# Autocrine growth regulation of W12 and GCA cells in culture

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**Abstract:** Two rat kidney cell lines transformed by two strains of ASV virus were investigated. It was demonstrated that these two lines (1) showed density-independent growth, (2) had a decreased requirement for serum in the culture medium, (3) had the ability to grow in a chemically defined medium (without serum), and the rate of this growth had increased with the increase in starting density of cells, and (4) had the ability of anchrage-independent growth, even without serum. These results confirmed autostimulation of growth of W12 and GCA cells. It was also shown that the crude conditioned media contained autocrine growth factors, which could be extracted with 1M acetic acid. The extracts (AEs) stimulated the growth of the parental cells and NRK-49F cells almost as well as 5% calf serum and the extraction resulted in several-fold purification of mitogenic substances. These substances were not only specific to parental lines, but also stimulated growth of other transformed lines and normal NRK-49F cells. Extracts from the conditioned media of W12 and GCA cells intensified the rate of anchorage-independent growth in the concentration-dependent manner. In AE-W12, two peaks of mitogenic activity were detected (F1, F2) and similarly in AE-GCA (F3, F4). Fractions F2 (~ 8 kDa), F3 (~25 kDa) and F4 (~ 12 kDa) were thermostable but F1 (~ 45 kDa) was thermolabile. All four fractions were sensitive to trypsin and DTT treatment, and were acid-stable. Using ELISA kit it was shown that W12 and GCA cells released TGFβ1 and GCA cells released very small quantities of bFGF. These results confirmed the autocrine regulation of growth in both cell lines.

**Key words:** W12 cells - GCA cells - Cell culture - Growth factors - Autocrine regulation

## Introduction

Hypothesis of autocrine regulation has become the basis for concept concerning the action of oncogene products and of peptide growth factors [35, 36]. Oncogenes assure the autonomy of cell growth not only by encoding autocrine growth factors or their receptors, but also by influencing the postreceptoral intensity of mitogenic signals caused by the growth factor or its receptor [40]. It has been shown that tumor cells produce and release almost all known types of PGFs [19].

Autocrine regulation involves factors stimulating and inhibiting cell growth (for instance TGFβ) [9, 22, 37]. It has been shown that oncogenic transformation might cause a loss of ability of transformed cells to synthesize, secrete and respond to autocrine inhibitors of cell growth [9, 36].

Autocrine regulation is not an exclusive property of transformed cells but also concerns normal cells, especially embryonic cells or mature, continuously renewing cells. Good examples are keratinocytes, which produce and release more than 10 different growth factors, which regulate their growth by the autocrine mechanism and growth of other skin cells on the paracrine way [11, 25].

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**Abbreviations used in the text:** AE-GCA: acetic acid extract of media conditioned by GCA cells; AE-W12: acetic acid extract of media conditioned by W12 cells; ASV: avian sarcoma virus; BMPs: bone morphogenetic proteins; BSA: bovine serum albumin; CFE: colony forming efficiency; CM-GCA: conditioned medium of GCA cells; CM-W12: conditioned medium of W12 cells; DMEM: Dulbecco's Modified Eagle Medium; DTT: dithiothreitol; FGF(s): fibroblast growth factor(s); HGF: hepatocyte growth factor; IGF(s): insulin-like growth factor(s); MIS: Mullerian inhibiting substance; NRK-49F: normal rat kidney cell line; PBS: phosphate-buffered saline; PGF(s): polypeptide growth factor(s) ; PSMF: phenylmethyl-sulfonyl fluoride; REF: rat embryo fibroblast; RSV: Rous sarcoma virus; SDS: sodium dodecyl sulfate; TCA: trichloroacetic acid; TGF(s): transforming growth factor(s); VVGF: vaccinia virus growth factor

Many tumor cells produce the same autocrine growth factors as their normal counterparts (*i.e.* cells of the same origin). Rizzino [31] has proposed a modification of the concept of autocrine regulation of cell growth. He attributes an important role of differentiation processes in the regulation of cell proliferation. According to this hypothesis, differentiation should restrict the ability of normal stem cells to become tumorigenic while defects of differentiation are responsible for the oncogenic processes. Defects in differentiation can block normal regulation of growth stimulators, what leads to continuous production of autocrine growth factors or transcription factors [26] and never-ending proliferation of cells that can not differentiate [31].

In the oncogenic transformation, a very important role is played by the oncogenic viruses. It was discovered that virally transformed cells produce polypeptide growth factors which regulate their own proliferation by autocrine mechanism [7]. The production of these PGF(s) was shown in many cells of different origin [13], but little is known about the production of autocrine growth factors by mammalian cells transformed by avian viruses. Such cells are a very useful model in research of viral transformation of cells. The aim of the present research was to establish if two rat kidney cell lines transformed by two different strains of ASV produce autocrine growth factors regulating cell proliferation *in vitro*.

## Materials and methods

**Cell cultures.** Normal rat kidney (NRK-49F) cells and PR-RSV transformed rat sarcoma (XC) cells were obtained from the Institute of Immunology and Experimental Therapy, Wrocław, Poland. The kidney cells transformed *in vitro* by SR-RSV-D virus (W12 cells) and by temperature-sensitive mutant LA339/B-77 (GCA cells) were obtained from the Institute of Molecular Genetics, Prague, Czech Republic.

Stock cultures of these cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% calf serum (Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml) and gentamycin (50 μg/ml). Cultures without serum were maintained in F-12+ medium, *i.e.* F-12 supplemented with $Na_2SeO_3$ (10 nM), transferrin (5 μg/ml) and bovine serum albumin (0.5 mg/ml). Cells were incubated in humidified atmosphere of 5% $CO_2$ at 37°C in Falcon dishes.

**Assessment of growth of W12 and GCA cells growing on the solid substratum.** $10^4$ cells in 1 ml of DMEM supplemented with 10% calf serum were put into the glass test-tube. After 24 hrs the medium was changed for 1 ml DMEM with different concentration of calf serum and/or tested factors. The number of cells after the cultivation (2, 4 and 6 days) was estimated by the method of Sugarman *et al.* [38]. Cells were washed 2 times with PBS, fixed with methanol and stained for 2 min with 0.5% crystal violet in methanol. The stain adsorbed by cells was eluted for 30 min by 0.1 M sodium citrate (pH 4.2) in 50% ethanol. Intensity of color was measured at 540 nm.

**Assay for DNA synthesis by cells growing on solid substratum.** $5×10^4$ of cultured cells in 1 ml DMEM with 10% calf serum were placed in glass test-tube for 24 hrs, then the medium was changed

for 1 ml DMEM or F-12/DMEM (1:1, v/v) without serum. After the next 24 hrs calf serum (0; 0.1%; 0.5%; 1%; 5% and 10%) or tested factors (in different, tested quantities) were added to the medium. After 8 hrs, $^3$H-thymidine (1 μCi for each test-tube) was added to the cultures for the next 16 hrs and then the cells were fixed for 1 hr in methanol: acetic-acid (1:3, v/v). Cells were next washed 2 times with 80% methanol, air-dried and digested with 0.25% trypsin (0.5 ml/test-tube, 0.5 hr, 37°C) and diluted in 1% SDS (0.5 ml/test-tube for 10 min). Aliquots of 0.8 ml from each test-tube were supplemented with 2.5 ml Bray's scintillator and the radioactivity incorporated by cells was determined in LKB-1211 Racbeta liquid scintillation counter.

