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Effects of Epidermal Growth Factor, Fibroblast Growth Factor, Retinoic Acid and Serum on Anchorage-dependent and Anchorage-independent Growth of HRRT Cells

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Abstract—*The effects of EGF, FGF, RA and serum on anchorage-dependent and anchorage-independent growth of HRRT cells were studied. The five different types of serum tested in the present work induced a dose dependent rise in anchorage-independent growth in aggregates. FCS, SBCS and RS also supported colony formation in soft agar, whereas BS and HS had no significant effect. EGF and FGF stimulated anchorage-dependent growth of HRRT cells in monolayers. The peptide growth factors were also found to induce phenotypic transformation of the nonneoplastic HRRT cells, as measured by anchorage-independent growth in soft agar as well as in aggregates. At equimolar concentrations EGF was much more effective than FGF. The stimulating effect of EGF and FGF on cell proliferation in the aggregate form was markedly inhibited by RA. Treatment of HRRT cells with the highest noncytotoxic concentration of RA,* 2×10^{-7} M, *reduced the stimulating effect of both growth factors by about* 60%.

INTRODUCTION

ACCUMULATING data indicate that the ability of mammalian cells to survive and proliferate in aggregates is closely correlated to their tumorigenic potential [1–5]. We have previously shown that assay of cell viability in an aggregation system using the untransformed HRRT cell line appears to be a reliable endpoint for *in vitro* studies on the initiation and promotion phases of cell transformation [6].

During the course of studies on chemical-induced carcinogenesis using the aggregation assay we observed that serum was a critical factor in the system. Serum is known to contain low levels of several transforming and growth-regulating factors [7–10], and studies have been initiated to obtain more information on the factors controlling the ability of mammalian cells to survive and proliferate in the aggregate form.

The purpose of the present work was to determine whether the anchorage-dependent growth of the HRRT cells in monolayers as well as the anchorage-independent growth in aggregates and in soft agar was affected by the growth factors EGF and FGF. The modulating effects of serum and retinoic acid have also been studied.

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The abbreviations used are: EGF, epidermal growth factor; FGF, fibroblast growth factor; RA, retinoic acid; NCS, newborn calf serum; FCS, fetal calf serum; SBCS, special bobby calf serum; HS, horse serum; RS, rabbit serum.

MATERIALS AND METHODS

Chemicals

Waymouth's medium and serum were obtained from Gibco Biocult, Paisley, U.K. EGF, FGF, RA and all other chemicals used in the present work were purchased from Sigma. Stock solutions of RA (10 mM) were made in ethanol and stored in freezing vials in liquid nitrogen. Dilutions were made immediately before use in medium such that the final concentration of ethanol was 0.2%. Control experiments were done in the presence of 0.2% ethanol. Stock solutions of EGF (5 μg/ml) and FGF (5 μg/ml) in medium were stored at −20°C. Dilutions were made immediately before use in medium.

Cells

The HRRT fibroblast rat kidney cell line used in the present work has been described previously [11]. The cells are grown in monolayer cultures on Waymouth's medium (MAD 82/3) supplemented with 4% NCS, penicillin (100 units/ml) and streptomycin (50 μg/ml). The cells are free of mycoplasma contaminations as tested by the method of Chen [12].

Colony formation in soft agar

10^5 cells were suspended in 1.5 ml of medium containing 10% FCS and 0.3% Difco Noble agar and laid over 7 ml of a 0.5% agar-medium basal layer in 50 mm Petri dishes. After 14 days of incubation at 37°C in an atmosphere of 5% CO_2 in air, colonies (>0.2 mm) were scored by direct visual counts using an inverted phase microscope equipped with a calibrated scale in one eyepiece.

Aggregation assay

The ability of HRRT cells to survive and proliferate in aggregates was assayed using a modification of the method of Steuer *et al.* [1], as described in a previous report [6]. Two milliliters of cell suspension (2×10^5 cells) were seeded in 35 mm Petri dishes having a solid bottom layer of agar (1% Difco Noble agar in 2 ml of growth medium containing 10% NCS). The dishes were incubated undisturbed at 37°C in a humidified atmosphere of 5% CO_2 in air. Viable cell counts (trypan blue exclusion) were performed in triplicate on trypsinized aggregates after 6 days incubation.

