A novel growth stimulating activity from BRL-3A cell conditioned medium

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Abstract. The R⁻ cell line is a 3T3-like cell line originating from mouse embryos with a homozygous disruption of the type 1 insulin-like growth factor receptor (IGF-IR) genes. Although R⁻ cells cannot grow at all in serum-free medium (SFM) supplemented by several known growth factors, either singly or in combination, they are able to grow in 10% serum, albeit at a reduced rate. These findings suggested that serum contains an unknown, or unidentified, growth factor that can promote cell growth even in cells devoid of IGF-IRs. In an effort to identify such growth factor, we searched, using R⁻ cells, for a growth and DNA synthesis stimulating activity in SFM conditioned by different cell lines. We found that the BRL-3A cell line secreted an activity capable of stimulating DNA synthesis and cell proliferation in R⁻ cells. This activity (which is concentration-dependent) can be collected and concentrated by ultrafiltration, it is heat-labile, proteinase K-sensitive and has a size larger than 10 kDa. Because of the resistance of R⁻ cells to stimulation by known growth factors, we believe that this activity is due to a novel polypeptide secreted by BRL-3A cells. Further characterization of the active component(s) is in progress.

R⁻ cell line is a fibroblast cell line, generated, by using the same protocol to generate 3T3 cell lines, from mouse embryos homozygous for a targeted disruption of the *Igf1r* genes (Baker *et al.* 1993, Liu *et al.* 1993), encoding the type 1 insulin-like growth factor receptor. R⁻ cells are able to grow in 10% serum, albeit at a reduced rate in comparison to mouse embryo fibroblasts with a wild-type number of IGF-IRs (Sell *et al.* 1993). However, R⁻ cells cannot grow at all in serum-free medium (SFM) supplemented with purified growth factors. Several known growth factors were tested singly or in association with other growth factors, and all failed to support the growth of R⁻ cells. They include the following growth factors: PDGF, EGF, IFG-I, IGF-II, insulin, b-FGF, a-FGF, TGF- α , TGF- β and HGF (Sell *et al.* 1994). With the exception of TGF- β , all these growth factors (singly or in appropriate combinations) support the growth of W-cells (a fibroblast cell line generated from mouse embryos littermates to the embryos with deleted *Igf1r* genes), or other 3T3-like cell lines. R⁻ cells are also refractory to transformation by SV40 large T antigen (Sell *et al.* 1993), and/or an activated and overexpressed Ras (Sell *et al.* 1994), by the bovine papillomavirus E5 oncoprotein (Morriane, DeAngelis & Baserga 1995), the human papillomavirus 16 E7 (Steller *et al.* 1996) and an activated c-src (Valentinis *et al.* 1997) and by over-expressed growth factor receptors, such as the EGF-R (Coppola *et al.* 1994), the PDGF- β receptor

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(DeAngelis, Ferber & Baserga 1995) and the insulin receptor (Miura *et al.* 1995, Morrione *et al.* 1997). All these conditions readily transform 3T3 cells and other mouse embryo fibroblasts with endogenous IGF-IRs. Re-introduction into R⁻ cells of a plasmid expressing a human IGF-IR cDNA restores growth in SFM supplemented by growth factors and the susceptibility to transformation.

IGF-I is the second growth factor for many cell types (reviewed by Baserga *et al.* 1997a), and, in fact, R⁻ cells overexpressing either the EGF-R (Coppola *et al.* 1994) or the PDGF- β receptor (DeAngelis *et al.* 1995) cannot even grow in SFM supplemented either by epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), indicating that the presence of a functional IGF-IR is required for the stimulation by other growth factors. These special features make R⁻ cells a unique cell line for the testing and isolation of novel growth factors which stimulate cell growth through pathways that are not shared with the known growth factor receptors, including the IGF-IR. The ability of R⁻ cells to grow in 10% serum, but not in SFM supplemented by known growth factors, does indeed suggest that they are stimulated by a not yet identified growth factor; however, since serum is a very complex mixture of stimulatory and inhibitory factors, we hoped that the use of conditioned media may facilitate the search for novel growth factors.

Using R⁻ cells to screen for growth and DNA synthesis stimulating activity in medium conditioned by six cell lines, WI-38, BALB/c 3T3, MCF-7, BRL-7, BRL-3A, T98G and T24H, we found that the last three cell lines could secrete an activity that stimulated the growth of R⁻ cells. We report here our preliminary data on the characterization of this activity from the CM of BRL-3A cells. It is heat-labile, proteinase K sensitive, has a size larger than 10 kDa and the stimulation is concentration-dependent. These results indicate that the CM of BRL-3A cells contains a polypeptide that has growth stimulatory activity on R⁻ cells, and therefore is not one of the better known growth factors. While its purification is in progress, we would like to present here its biological characterization.

MATERIALS AND METHODS

Cell lines and culture conditions

R⁻ cells

All experiments used as the test cell line, R⁻ cells (Sell *et al.* 1993, 1994), which are 3T3-like cells derived from mouse embryos with a targeted disruption of the IGF-IR genes (Baker *et al.* 1993, Liu *et al.* 1993). These cells have been repeatedly described and characterized in previous papers from this laboratory (Coppola *et al.* 1994, Miura *et al.* 1995, Rubini *et al.* 1997). They grow in 10% fetal bovine serum (FBS), but they do not grow at all in SFM supplemented with purified growth factors.