**Assay for anchorage-independent colony formation.** 1.5 ml of DMEM containing 0.6% agar, supplemented with calf serum (0-10%) was layered on a 35 mm plate. 1.5 ml of 0.3% agar (in the same medium as in the lower layer) with cells at concentration ranging from $1×10^3$ to $9×10^4$ per dish and the tested factors (at different concentrations) were layered onto the lower layer. Cells were incubated for 7 days in a humidified atmosphere containing 5% $CO_2$ at 37°C. Colonies larger in diameter than 30 μm were counted under a microscope and CFE (colony forming efficiency) was then estimated.

**Assay for DNA synthesis in soft agar.** 0.5 ml of DMEM containing 0.3% agar, 1-10% calf serum and cells at concentration from $1×10^3$ to $9×10^4$ per ml were layered onto 0.5 ml preformed base layer of 0.6% agar in the same medium in 10 cm test-tube. Cultures were incubated at 37°C for 1 to 5 days prior to a 24-hr pulse-labeling with 1 μCi of $^3$H-thymidine. After the pulse, agar was diluted with 4 ml of warm PBS and cells were centrifuged at $1000×g$ for 15 min. Then the cells were washed 2 times with warm PBS (2×4 ml) and precipitated 3 times with 2 ml of 5% TCA. The pellet which appeared after triple centrifugation was dried and diluted in 0.5 ml of hot 0.2 M NaOH. 0.3 ml of this dilution was neutralized by 0.2 M $KH_2PO_4$ and 2.5 ml of Bray's scintillator was added. Radioactivity was then determined in LKB-1211 Racbeta liquid scintillation counter.

**Assay for the activity of conditioned media (CM) of W12 and GCA cells.** W12 and GCA cells were cultured in the Roux's bottles up to the monolayer stage. Then the medium was removed, cells were washed 2 times with 50 ml of PBS and fresh DMEM without serum was placed in bottles. After 48 hrs of incubation, conditioned media were pooled from 20 bottles for each cell line, supplemented with PSMF (50 mg/l of medium) and centrifuged ($1000×g$ for 15 min).

Activity of such a crude medium was estimated as follows: W12 and GCA cells were growing in the tissue culture dishes from 3 different densities ($1×10^6$; $2.5×10^6$ and $5×10^6$) in DMEM with 5% calf serum. After 24 hrs of incubation, the media were centrifuged ($1000×g$, 15 min) and supplemented with fresh DMEM (in relation 3:1) without serum. Such mixtures were tested for stimulating growth of W12 and GCA cells cultured on the solid substratum. W12 and GCA cells growing without serum and without conditioned media were the controls.

**Extraction of growth factors from the conditioned media with 1M acetic acid.** Conditioned media (1500-2000 ml) were dialyzed for 72 hrs using Kalle A.G. membranes (permeable for molecules with m.w. up to 2 kDa) against 0.1 M acetic acid at 4°C. Nondialyzable part of conditioned media was dried by evaporation at 37°C. Dry matter was diluted in 1 M acetic acid in 0.01 of the starting volume. This solution was centrifuged ($1500 × g$, 15 min) the supernatant was dialysed for 48 hrs against 0.2 M acetic acid using membranes mentioned above. Nondialyzable extract was centrifuged ($1500 × g$, 15 min) and the supernatant was lyophilized.

In all fractions obtained during this procedure, estimation of protein content was done by the method of Bradford [3].

**Assay for activity of acetic acid extracts (AEs) of the conditioned media of W12 and GCA cells.** The activity of acetic acid extracts

was determined by incorporation of $^3$H-thymidine by cells growing on the solid substratum. The parental cells (*i.e.* cells from which the conditioned medium was obtained) and additionally NRK-49F cells for comparison were used as the target cells. Acetic acid extracts were used at concentration from 0 to 20 µg of protein per ml of medium. Results were presented as percentage of values observed in the control, which was a sample without acetic acid extracts and without serum.

The acetic acid extracts were tested for specificity of their action by evaluating their influence on additional cell lines. Influence of AE-W12 was tested on GCA and XC cells, and of AE-GCA on W12 and XC cells. The incorporation of $^3$H-thymidine by cells growing on the solid substratum was measured. The protein concentration in the used extracts was 15 µg/ml. Results were presented as percentage of values found in the control (sample without extracts and without serum).

**Isolation and characterization of polypeptide fractions present in AEs.** Separation of polypeptides contained in AEs from conditioned media of both cell lines was performed on the column (57×2 cm) filled with Bio-Gel P-60. The gel was equilibrated with 1M acetic acid during 24 hrs and calibrated with following standard proteins: ovalbumin (45 kDa), chymotrypsinogen (25 kDa), cytochrome c (12.5 kDa) and insulin A (6 kDa). Lyophilizates of AEs were dissolved in 1M acetic acid to obtain 2 ml solution which contained 2 mg protein. Such sample was put on the column. Separation was carried out in 1M acetic acid (1ml/min). The fractions (19 ml) were collected. After estimation of protein content (by Bradford's method), fractions were lyophilized. Lyophilizate of each fraction was dissolved in DMEM and added to the medium of target (parental) cells growing on the solid substratum (glass test tubes) for estimation of their biological activity by $^3$H-thymidine incorporation. All fractions which significantly stimulated growth of target cells were pooled and their biological activity at different concentrations was estimated.

Fractions stimulating cell growth were tested for the influence of temperature on their biological activity. Sample of each fraction was dissolved in PBS (to concentration of 500 ng protein/ml ) and were placed in water bath at 56°C for 30 min or at 100°C for 3 min.

The influence of trypsin was tested by incubation of the fractions with this enzyme (50 µg/ml, 37°C, 3 hrs). Then trypsin was inactivated with trypsin inhibitor from bovine lungs (150 µg/ml). Fractions incubated with trypsin and its inhibitor in the same quantities as in the main experiment served as a control.

The resistance for reducing agents was tested by incubation of the studied fractions with 65 mM dithiothreitol (DTT) at room temperature during 1 hr. The control samples were incubated in the same conditions but without DTT. All samples were then dialyzed for 48 hrs using A.G. Kalle membranes (permeable for molecules with m.w. up to 2 kDa) against PBS with BSA (100 µg/ml) at 4°C.

Biological activity of all fractions treated as mentioned above was evaluated by $^3$H-thymidine incorporation by W12 and GCA cells growing on the solid substratum (glass test tubes).

**ELISA test for TGFβ$_1$, TGFβ$_2$ and bFGF.** Crude conditioned media (CMs), acetic acid extracts (AEs) and fractions F1-F4 were tested for the presence of some growth factors. The ELISA kits (from R&D Systems) for TGFβ$_1$, TGFβ$_2$ and bFGF were performed following the manufacturer's instructions. The minimal detectable concentration was: 3 pg/ml (bFGF) and 7 pg/ml (TGFβ$_1$ and TGFβ$_2$).

## Results

### *Anchorage-dependent growth of W12 and GCA cells*

For the purpose of evaluating autonomy of growth of the studied cells, the influence of different concentrations of

calf serum in the medium on growth of these cells was tested during 6-day culture (Fig. 1A,C). It appeared that W12 and GCA cells almost did not grow in DMEM without serum. Five percent was the optimal concentration of calf serum in the culture medium; 10% calf serum did not significantly influence the kinetics of growth of the cells.

The doubling time of W12 cells cultured in the presence of 0.5%, 5% and 10% calf serum was, respectively: 80, 28 and 24 hrs, and for GCA cells 22 hrs (5% calf serum) and 20 hrs (10% calf serum).

Incorporation of $^3$H-thymidine after 24-hr incubation of cells of both lines with different concentrations of calf serum showed that 5% calf serum induced significant increase in isotope incorporation, and a further slight increase was observed for 10% calf serum (Fig. 1 B, D).