RESULTS

Data from previous studies have demonstrated that serum provides factors which promote anchorage-independent growth of various types of cells in soft agar [7–10]. It was of interest to determine if a similar effect of serum could be observed on the ability of cells to survive and proliferate in the aggregate form. Table 1 shows the effect of increasing concentrations of five different types of serum on anchorage-independent growth of HRRT cells in aggregates. It appears that no viable cells were found after incubation for 6 days in serum-free medium. With all types of serum tested, cell survival and growth in the aggregate form was stimulated in a concentration-dependent manner. Furthermore, the effect obtained varied markedly with the type of serum used. In particular, FCS and SBCS induced a considerable increase in the growth of HRRT cells. FCS and SBCS were also found to be effective in supporting colony formation by the HRRT cell line in soft agar (Fig. 1). On the other hand, NBC and HS did not induce colonies in soft agar, even at concentrations as high as 20%.

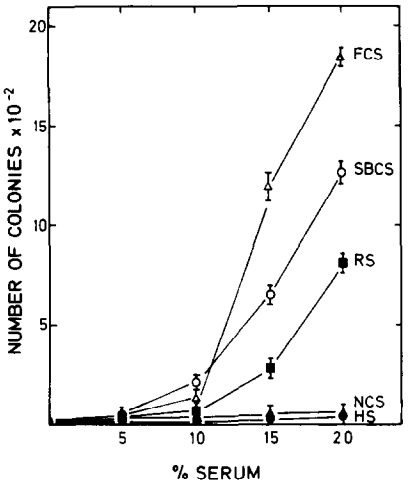

Fig. 1. Effect of serum on colony formation in soft agar. Cell cultures in the exponential phase of growth were trypsinized (0.05% trypsin and 0.02% EDTA) at 37°C for 10 min. After centrifugation the cells were suspended in medium containing various concentrations of serum, and seeded in soft agar. All experiments were carried out twice. Points are means ± standard deviation of six cultures.

The results presented above seem to indicate that anchorage-independent growth of HRRT cells in the aggregate form, as well as in the soft agar system, is strongly influenced by factors present in various amounts in different types of serum. Growth factors, defined as polypeptides that stimulate cell proliferation, are major growth regulatory molecules for cells in culture, and probably also for cells *in vivo*. Nontransformed cells show an absolute requirement for growth *in vitro*, and generally more than one factor is required.

The results in Fig. 2 show the effect of the growth factors EGF and FGF on proliferation of the untransformed HRRT cell line in monolayers. It appears that at all concentrations of serum tested, 5 ng of EGF per ml of medium induced a marked stimulation in cell growth, whereas FGF had only a moderate effect at this concentration. Neither EGF nor FGF were able to support growth in monolayers in serum-free medium, indicating the additional need of other factors present in serum for *in vitro* growth of HRRT cells.

EGF [13–15] as well as FGF [16] have previously been shown to induce anchorage-independent

Table 1. Modulating effect of serum on cell growth in aggregates

Concentration of serum (%)	No. of viable cells ($\times 10^4$) NCS	FCS	RS	HS	SBCS
0	0	0	0	0	0
5	3.1 ± 0.6	9.2 ± 2.1	8.3 ± 1.4	9.7 ± 2.1	8.3 ± 1.3
10	4.1 ± 1.3	26.3 ± 3.2	15.3 ± 2.3	17.2 ± 2.4	24.7 ± 2.5
20	12.2 ± 2.9	32.1 ± 2.7	19.7 ± 1.7	21.3 ± 2.1	30.1 ± 3.4

2×10^5 cells in 2 ml of medium containing the indicated types and concentrations of serum were added to Petri dishes as described in Materials and Methods. Viable cells were counted after 6 days incubation. The figures are mean ± standard deviation of six dishes.

