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Clonal growth of bladder cancer cells in a double layer soft agar assay: needles addition of multiple culture supplements*

K. Lee[1], P. Smith, G. H. Weiss, and A. T. K. Cockett

University of Rochester School of Medicine, Department of Urology, Rochester, NY, USA

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Summary. The necessity of multiple culture supplements, characteristic of Hamburger and Salmon (H&S) soft agar assay (1977), for the clonal growth of bladder cancer cells was systematically investigated. One murine and 6 human cell lines were cultivated in different culture supplemented conditions based on the H&S method. All cell lines successfully formed colonies in the absence of supplements. The inclusion of 2-mercaptoethanol to supplement-free medium stimulated the clonal growth of murine (MBT-2) cells, whereas it did not affect the growth of human cell lines. DEAE-dextran suppressed the clonal growth of 5 out of 7 lines. Tryptic soy broth stimulated the growth of 2 human cell lines, whereas it suppressed the other 2 cell lines. The chemosensitivity of MBT-2 cells was not affected by these supplements.

Key words: Double layer soft agar assay – Culture supplements – Bladder cancer

During the past decade the Hamburger and Salmon (H&S) soft agar assay [6, 7, 16] had received widespread attention as a possible test for predicting the clinical response of individual human tumors to specific drugs. Although routine clinical use of this assay is hampered by many intrinsic problems [2, 12, 15], currently it is generally accepted that this assay is predictable of tumor response in man, and therefore it plays an important part in preclinical cancer research.

In our earliest experience with murine bladder cancer cells, tumor cells grew poorly in the doulbe layer soft agar media as prepared by the H&S method [16]. Unexpectedly, exclusion of supplements from the media yielded more tumor colonies. The necessity of multiple culture supplements which are characteristic of the H&S method for the clonal growth of bladder cancer cells was systematically investigated.

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Materials and methods

Cell lines and culture medium

One murine and six human bladder cancer cell lines grown in monolayer culture were used. MBT-2 is a carcinogen (FANFT) induced poorly differentiated transitional cell carcinoma (TCC) of C3H mice [11, 17]. Human bladder cancer cell lines were purchased from American Type Culture Collection (Rockville, MD). The J-82, T-24, and TCCSUP are undifferentiated TCC; RT-4 and 5637 are well differentiated TCC; SCaBER is a squamous cell carcinoma of the bladder.

The MBT-2 and 5637 cells were grown in RPMI 1640 medium with 10% heat inactivated fetal bovine serum (HI-FBS). The RT-4 and T-24 cell line were cultivated in McCoy's 5A medium with 10% HI-FBS. The culture medium for J-82, TCCSUP and SCaBER cell lines consisted of Eagle's minimum essential medium with 10% HI-FBS and non-essential amino acids (plus sodium pyruvate for TCCSUP).

Tumor cells from subconfluent flasks were used throughout the experiments. Single cell suspensions were produced by incubating the cells in 0.25% trypsin/EDTA (Gibco; Grand Island, NY) in Ca^{++} and Mg^{++} free Hank's balanced salt solution (HBSS) at room temperature for 2 min (MBT-2, T-24 and TCCSUP)or at 37°C for 15 min (5637, RT-4, J-82 and SCaBER). Tumor cell suspensions produced in this manner were either used for the experiments immediately or subcultivated.

The colony stimulating activity of horse serum (HS; Gibco) was vastly different from lot to lot. Among 4 different lots of HS we tested, one lot of HS (Lot 25p2171) stimulated clonal growth of all cell lines and others suppressed all of them: "the colony stimulating" lot of HS was used for the experiments. Unlike the HS, HI-FBS from 4 different lots showed a similar growth promoting activity.