The obtained data allowed to draw the regression curve between the increase in the percentage of cell number after 48 hrs and of $^3$H-thymidine incorporation after 24 hrs. Both parameters correlated very well, with correlation coefficient 0.913 for W12 cells and 0.926 for GCA cells (Fig. 2).

Since both cell lines revealed lower requirement for serum than normal cells, it was interesting if they would be able to grow in the absence of serum. For testing this, a rich, nutritive F-12/DMEM+ medium was used. It appeared that cells of both lines are able to grow in such a chemically defined medium (Fig. 3). The doubling time for cells growing in this medium from the starting density of 1×10$^4$ cells/ml was 58 hrs (W12) and 104 hrs (GCA); it was 2 and 5 times longer respectively, than for these cells growing in DMEM with 5% calf serum.

In F-12/DMEM+ medium, W12 and GCA cells incorporated 163% and 155% $^3$H-thymidine, respectively, as compared to these cells growing in DMEM without serum (Table 1).

W12 and GCA cells grow in a medium without serum probably as a result of autocrine regulation. If this is true, the rate of growth in chemically defined medium ought to depend on the starting density of cells. For this purpose, the kinetics of growth of W12 and GCA cells were tested by measuring incorporation of $^3$H-thymidine pulse by cells growing on the solid substratum in F-12/DMEM+ medium without serum. During a 5-day culture, a continuous increase in incorporation of the isotope, dependent on the starting density of cells was observed (Fig. 4). Assuming incorporation of isotope on day "0" as 100%, the increase in incorporation by cells growing from three starting densities: 1×10$^4$, 3×10$^4$ and 6×10$^4$ cells/ml was for W12 cells, respectively: 210%, 310% and 364%, and for GCA cells: 257%, 281% and 329%. Such a dependence of cell growth on starting density suggests that the endogenous factors are the stimulators of growth of the tested cells.
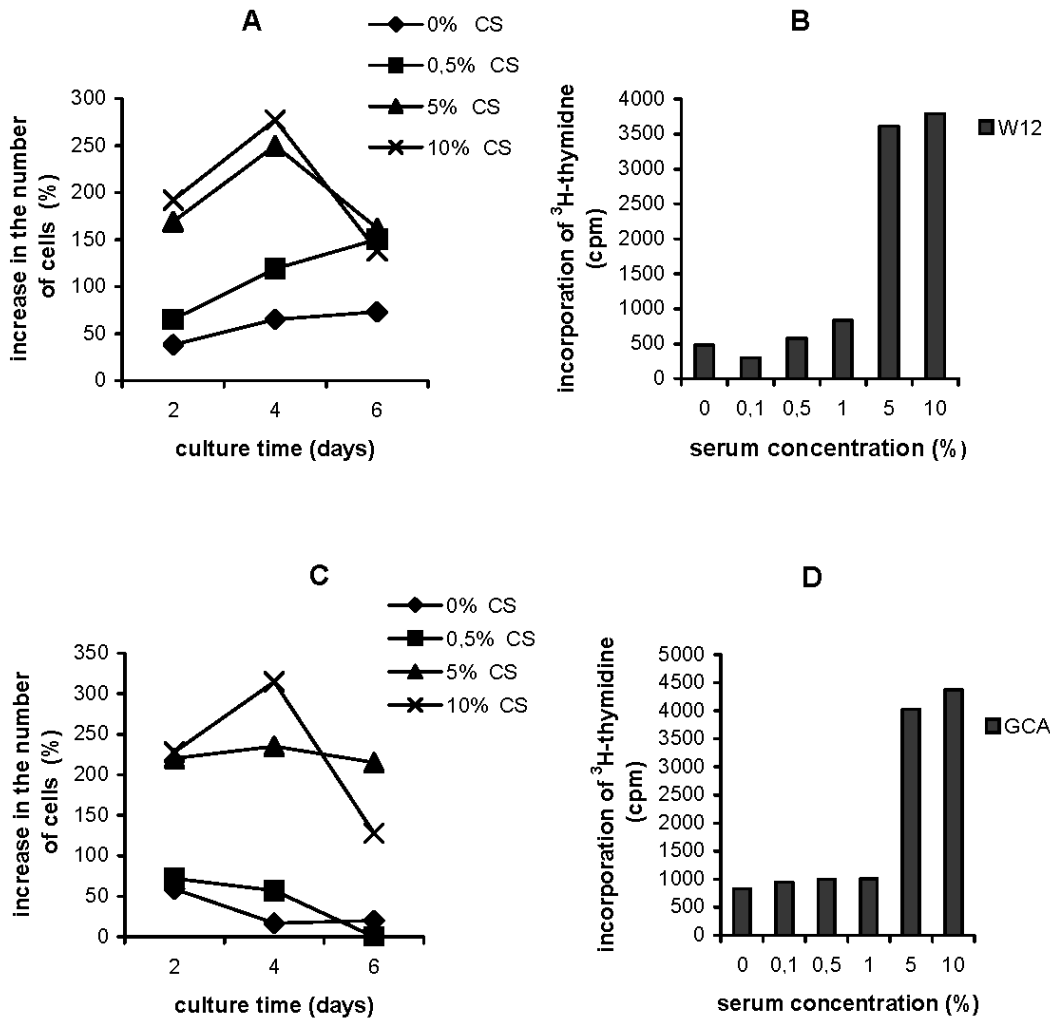
**Fig. 1.** Influence of calf serum (CS) on growth of W12 and GCA cells on the solid substratum. Increase in the number of W12 (**A**) and GCA (**C**) cells starting from the initial density $1 \times 10^4$ cells/ml during 6-day incubation in DMEM supplemented with 0%, 0,5%, 5% and 10% calf serum. Incorporation of $^3$H-thymidine by W12 (**B**) and GCA (**D**) cells growing in DMEM supplemented with 0%, 0.1%, 0.5%, 1%, 5% and 10% calf serum. Means were calculated for n=4 (A,B), n=8 (C) and n=6 (D). SD was <15%.
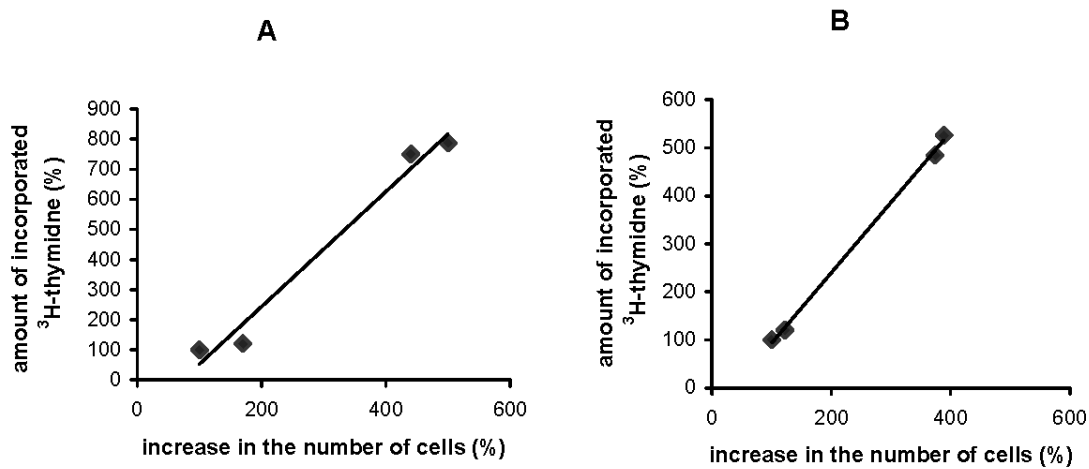