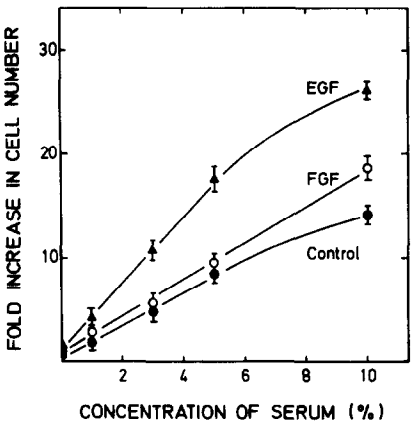

Fig. 2. Effect of EGF and FGF on cell growth in monolayers at various serum concentrations. Multiwell plastic plates were inoculated with 2.5×10^4 *cells/well in* 2 ml *of medium with* 4% *NCS and incubated in a humidified atmosphere of* 5% CO_2 *in air. After* 24 h *the medium was replaced with fresh medium containing EGF* (5 ng/ml) *or FGF* (5 ng/ml), *and various concentrations of serum as indicated. Cell counts were performed with an automatic particle counter after* 5 *days of incubation. Each point represents the mean from four wells,* ± *standard deviation.*

growth in soft agar of various nontransformed cells. Similar results were obtained with the HRRT cell line in the present work. Both EGF (Fig. 3A) and FGF (Fig. 4A) induced a dose-dependent rise in the colony forming ability in soft agar. At equimolar concentrations EGF was about 10 times more potent than FGF. It also appears from Fig. 3B and Fig. 4B that the rise in colony formation induced by the growth factors was dependent on the serum concentration in the medium. Below 5% serum EGF and FGF had no significant effect on soft agar growth of HRRT cells.

Interestingly, in the present work it was found that EGF as well as FGF had a dramatic effect on the anchorage-independent growth and survival of HRRT cells in aggregates. The data in Fig. 5 demonstrate that both growth factors induced a dose-dependent increase in cell number in the aggregation assay system. In the presence of 10 ng/ml of EGF a sixfold enhancement in the number of viable cells was found as compared to control cultures without added growth factor. Similar to the results obtained in the soft agar system, a considerably smaller effect was found with FGF. Thus, with 100 ng per ml of medium, a fourfold increase in cell number was found after 6 days of incubation. When EGF and FGF were removed from the medium, HRRT cells retained their original normal growth pattern in the aggregation system.

It should be noted that the stimulating effect of the growth factors on the aggregation system is not only due to increased cell survival. All experiments were started with 10^5 cells per ml of medium. After 6 days of incubation the number of viable cells in control cultures was about 0.3×10^5 cells per ml, whereas with 10 ng of EGF and 100 ng of FGF per ml of medium, the mean cell number was 1.9×10^5 and 1.2×10^5 cells per ml, respectively. Thus, the growth factors induced a marked increase in proliferation of HRRT cells in aggregate form.

It has previously been observed that retinoic acid inhibits the phenotypic transformation of nonneoplastic cells by peptide growth factors, as measured by colony formation in soft agar [14, 16, 17]. From the results in Fig. 6 it appears that retinoic acid blocked anchorage-independent growth of HRRT cells in aggregates in a dose-dependent manner. The EGF- and FGF-induced stimulation was completely inhibited by 10^{-5} retinoic acid and showed an IC_{50} of about 10^{-7} M. It should be noted that retinoic acid at concentrations above 2×10^{-7} M was found to inhibit proliferation in monolayers of HRRT cells (data not shown). At this nontoxic concentration, retinoic acid reduced the stimulating effect of both EGF and FGF on growth in aggregates by about 60% at various concentrations of the growth factors (Fig. 5).