Soft agar assay

The H&S soft agar assay was performed according to the method described details by Soehnlen et al. [16]. Each culture supplement (SIGMA, St. Louis, MO) was dissolved in distilled water (insulin was dissolved in 0.01 mM HCl) to make a suggested concentration of stock solution. When any of the supplements were omitted, HBSS was substituted. Agar (Difco, Detroil, MI) was dissolved in distilled water at 3% and autoclaved. An under-layer of one ml of 0.5% agar medium was plated in a 35 mm-well (six-well plate; Falcon, Lincoln

Table 1. Influence of full supplements on clonal growth of MBT-2 cells

Medium conditions		Mean colony counts[c]		
Supplements[a]	Sera[b]	Expt. 1	Expt. 2	Expt. 3
(−)	FBS/FBS	49	72	212
(+)	FBS/FBS	5	2	4
(−)	HS/Mix	106	132	ND
(+)	HS/Mix	157	136	ND

[a] Full supplements (+) or HBSS (−) were added to the basic medium
[b] FBS/FBS, HI-FBS for both layers; HS/Mix, HS for top- and mixture of HI-FBS and HS for under layer
[c] Number of MBT-2 cells plated was 10^4 to 2×10^4 per well
ND = not determined

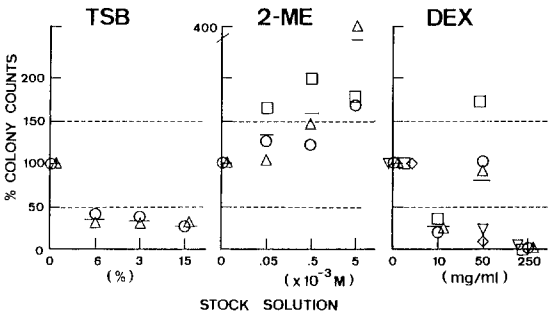

Fig. 1. Influence of the inclusion of supplement on clonal growth of MBT-2 cells. Supplement-free H&S medium with HI-FBS was included with tryptic soy broth (TSB), 2-mercatoethanol (2-ME), or DEAE-dextran (DEX). Different symbols represent results of separate independent experiments; horizontal bar, mean of separate experiments

Park, NJ) in triplicate and allowed to gel at room temperature for 1-h. Five $\times 10^3$ to 10^5 viable tumor cells per well (trypan blue exclusion method) were suspended in one ml of 0.3% agar medium and were then added to each 35 mm well on top of the 0.5% agar layer. Plates were incubated at 37°C in 5% CO_2 and in a 100% humidified atmosphere for 10 to 31 days.

Chemosensitivity test

Chemosensitivity of MBT-2 cells in different medium conditions was investigated. Adriamycin (ADM), cisplatin (DDP) and mitomycin C (MMC) were purchased from SIGMA. JM-8 is a kind gift of Bristol Laboratories (Syracuse, NY). The drugs were dissolved in 0.9% NaCl or H_2O (MMC), aliquoted and stored at -70°C until used. The cells were exposed to either drug free medium (McCoys wash) or medium containing the drugs at 37°C for 1 h, washed × 3, then plated [16].

Evaluation

Colonies of more than 50 cells were scored with an Olympus IMT-2 inverted microscope at ×40 and ×100. Only experiments giving between 30 and 300 colonies per well in the controls were included. The results were reported as mean colony count or percent mean colony count of control. Standard deviation was usually less than 10% (always less than 25%) of mean value and is not shown in the result section. A more than 50% increase (or decrease) in colony was regarded as stimulation (or suppression) of clonal growth.

Results

Clonal growth of MBT-2 cells

The influence of inclusion or exclusion of whole supplements of clonal growth of MBT-2 cells are shown in Table 1. The inclusion of all supplements inhibited clonal growth of MBT-2 cells when HI-FBS was used for both layers. However, the use of HS overcame the detrimental effects of full supplementation: the MBT-2 cells successfully grew both in the full- and unsupplemented medium without discrimination when HS was used. Because of this observation HI-FBS was used for both layers to investigate the influence of individual supplements on thegrowth of MBT-2 cells.

An individual supplement was added to supplement-free medium and mean colony counts in the test condition was compared to that in the supplement-free medium. Addition of tryptic soy broth suppressed clonal growth of MBT-2 cells in a manner dependent on the concentration (Fig. 1). By contrast, 2-mercaptoethanol (2-ME) stimulated the colony formation of MBT-2 cells over a wide range of concentration. DEAE-dextran showed a tri-phasic dose response curves (Fig. 1). The response of MBT-2 cells at the original concentration of dextran (50 mg/ml stock solution) was varied from experiment to experiment possibly due to a narrow range of effective concentration. Addition of other supplements, i.e., vitamin C, insulin (from bovine pancreas; SIGMA, I 5500), sodium pyruvate, serine, asparagine or $CaCl_2$ to the supplement-free medium did not affect the CEs in at least 2 separate determinations (data not shown).