**Fig. 2.** Regression curve between the increase in the cell number after 48 hrs of cultivation and the increase in $^3$H-thymidine incorporation after 24 hrs incubation estimated for W12 (**A**) and GCA (**B**) cells.
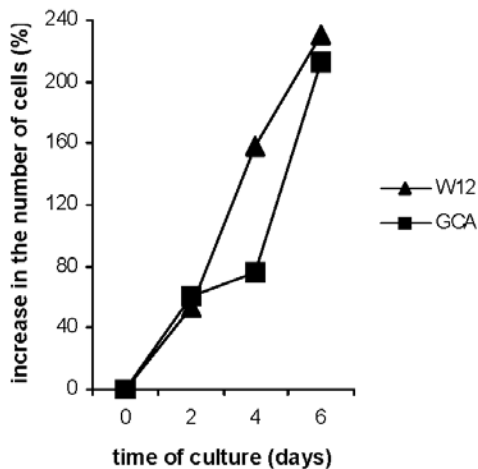
**Fig. 3.** Increase in the number of W12 and GCA cells during 6-day culture in F-12/DMEM+. The initial density was $10^4$ cells/ml. Means were calculated for n=4. SD was <15%.

**Table 1.** Incorporation of $^3$H-thymidine by W12 and GCA cells ($5 \times 10^4$ cells/ml) growing on the solid substratum, after 24-hr incubation in media without serum (DMEM or F-12/DMEM+)

| Medium | DMEM | | F12/DMEM+ | |
|---|---|---|---|---|
| Cell line | cpm ± SD | % | cpm ± SD | % |
| W12 | 481 ± 25 | 100 | 785 ± 42 | 163 |
| GCA | 831 ± 37 | 100 | 1288 ± 65 | 155 |

Means±SD were calculated for n=4

## Anchorage-independent growth of W12 and GCA cells

The clonal growth in semisolid medium very well correlates with the neoplastic growth *in vivo*. This ability of W12 and GCA cells was verified by performing a clonal test in 0.3% agar in DMEM (Fig. 5A,B). Both cell lines formed colonies >30 µm in diameter when the concentration of serum was at least 5%. The starting density of cells is very important for colony forming efficiency (CFE), the percentage of cells in an inoculum which give rise to multicellular colonies in semisolid medium. The threshold density was $6 \times 10^3$ cells/ml for W12 cells and $3 \times 10^3$ for GCA cells [18]. Maximal CFE was observed in cells growing from the starting density of $6 \times 10^4$ cells /ml in DMEM with 10% calf serum; it was 5.8% for W12

cells and 6.2% for GCA cells. In the case of higher starting density of cells ($9 \times 10^4$/ml), a decrease in CFE was observed.

In the F-12/DMEM+ defined medium, W12 and GCA cells formed colonies from the starting density of $3 \times 10^4$ cells/ml, and maximal CFE was 4.5% for W12 and 4.8% for GCA cells (at starting density $6 \times 10^4$ cells/ml), *i.e.* only slightly lower than in DMEM with 10% calf serum.

These results have shown that W12 and GCA cells are capable of clonal growth in a medium without serum, and the rate of this growth only slightly depends on exogenous growth factors. It confirms a hypothesis of autostimulation of growth in both cell lines, but does not prove, if it is auto- or intracrine regulation.

## Mitogenic activity of conditioned media (CM) of W12 and GCA cells

In order to confirm the release of growth factors by W12 and GCA cells, the biological activity of their crude conditioned media was tested. For both cell lines, a weak stimulation of DNA synthesis was shown. That stimulation was proportional to the density of cells, which
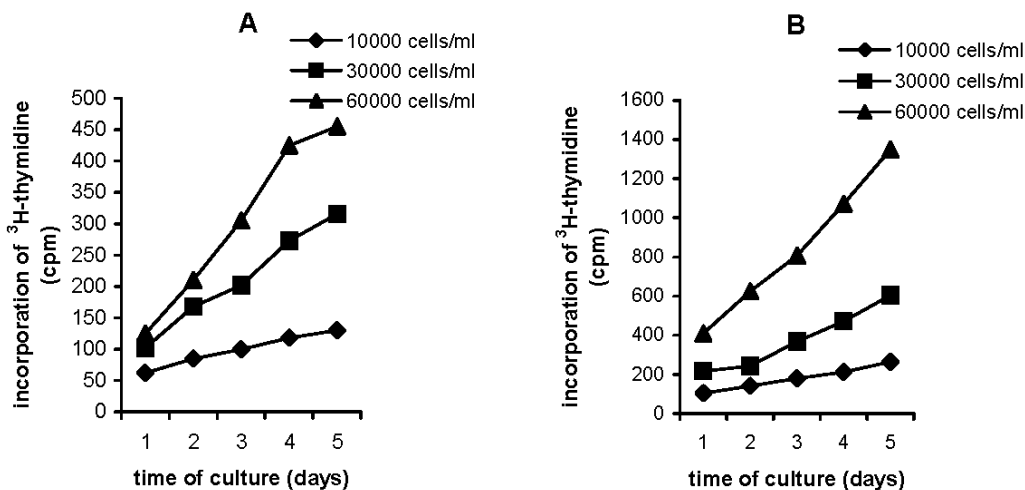


**Fig. 4.** Kinetics of $^3$H-thymidine incorporation by W12 (**A**) and GCA (**B**) cells growing on the solid substratum in F-12/DMEM+ during 5 days, starting from the initial densities of: $1 \times 10^4$, $3 \times 10^4$ and $6 \times 10^4$ cells/ml. Means were calculated for n=4. SD was <15%.

**A**



**B**
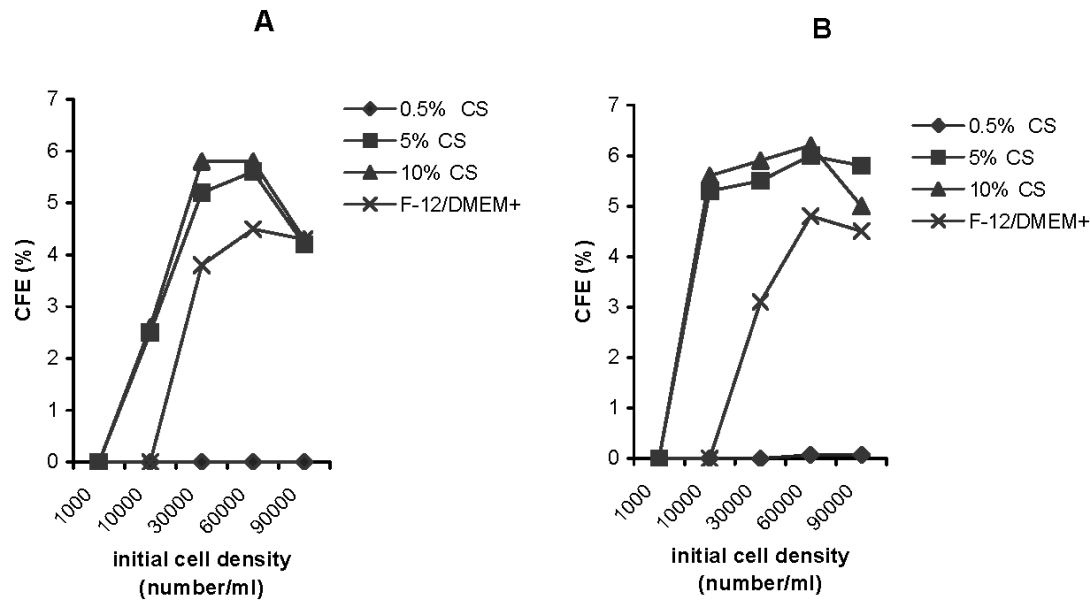
**Fig. 5.** Clonal growth of W12 (**A**) and GCA (**B**) cells in 0.3% agar depending on initial cell density ($1 \times 10^3$, $1 \times 10^4$, $3 \times 10^4$, $6 \times 10^4$ and $9 \times 10^4$) and on concentration of calf serum in DMEM (0.5%, 5%, 10% or without serum in F-12/DMEM+). Means were calculated for n=9. SD was <20%.