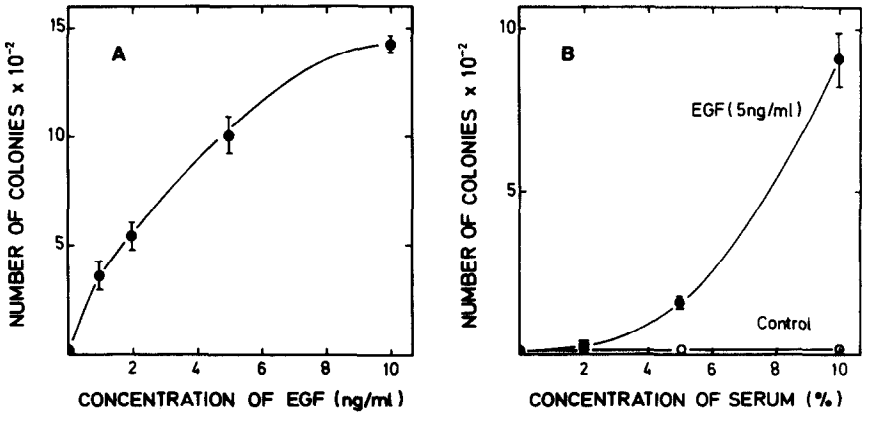

Fig. 3. Effect of EGF on colony formation in soft agar. Single cells suspended in medium containing various concentrations of EGF and FCS as indicated were seeded in soft agar. The points are the mean ± *standard deviation of the number of colonies from eight cultures.*

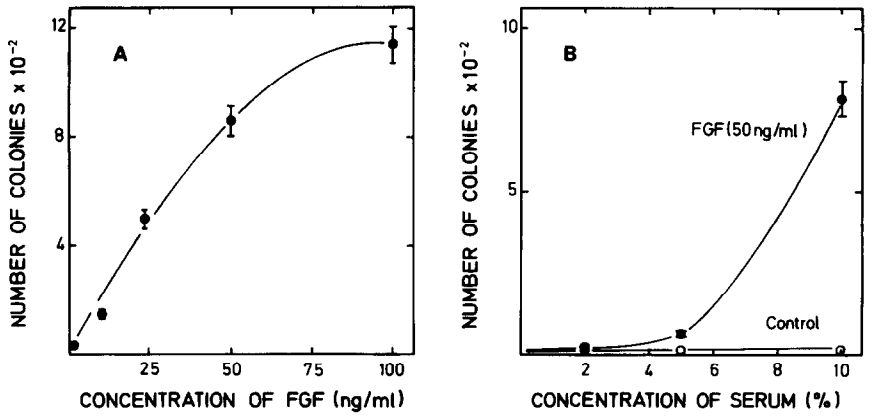

Fig. 4. Effect of FGF on colony formation in soft agar. Single cells suspended in medium containing various concentrations of FGF and FCS as indicated were seeded in soft agar. The points are the mean ± standard deviation of the number of colonies from eight cultures.

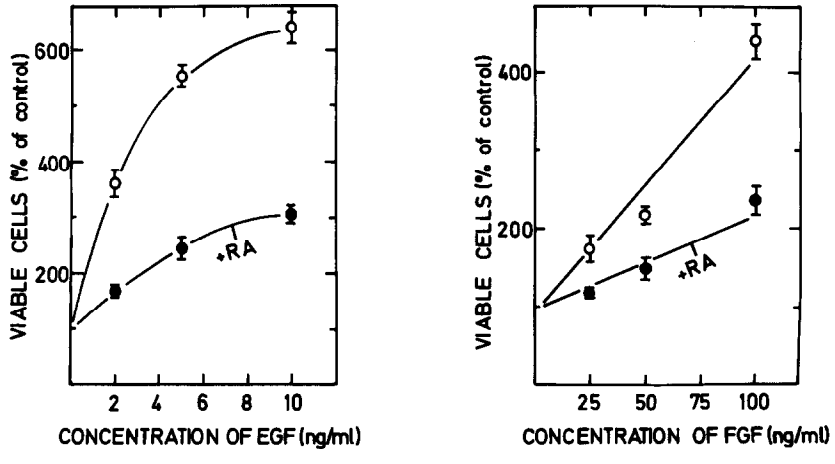

Fig. 5. Effect of EGF, FGF and RA on survival and growth of HRRT cells in aggregates. 2 × 10^5 cells suspended in 2 ml of medium containing the indicated concentrations of EGF or FGF were seeded in Petri dishes as described in Materials and Methods. The concentration of RA was 2 × 10^{-7} M. The points are the mean ± standard deviation of three different experiments. In each experiment triplicate cultures were prepared for each concentration of growth factor. The total number of viable cells in control cultures after 6 days of incubation varied from 3 to 4 × 10^4.