Clonal growth of human bladder cancer cells

The clonal growth of human bladder cancer cell lines was also affected by differences in sera. Among 6 human cell lines we tested, TCCSUP, 5637 and SCaBER grew poorly in the H&S medium when HI-FBS was used for both layers. Three other cell lines grew soley in HI-FBS without the HS, though they grew better in the presence of HS than in HI-FBS alone (data not shown).

Human bladder cancer cells were cultivated in several different medium conditions which have been shown to influence the growth of MBT-2 cells (Table 1, Fig. 1). Figure 2 shows the results: mean colony counts in the test conditions are reported as percent of that in supplement-free medium with mixture of HI-FBS and HS (condition A). The inclusion of all the supplements (condition B) stimulated clonal growth of 5637 and SCaBER cells, whereas it suppressed RT-4 and TCCSUP cells. The inclusion of 2-ME (condition C) showed neither detrimental nor beneficial effects to the growth of all human

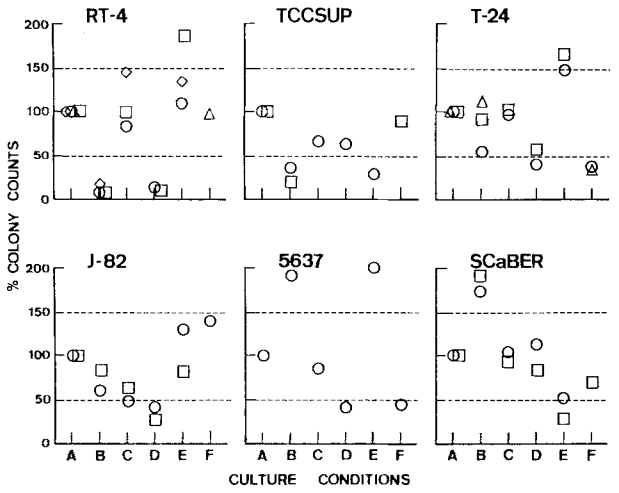

Fig. 2. Influence of different culture conditions on clonal growth of human bladder cancer cell lines. Supplement-free H&S basic medium (A) was used with complete supplementation (B), 2-mercatoethanol (C), DEAE-dextran (D), or tryptic soy broth (E): in condition A to E, horse serum was used for top- and mixture of HI-FBS and HS was used for under layer. In condition F, customary monolayer culture medium with HI-FBS was used for the both layers

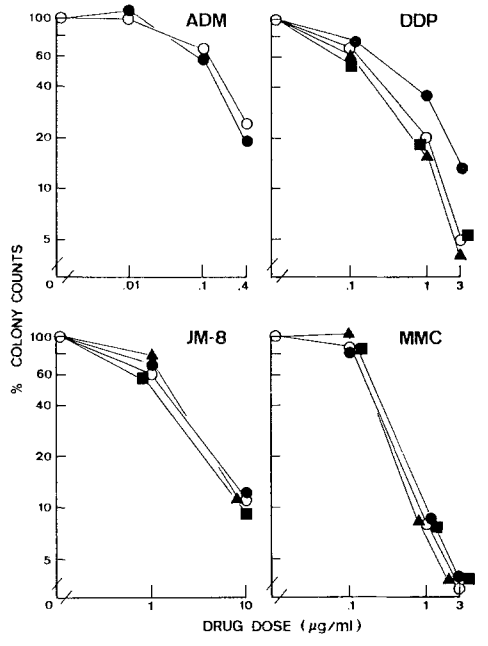

Fig. 3. Influence of the inclusion of supplement on chemosensitivity of MBT-2 cells. Following 1-h exposure of MBT-2 cells to adriamycin (ADM), cisplatin (DDP), JM-8, or mitomycin C MMC), the cells were cultivated in different culture medium. Unsupplemented medium (with HI-FBS) (○) was used with 2-mercaptoethanol (▲), DEAE-dextran (●), or tryptic soy broth (■)

bladder cancer cell lines. The inclusion of DEAE-dextran (condition D) suppressed clonal growth of 4 of six cell lines or 67% with no beneficial effect on the rest of cell lines. Tryptic soy broth showed inconsistent effects on bladder cancer cells (condition E); it stimulated clonal growth of T-24 and 5637 whereas it suppressed the growth of TCCSUPand SCaBER cells. Each cell line was cultivated in their customary monolayer culture medium (condition F): e.g., McCoy's 5A with HI-FBS was used for both layers for RT-4 cells. All cell lines showed clonal growth in their culture medium despite HS was not used.