**A**



**B**
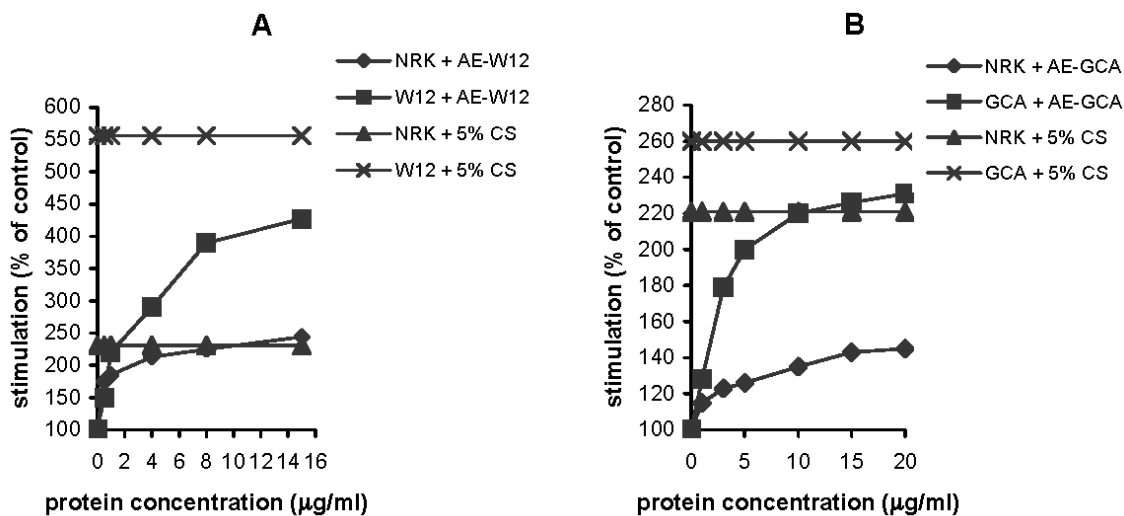
**Fig. 6.** Activity of acetic acid extracts (AE) of media conditioned by W12 (**A**) and GCA (**B**) cells evaluated by $^3$H-thymidine incorporation by NRK-49F and W12 or GCA cells growing on the solid substratum in DMEM without serum. Means were calculated for n=3. SD was <15%.

produced the tested crude conditioned media (Table 2). The low level of the stimulation was probably the result of low concentrations of the cell growth stimulators contained in the crude media; moreover the media are partially consumed by cells growing in them during a 48-hr incubation.

This experiment confirmed the presence of autocrine growth factors in the conditioned media of tested cells and justified an attempt to purify these factors.

The majority of known, so far, growth factors of somatic cells are thermo- and acid-stable (except FGFs

and HGF), thus extraction with 1M acetic acid was used for preliminary purification of CM components.

### *Influence of acid extracts (AEs) of CM on anchorage-dependent growth of W12 and GCA cells*

The mitogenic activity of extracts (AEs) was estimated as concentration-dependent stimulation of DNA synthesis in NRK-49F cells and W12 or GCA cells growing on the solid substratum. Results (Fig. 6 A,B) showed a

**Table 2.** Stimulation of incorporation of $^3$H-thymidine (in % of control) in the W12 and GCA cells evoked by conditioned media of these cells growing from 3 starting densities
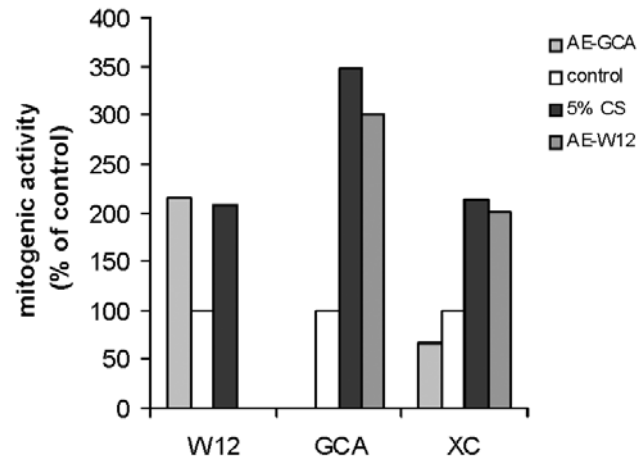
| Conditioned medium | Stimulation of incorporation of $^3$H-thymidine (%) Starting cell density | | |
|---|---|---|---|
| | $1 \times 10^6$ | $2.5 \times 10^6$ | $5 \times 10^6$ |
| CM-W12 | $120.0 \pm 18.0$ | $136.0 \pm 17.0$ | $138.0 \pm 18.1$ |
| CM-GCA | $103.0 \pm 10.5$ | $124.0 \pm 16.1$ | $126.0 \pm 16.9$ |

This test estimating activity of conditioned media was performed on W12 and GCA cells (respectively) growing on the solid substratum in DMEM without serum. The controls were cultures of W12 and GCA cells growing in DMEM without serum and without conditioned media. Means±SD were calculated for n=3.



**Fig. 7.** Influence of acetic acid extracts (AE, 15 µg/ml) of CM-W12 and CM-GCA on $^3$H-thymidine incorporation by nonparental cells (W12, GCA and XC) growing on the solid substratum in DMEM without serum. The target cells growing in DMEM without serum served as controls. Means were calculated for n=3. SD was <15%.

significant, concentration-proportional stimulation of DNA synthesis in the transformed cells (parental for the extracts) and in the cells of normal cell line (NRK-49F). AE-W12 at concentration of 15 µg of protein/ml stimulated W12 cells 2 times stronger (427% of control) than NRK-49F cells (244% of control) and AE-W12 was only 20% less effective than 5% calf serum (556% of control) (Fig. 6A). ED$_{50}$ calculated from this graph (2.8 µg/ml) showed 8-fold purification of growth factors in comparison with the crude CM-W12.

AE-GCA (Fig. 6B) had a stronger stimulatory effect on the growth of GCA cells (231% of control) than of NRK-49F cells (145% of control) and acted a little weaker than 5% calf serum (260% of control). ED$_{50}$ (2.5 µg of protein/ml) showed 13-fold purification of growth factors in comparison with the crude CM-GCA.

For the purpose of verifying the specificity of action of growth factors produced by W12 and GCA cells, we tested the influence of AEs on the anchorage-dependent growth of different cells: AE-W12 on GCA and XC cells, and AE-GCA on W12 and XC cells. Results (Fig. 7) showed that AE-W12 stimulated growth of GCA cells (300% of control) and XC cells (201% of control) and this influence was similar to that of 5% calf serum. AE-GCA stimulated growth of W12 cells (214% of control) similarly to 5% calf serum but unexpectedly inhibited growth of XC cells (66% of control). This experiment confirmed the presence of mitogenic substances, stimulating DNA synthesis in the normal and in the virally-transformed cells in conditioned media of both cell lines.