DISCUSSION

The HRRT cell line used in the present study was derived from a hereditary renal rat tumor. However, the cells are not able to form colonies in soft agar, and do not form tumors in nude mice [11]. Thus, the cells lack properties generally considered to be typical of transformed cells, and behave as an untransformed cell line. In a previous report we have shown that the aggregation assay system using the HRRT cell line may be a useful model system for studies on the initiation and promotion phases of cell transformation [6]. Moreover, since the aggregation properties appears to occur before the appearance of other conventional criteria of cell transformation, the assay may prove to be a valuable short-term test for carcinogen screening *in vitro*.

The ability of cells to produce colonies in soft agar is one of the criteria commonly used to determine malignant transformation. Accumulating data indicate that the ability of mammalian cells to proliferate in aggregates, when suspended in liquid medium above an agar base, is closely correlated with their tumorigenic potential [1–5]. The data in the present report show that the growth factors EGF and FGF reversibly induced the nonneoplastic fibroblast indicator HRRT cell line to express a transformed phenotype. Thus, both peptides had a marked stimulating effect on the ability of HRRT cells to form colonies in soft agar as well as on survival and proliferation in the aggregate form. Furthermore, the results also demonstrate that retinoic acid is a potent inhibitor of the EGF and FGF induced phenotypic transformation of HRRT cells, as meas-

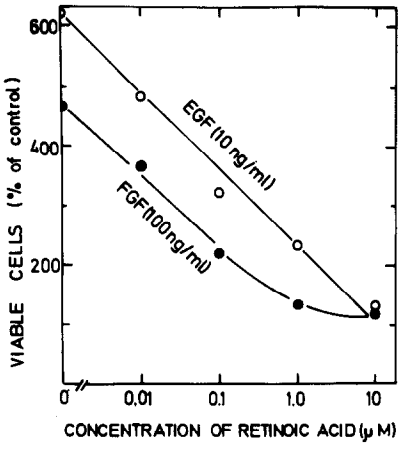

Fig. 6. Inhibiting effect of RA on EGF and FGF induced stimulation of growth in aggregates. 2×10^5 *cells in* 2 ml *of medium containing EGF* (10 ng/ml) *or FGF* (100 ng/ml) *and the indicated concentrations of RA were seeded in Petri dishes as described in Materials and Methods. Points are means ± standard deviation from three individual experiments, for a total of nine dishes per point.*

ured by anchorage-independent growth in aggregates. This observation is in agreement with the findings in a number of other systems. Retinoids have previously been extensively studied for antineoplastic activity *in vitro* [14, 16–20] and *in vivo* [21–24]. While most studies indicate that retinoic acid inhibited carcinogenesis, recent reports have shown an enhancing effect in various model systems [25–29].

The role of EGF and FGF in the expression of the transformed phenotype of HRRT cells, as well as the mechanisms of the protective action of retinoic acid, are unknown. However, evidence that growth factors might be directly involved in malignant cell transformation has recently accumulated. Thus, peptide growth factors including EGF and FGF have been found to induce phenotypic transformation of various nontransformed cell lines [13–17], and it has become evident that cells transformed by viruses and cells stimulated by growth factors are strikingly familiar.

We have previously shown that measurements of cell viability in the aggregation assay system, using the nonneoplastic HRRT indicator cell line, are a reliable endpoint for *in vitro* studies of cell transformation [6, 30]. The strong response to peptide growth factors observed in the present work, as well as previous data on chemical carcinogens [6, 30], seem to suggest that the aggregation assay may be a useful model system for studies on mechanisms involved in malignant cell transformation.

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