFA could not satisfy the requirement of the cells for EGF. CFA effects were, on the contrary, potentiated by EGF, suggesting that colony-stimulating activity in CFA resides largely within a (class of) growth factor(s) that act synergistically with EGF.

The experiments with NRK cells show colony growth stimulation with a pattern very similar to that of tumor cell colony growth stimulation, including the potentiation of CE by EGF. Since 1.25 ng/ml EGF, enhances the CE of NRK cells already increased by 50% CFA, it can be concluded that the amount of EGF present in CFA may well be at or below normal physiological levels (e.g., 1 ng/ml FCS) and would not be optimally stimulatory.

However, since CFA stimulates NRK colony growth in the absence of additional EGF other TGFs which may be less effective than EGF on a molar basis [15] are likely to be present in CFA in amounts sufficient to effect stimulation of NRK colony formation. As argued in a recent publication [29], the compact morphology of NRK colonies would be induced by TGF β + EGF-like activity, while

the presence of the dispersed colony type points to yet a third growth factor type. The presence of TGF-like molecules in human CFA is yet to be demonstrated by extensive purification procedures in order to be able to determine their specificity and possible origin. An interesting issue to be investigated will be whether tumor specific TGFs, as found in urine of cancer patients [30], are present in CFA. Whatever the amount and type of growth factors found to be present in CFA, our experiments suggest that the balance in human CFA is generally in favor of stimulating activity [20].

Apart from growth factor effects on the CE of tumor cells, our results show that purified BSA increased CE and influenced colony morphology of tumor cells possibly by enhancing cell adhesion. BSA, which does not contain transforming activity itself, according to our experiments probably does not act by fatty acid suppletion to the cells [31] since FCS was present in the agar underlayer to provide essential low-density lipoprotein. Furthermore, in our agar system we have no indication that BSA acts as a major radical scavenger. Since purified but not crude fraction-V BSA lowers the CFA requirement for an optimal CE, it is also unlikely that the binding of toxic substances in CFA is essential for the BSA effect. Rather, BSA may modulate growth factor effects in a manner whereby lower concentrations are required for optimal colony growth.

In conclusion, the present results show that a minimal essential growth medium supplemented with CFA, charcoal-treated BSA and EGF is likely to confer an optimal composition to the agar culture for expression of tumor cell colony formation. The results further strongly suggest that analysis of CFA growth factor constitution will provide more information as to the requirements for tumor cell growth.

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