**A**

**B**
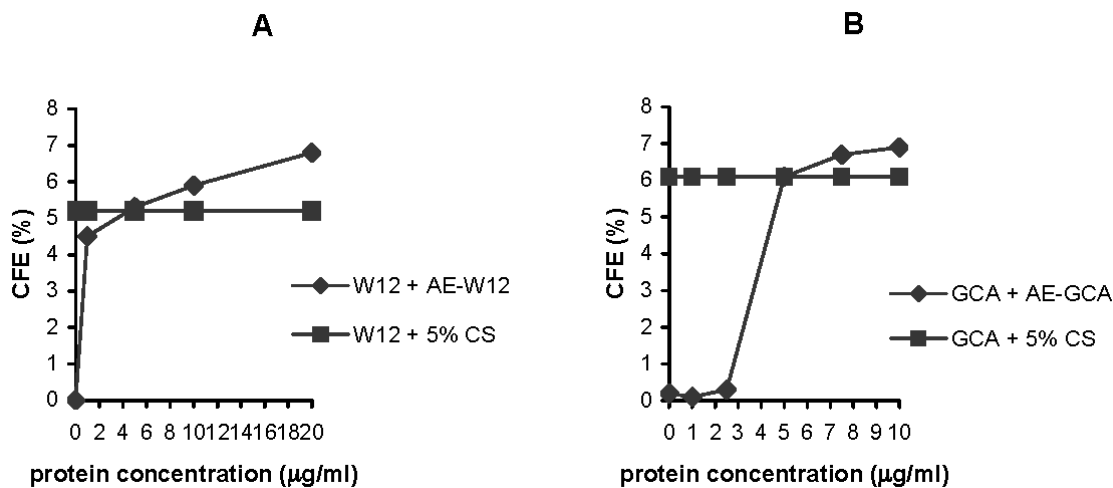


**Fig. 8.** Influence of acetic acid extracts (AE) of CM-W12 (**A**) and CM-GCA (**B**) on colony (> 30 µm in diameter) forming efficiency (CFE) by W12 (**A**) and GCA (**B**) cells in semisolid DMEM with 2% calf serum. Means were calculated for n=3. SD was <20%.
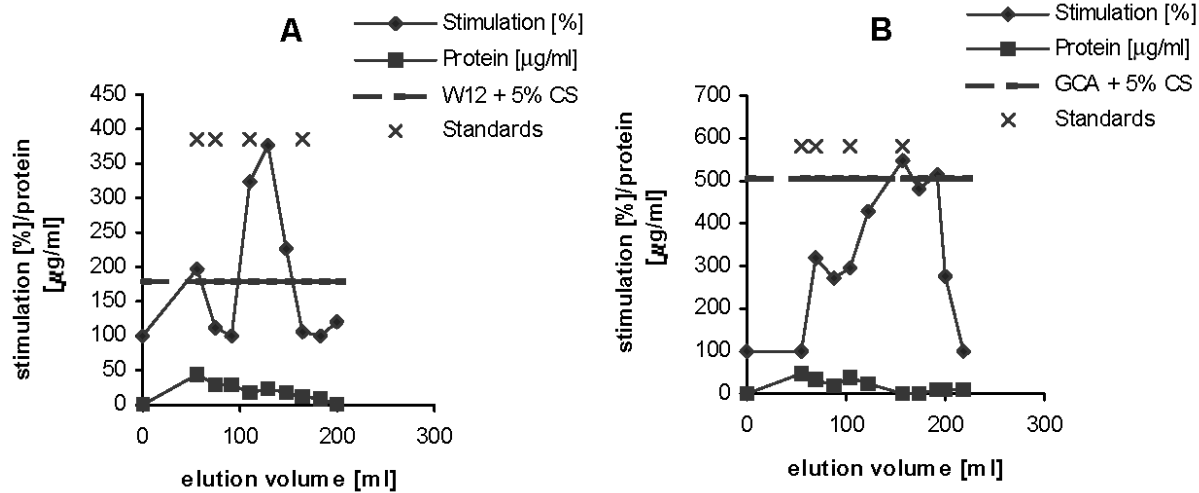
**Fig. 9.** Separation of acetic acid extracts from W12 (**A**) and GCA (**B**) cells on the Bio-Gel P-60 column in 1M acetic acid. Protein content was estimated by the Bradford's method and the activity of separated fractions by assessing DNA synthesis stimulation (^{3}H-thymidine incorporation) in the W12 or GCA cells growing on the solid substratum in MEM without serum. The column was calibrated with the following standard proteins: ovalbumin (45 kDa), chymotrypsinogen (25 kDa), cytochrome c (12.5 kDa) and insulin A (6 kDa)(respectively, from left to right). Means were calculated for n=9. SD was <15% .
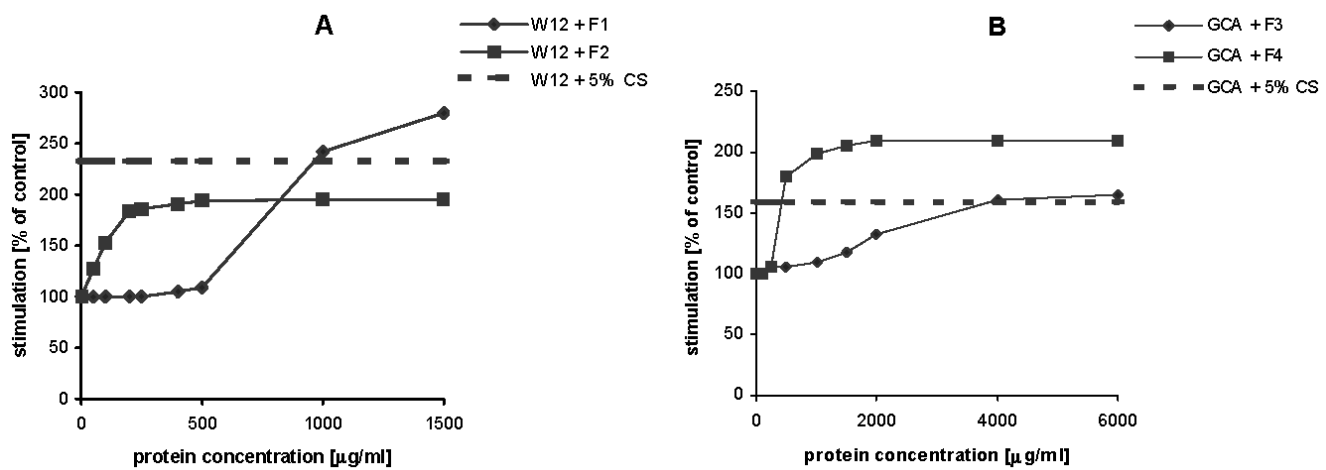


**Fig. 10.** The activity of fractions obtained from CM-W12 (**A**) and from CM-GCA (**B**) evaluated by ^{3}H-thymidine incorporation by W12 (**A**) and GCA (**B**) cells growing on the solid substratum in MEM without serum. Means were calculated for n=4. SD was <15%.

## Influence of AEs on anchorage-independent growth of W12 and GCA cells

The clonal growth of tested cells in semisolid agar in DMEM with 2% calf serum and different concentrations of AEs was assessed. W12 formed colonies in soft agar proportionally to the concentration of AE-W12 (Fig. 8A) and, similarly, AE-GCA influenced in a concentration-dependent manner colony formation by GCA cells (Fig. 8B). For both cell lines CFE values were similar to CFE for 5% calf serum (5.2% for W12 and 6.1% for GCA cells) at AE concentration of at least 5 μg prote-

in/ml. This experiment confirmed that mitogenic substances contained in the conditioned media of the tested cells also stimulated the rate of anchorage-independent growth.