Chemosensitivity test

Following exposure of MBT-2 cells to the cytotoxic agents for 1 h, cells were cultivated in different culture medium. Figure 3 depicts the results. Addition of 2-ME or tryptic soy broth to the supplement-free medium did not alter the chemosensitivity of the cells. The presence of DEAE-dextran provided some protection for MBT-2 cells from DDP cytotoxicity, but the difference was relatively small.

Discussion

Very few attempts have been made to the examine in detail how multiple culture supplements, characteristic of the H&S method, influence the clonal growth of tumor cells. To our knowledge, only Endresen et al. [3] investigated in detail the necessity of multiple supplements in the H&S method using a cell line (KN cell). They demonstrated that multiple culture supplements were unnecessary or even detrimental to the clonal growth of KN cells.

Most supplements in the H&S method i.e., Na pyruvate, serine, $CaCl_2$ vitamin C, asparagine, and insulin are present in the basic media (a physiologic level of insulin is present in the 10% serum [10]). The addition of these supplements to basic median did not affect the growth of MBT-2 cells in agreement with findings of Endresen et al. [3]. The influence of these individual supplements on the human cell lines was not tested, though all human cell lines successfully grew in the supplement-free medium, suggesting that the addition of these supplements are not essential for human bladder cancer growth.

In contrast, several supplements significantly affected the clonal growth of bladder cancer cells. The role of DEAE-dextran, a water-soluble polycation, in this assay might alter cell physiology by reacting with the negatively charged cell surface [13] and/or neutralize a potential cell inhibitor (acidic polysaccharide) released from agar by autoclaving[4]. Our data indicate, however, that the inclusion of DEAE-dextran has no beneficial effects on the bladder cancer growth. Tryptic soy broth might be a nutrional manipulation of amino acid for cancer cells, though the response of bladder cancer cells to it was varied. It may be possible to tailor the culture medium for individual cell types (inclusion or exclusion of tryptic soy broth). However, the growth promoting effect was relatively small (at most 2 fold increase in the CE; Fig. 2) and all cell lines were able to grow without tryptic soy broth. A 2-ME exerts its growth promoting effect on malignant lymphoid cells [9]. A number of authors have excluded 2-ME from their assay and have shown successful colony formation from variety of tumor types. Endresen et al. [3] reported the adverse effect of 2-ME on clonal growth of

KN cells. To our surprise, the inclusion of 2-ME stimulated the growth of MBT-2 cells over a large range of concentration (Fig. 1), whereas it had no influence on the growth of human cell lines.

The ability of cells to grown and form colonies in soft agar (anchorage independent cell growth) is considered to provide the accurate assessment of tumor cell survival. The H&S assay in cancer research has two important roles, a preclinical investigational use and a possible clinical chemosensitivity testing. In preclinical cancer research, the use of established cell lines overcomes a major intrinsic problems of this assay, i.e., the requirement of a large number of vialble single cells [2, 12, 15]. Our data directly applies to the modification of this assay in preclinical use. All the culture supplements in the H&S method are not essential for the clonal growth of established bladder cancer cells. Therefore, they can be omitted and this significantly simplifies the assay procedures. Our chemosensitivity data suggest that such modification may not influence the chemosensitivity of tumor cells. Some supplements could stimulate the clonal growth of certain cell types, though the growth promoting effect when present was relatively small.

There are no available data which indicate the necessity of the multiple culture supplements for the clonal growth of primary bladder tumors from patients. Many modifications of this assay have been reported to improve the CEs [1, 5, 18–21], however, to impact the problem associated with growth of primary (bladder) tumor specimen in this assay, logarithmic increase in the CEs might be required. In this context, the inclusion or exclusion of supplements may not affect the overall successful rate of this assay, thus they could be omitted.

In conclusion, multiple culture supplements used in the H&S method are not mandatory for clonal growth of bladder cancer. These supplements can be eliminated from the medium without altering chemosensitivity of bladder cancer cells.

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Dr. Kan-ei Lee
Box 656
University of Rochester School of Medicine
Urology Department
601 Elmwood Avenue
Rochester, NY 14642
USA