## Isolation and preliminary identification of mitogenic fractions obtained from AEs

Acetic acid extracts of W12 and GCA cell-conditioned media were separated on Bio-Gel P-60 column (Fig. 9A,B). In the AE-W12, two peaks of mitogenic activity were detected. The first peak (F1) was present in the

**Table 3.** Influence of temperature, dithiothreitol and trypsin on mitogenic activity of the factors released by W12 and GCA cells

| Treatment | Mitogenic activity ( % of control) ± SD | | | |
|---|---|---|---|---|
| | CM-W12 | CM-W12 | CM-GCA | CM-GCA |
| | F1 | F2 | F3 | F4 |
| 56˚C, 30 min. | 34±4 | 94±11 | 91±10 | 86±10 |
| 100˚C, 3 min. | 23±3 | 89±9 | 93±9 | 92±11 |
| dithiothreitol (65 mM) | 13±2 | 15±2 | 17±2 | 14±2 |
| trypsin (50 μg/ml) | 23±3 | 36±4 | 38±5 | 32±3 |

The fractions were used at concentration of 1 $ED_{50}$/ml. Cultures with addition of untreated fractions had served as a control. Mitogenic activity was evaluated by incorporation of $^3$H-thymidine by cells growing on the solid substratum. Means±SD were calculated for n=3.

elution volume 0-60 ml (m.w. > 45 kDa), and the second one (F2) between 110-150 ml (m.w. approx. 8 kDa). The mitogenic activity of F1 and F2 was then evaluated in relation to concentration (Fig. 10A). The ED50 values calculated from the obtained data were 800 ng/ml for F1 and 90 ng/ml for F2. In the AE-GCA, also two peaks of mitogenic activity were detected: F3 between 60-90 ml (m.w. 25 kDa) and F4 between 105-200 ml (m.w.<12 kDa). $ED_{50}$ for F3 was 2000 ng/ml and for F4 400 ng/ml (Fig. 10B)

Purification of 1500 ml conditioned medium from W12 or GCA cells yielded 340 μg F1, 86 μg F2, 208 μg F3 and 334 μg F4. The obtained fractions were purified: F1 28-fold, F2 250-fold, F3 17-fold and F4 85-fold as compared to crude conditioned medium. Fractions F2, F3 and F4 were thermostable but F1 was thermolabile. All four fractions were sensitive to trypsin and dithiothreitol treatment, and were acid-stable because they retained their biological activity under the conditions of purification in 1M acetic acid, pH <3.0 (Table 3).

Using ELISA kits it was shown that W12 and GCA cells released TGFβ1, which was present in CM-W12, CM-GCA, AE-W12, AE-GCA, F1 and F3, and much less in F2 and F4 (Table 4). The total volume of conditioned medium used for single purification procedure (1500 ml) contained 3.9 μg TGFβ1 in CM-W12 and 3.1 μg in CM-GCA.

ELISA test for TGFβ2 was also performed in order to check whether the other member of TGFβ-family was produced by W12 and GCA cells. The tested cells did not release TGFβ2 at all (it was not detected in any fraction).

ELISA test for bFGF showed that W12 cells did not release this factor at all, but very little quantities of bFGF were detected in the crude CM-GCA (100 pg/ml). It was not detectable in other fractions derived from CM-GCA.

## Discussion

Cells releasing autocrine growth factors *in vitro* show a decreased requirement for serum in the culture medium. Serum is a source of not only many different growth factors, but also binding proteins, lipids and factors increasing adhesion of cells to the substratum. Normal cells require 10% calf serum in the medium, W12 cells grow in 0.5% calf serum but 5% is enough to promote unlimited proliferation (likewise for GCA cells). Both cell lines also grow in the defined medium (without serum). That decreased requirement of W12 and GCA cells for serum means an independence of cell growth of exogenous growth factors suggesting that both cell lines produce autocrine growth factors.

The kinetics of growth of both cell lines in defined medium showed that the rate of growth increased with the increase of the initial density of cells. Such kinetics may be explained by an increasing concentration of autocrine growth factors, released by these cells to the culture medium. Another characteristic property of transformed cells, the loss of density-dependent contact inhibition of W12 and GCA cells, was shown earlier [18].

**Table 4.** Quantity of TGFβ1 in the fractions obtained from conditioned media of W12 and GCA cells

| Stage of purification | W12 (pg/ml) | GCA (pg/ml) | Volume of fraction (ml) | Quantity of TGFβ1 in the fractions derived from W12 (ng/fraction) | Quantity of TGFβ1 in the fractions derived from GCA (ng/fraction) |
|---|---|---|---|---|---|
| Conditioned medium (CM) | 2610 | 2070 | 1500 | 3915 | 3105 |
| Acetic acid extract (AE-) | 379440 | 41200 | 4.2 | 1594 | 173 |
| F1 | 4350 | - | 8.0 | 35 | - |
| F2 | 1850 | - | 8.0 | 15 | - |
| F3 | - | 10620 | 8.0 | - | 85 |
| F4 | - | 780 | 8.0 | - | 6 |

ELISA test from R&D Systems was performed following the kit instruction. The minimal detectable concentration of TGFβ1 was 7 pg/ml. Means were calculated for n=2.

W12 and GCA cells grow very well in the semisolid medium showing their ability to anchorage-independent growth - the feature of transformed cells [34]. These cells form colonies in soft agar in the same conditions as on the solid substratum; their CFE increases in the presence of 5% calf serum in 0.3% agar, for the initial density not lower than $10^4$ cells/ml. The decrease in CFE for culture growing from initial density of $9\times10^4$ cells/ml may be explained by (1) quicker exhaustion of nutritive medium components (2) increase in concentration of some cell growth inhibitors presumably produced by these cells, and/or (3) accumulation of toxic metabolites of cells in their semisolid surrounding.

Most mammalian cells of solid tissue origin display better growth and viability when attached to a surface or substratum than when maintained in a single-cell suspension devoid of such contacts. By contrast, established cell lines known to be tumorogenic are frequently either anchorage-independent or display a much reduced anchorage requirement. This had led to the hypothesis that the loss of anchorage dependence in culture accompanies and can be used as an index of oncogenic transformation [34]. Anchorage independence very well correlates with the neoplastic growth *in vivo* [6] and is used in the estimation of TGFs activity [32].

Since counting of colonies may be not very precise, the incorporation of $^3$H-thymidine to DNA of cells growing in soft agar is very often estimated. This method is used to test anticancer drugs [14].

W12 and GCA cells are capable of anchorage-independent growth in the semisolid medium without serum, but they require higher initial density ($3\times10^4$ cells/ml) than in the medium with serum. These results show that the threshold density of tested cells in the semisolid medium without serum is higher than in the medium with serum. Maximal CFE for both cell lines is only insignificantly lower than in DMEM with 10% calf serum. It confirms the significance of serum as a complete source of different growth factors. Decrease in CFE for the initial density of $9\times10^4$ cells/ml for both lines growing in semisolid F12/DMEM+ may be due to a stronger negative influence of cell metabolites on proliferation, because this medium does not contain serum, which plays a role of factor attenuating the influence of culture environment changes. The influence of serum on anchorage-independent growth of W12 and GCA cells is probably caused by the presence in serum of different mitogenic factors (EGF, PDGF, IGF-I, *etc.*) and other factors taking part in the growth regulation (insulin, TGF-β, transferrin, *etc.*) [10]. So far, little is known about the mechanism of acquiring the ability of anchorage-independent growth by transformed cells. Edelman's hypothesis assumes that the growth factor receptors present on the cell surface as well as microtubules and microfilaments together participate in the induction of DNA synthesis by growth factors [8]. It means that the phenome-

non of anchorage-independent cell growth may involve the cooperation of cytoskeleton with growth factors switched on after transformation of the cells [12].

The oncogenic transformation leads to a decrease in quantity or complete loss of stretch fibers associated with depolymerization of F- into G- actin and in consequence with the decrease in ability to create fibronectin fibers on the cell surface. It causes a change in the shape of the cell. Simultaneously, the quantity of transmembrane linkages between the extracellular fibronectin with subplasmalemmal microfilaments decreases, resulting in a decreased ability of cells to adhere to the substratum [12].

It is known that a transforming protein of RSV (pp60[src]) is linked to the cytoskeleton network and may influence depolymerization of actin by the phosphorylation of its tyrosine residues. It was shown that pp60[src] is present on the inner surface of the cell membrane and in the adhesion plaques; vinculin and talin, which are present in the plaques, are the candidates for phosphorylation. Pp60[src] forms a complex with vinculin, causing disorganization of actin filament network. In the place of cell-substratum contact, the products (possessing activity of tyrosine kinase) of other oncogenes (c-abl, c-yes) were detected. Pp60[src] probably causes the phosphorylation of protein linking microtubules with microfilaments (MT-MF), leading to the separation of these structures and to disorganization of cytoskeleton.

Changes in phosphorylation of cytoskeleton caused by the oncogene of RSV may be the reason for the altered cell adhesion after viral transformation and in consequence acquirement of ability of anchorage-independent cell growth [12]. Effect of action of viral transforming protein may be reinforced by the growth factors released by cells to their environment, especially when these factors act synergistically [27].

The next confirmation of the release of autocrine growth factors by W12 and GCA cells was obtained earlier in the experiment of coculture of the RSV-transformed cells with NRK-49F as indicator cells. The results suggest that W12 and GCA cells release factors stimulating the anchorage-independent growth of indicator cells [18].

In the next phase of studies, the activity of crude conditioned media was tested. It appeared that stimulation of growth of W12 and GCA cells depending on the concentration of conditioned medium was relatively weak. It may be due to (1) the presence of many peptides with opposite action in the crude conditioned medium, (2) low concentration of growth stimulators in crude conditioned medium before their purification and concentration, (3) the presence of cell metabolites, which have an inhibitory influence on cell proliferation, or, (4) partial consumption of nutritive substances present in crude conditioned medium by cells growing in it during 48-hr incubation; addition of 25% fresh culture medium had to partially supplement the nutritive components in the medium.

The next stage of investigations was the isolation, separation and purification of substances released by W12 and GCA cells to the culture medium. The conditioned media were extracted with 1M acetic acid. The mitogenic activity of these acetic acid extracts (AEs) was tested using the method of $^3$H-thymidine incorporation by cells growing on the solid substratum. This parameter showed a good correlation with the increase in the number of cells during long-lasting culture and was not so sensitive to small environmental changes as the tests in semisolid media. The use of this method led to a quicker estimation of the effect of the obtained extracts and to better reproducibility of results. The increase in DNA synthesis by cells growing in DMEM without serum was proportional to the concentration of AE-W12 or AE-GCA, providing evidence for the presence (in conditioned media of both cell lines) of factors stimulating proliferation of cells. Their activity is specific not only to cells parental for AE because these factors also stimulate growth of NRK-49F and other tumor cell lines, although their maximal activity was demonstrated on the parental cells. Therefore, in the following stages of this study the estimation of activity of purified factors was limited to their influence on the cells of parental lines. The weak specificity of the investigated factors is the property characteristic for the majority of polypeptide stimulators of growth. It is related to the presence of growth factor receptors on the surface of different types of cells.

The influence of the increasing concentrations of AE-W12 on W12 cells (and AE-GCA on GCA cells) growing in soft agar as anchorage-independent was also investigated. For both cell lines a strong stimulation of colony forming ability of target cells was shown. The CFE was also increased, achieving (for maximal concentrations of AEs) values higher than after stimulation by 10% calf serum. Growth factors present in the conditioned media of W12 and GCA cells are not only mitogens for cells growing on the solid substratum but also very well stimulate the anchorage-independent growth, what is typical mainly of TGFs.

AEs reveal not only the mitogenic activity. It was shown earlier that morphological changes (in shape and pattern of growth in culture) of REF cells, characteristic for transformed cells, appeared after incubation with AE-GCA. The surface alterations of REF cells such as an appearance of membrane ruffles, finger-like structures, the increase in the number of coated pits and blebs were also described. These alterations demonstrate that growth factors present in AE-GCA have another biological activity characteristic of many polypeptide growth factors [39].

The experiments discussed above have confirmed the autocrine regulation of proliferation of W12 and GCA cells. Such regulation was supported by purification of the AEs followed by preliminary isolation and identification of autocrine growth factors released by W12 and GCA cells.

Polypeptides present in AE-W12 and AE-GCA were separated on Bio-Gel P60 column and 4 different fractions were obtained. They were also purified: from18- to 250-fold (fraction F2, purification comparable to that after one-step HPLC) [1]. Molecular weights of these fractions evaluated according to elution volume are not very precise, because some polypeptides show tendency to retard the elution from the column [42]. Biological activity of F2 and F4 and their molecular weights (8-12 kDa) can suggest that TGF$\alpha$ may be present there [24]. Fraction F1 was eluted in empty volume of used column. There are factors stimulating cell transformation that possess high molecular weight: 190 kDa, 250 kDa [20], 100 kDa [28] and 75kDa [23]. In this fraction, TGF$\beta$ can also be present. Molecular weights of F3 fraction (25 kDa) correspond to TGF$\beta$ or TGFe (epithelial type, m.w. 22-25 kDa); and other factors were also detected in this range of molecular weight (16-28 kDa)[21]. Our procedure eliminated peptides with molecular weight below 2 kDa and acid-labile factors. All four fractions were thermo- and acid-stable, what agrees with the features of the majority of PGFs [4]. The sensitivity of the obtained fractions to dithiothreitol and trypsin was expectedly high, indicating that for full activity of these factors their peptide and disulphide bonds must be intact.

ELISA test confirmed the release of TGF$\beta_1$ by both cell lines, in quantities (2.6 $\mu$g TGF$\beta_1$/l in CM-W12 and 2.0 $\mu$g/l in CM-GCA) comparable to those detected in the conditioned media of other virally transformed cells (2.4-8.4 $\mu$g TGF$\beta$/1500 ml CM) [2, 17]. The investigated cells seem to release only TGF$\beta_1$, although some cell types such as skin cells [29] and Schwann cells [33] produce both TGF$\beta_1$ and TGF$\beta_2$. Surprisingly, very small quantities of bFGF were found CM-GCA, in spite of its acid-lability.

At first, it was supposed that only Retroviridae, by transforming the cells, confer upon them the ability to produce transforming growth factors. Later, secretion of growth factors with TGF activity was detected in cells transformed by other viruses, such as SV-40 [15, 28] and Polyoma virus [16]. Vaccinia virus [30], Shope fibroma virus [5] and Myxoma virus [41] encode peptides which are similar to EGF and bind to EGF receptors. This study adds avian sarcoma virus to that list.

Future investigations should allow further isolation and identification of other autocrine factors released by W12 and GCA cells.

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