For the production of conditioned medium (CM), the following cell lines were used: WI-38, BALB/c 3T3, MCF-7, BRL-3A, T98G and T24H. WI-38 is a human lung diploid cell line derived by L. Hayflick from normal embryonic (3 months gestation) lung tissue (Hayflick 1965). BALB/c 3T3 have been grown in our laboratory for several years. MCF-7 is a human breast adenocarcinoma cell line derived from a pleural effusion (Soule *et al.* 1973). BRL-3A is a cell line originated as a clone from a 5-week-old female Buffalo rat liver (BRL). Serum-free medium conditioned by the BRL-3A cells produces a family of polypeptides termed multiplication-stimulating activity or MSA which have been reported to be related to somatomedins and stimulate [³H]-thymidine incorporation into DNA in chick embryo fibroblasts and human skin fibroblasts (Dulak & Temin 1973a,b, Dulak & Shing 1977, Nissley *et al.* 1977). T98G is human glioblastoma cell line derived from a glioblastoma multiforme tumour from

a 61-year-old male (Stein 1979). T24H, is an NIH/3T3 cell line transfected with T24 oncogene carrying a point mutation at codon 12 of H-ras oncogene (Srivastava *et al.* 1985). All these cell lines were maintained routinely in DMEM medium containing 10% fetal bovine serum except for BRL-3A, which is maintained in F-10 medium plus 10% fetal bovine serum.

Preparation of conditioned medium

BRL-3A cells (1×10^6) were plated in 10-cm petri dishes in 10 ml of F-10 medium containing 10% FBS and were incubated at 37°C in a humidified CO₂ incubator. After reaching subconfluence, the cells were washed with three times Hank's solution, then incubated in 10 ml of SFM. The following day this medium was discarded, and the cells were washed with Hank's solution, again three times, then fresh SFM was added. The medium from these cultures was repeatedly collected and pooled, unless otherwise noted. Conditioned medium from cultures of BRL-3A cells was harvested for a period of 1 month, after which the cultures were discarded and replaced by new cultures. Collected medium was sterilized using 0.2 µm filter and stored frozen at -20°C until needed. In the primary screening of other cell lines for the identification of stimulating activity in the CM, the same procedure was followed.

Determination of cell growth

For all experiments on stimulation of cell proliferation, the cells were seeded at a density of $5 \times 10^3/\text{cm}^2$ in medium containing 10% FBS and allowed to attach for 24 h. After washing three times with Hank's solution, the cultures were placed in serum-free DMEM medium (SFM), and then treated as described in the respective experiments (see Results). The cell numbers were at time 0 and after 3 days in culture. For Figure 1 only, the SFM was also supplemented with 0.1% bovine serum albumin, and 50 µg/ml transferrin.

DNA synthesis

R⁻ cells were seeded at a density of $5 \times 10^3/\text{cm}^2$ on coverslips in DMEM medium containing 10% FBS and allowed to attach for 24 h. The cultures were then made quiescent for 96 h in SFM prior to the addition of growth factors or CM. Tritiated thymidine (0.5 µCi/ml) was added at the same time as growth factors or CM, and the incubations were continued for 24 h. The cells were then fixed in cold methanol and autoradiographed by standard procedures. The percentage of labelled cells was determined by scoring a total of 1000 cells. DNA synthesis stimulating activity was expressed in percentage of labelled nuclei.

Ultrafiltration and protein assay

YM10 membrane disks (Amicon) were used to concentrate and separate the BRL-3A cell conditioned medium into two different fraction sizes, i.e. smaller than 10 kDa and larger than 10 kDa. Protein concentrations of all samples were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using BSA as the standard.

Biochemical characterization of CM

To observe the effect of heat on the growth stimulating activity of BRL-3A CM, 2 ml each of the original BRL-3A CM were pre-heated for 30 min at 65°C, 85°C, and 100°C, respectively, then used to treat the quiescent R⁻ cells at the same time as addition of unheated CM or 10% FBS or SFM only. To test the effect of proteinase K on this activity, 1 ml each of BRL-3A CM was pre-incubated at 37°C for 20 min either with or without 100 µl of proteinase K beads (PKB, 1 mg/ml, Sigma Chemical Company, St. Louis, MO), which were

then removed from the CM after the pre-incubation, then mixed with SFM, at a ratio of 1:1, and used to treat the quiescent R⁻ cells at the same time as addition of 10% FBS or SFM only. Phenylmethylsulfonyl fluoride (PMSF, Sigma), a proteinase inhibitor, was used at a final concentration of 20 μ M to monitor the specific effect of proteinase K on CM.

RESULTS

Screening of conditioned medium from different cell lines for growth stimulating activity on R⁻ cells

To identify this putative novel growth factor, we screened six cell lines for their ability to secrete in the conditioned medium a growth stimulating activity (Figure 1). R⁻ cells were plated and made quiescent as described in Materials and Methods. The cells were left in SFM, or were supplemented with either 10% FBS, or PDGF (5 ng/ml) and EGF (20 ng/ml) (P+E), or with conditioned medium added at a ratio of 1:1, from cell lines WI-38, BALB/c 3T3, MCF-7, BRL-3A, T98G and T24H (see Materials and Methods for the description of these cell lines and the production of conditioned medium). When compared to SFM, the conditioned media from cell lines BRL-3A, T98G and T24H were found to stimulate the growth of R⁻ cells substantially, the effect being close to that of 10% FBS, while the conditioned media from other three cell lines, WI-38, BALB/c 3T3, and MCF-7, were found

Figure 1. Screening of conditioned medium from different cell lines for growth stimulating activity on R⁻ cells. R⁻ cells were plated in triplicate and treated as described in Materials and Methods. Cell counting was done on triplicate cultures after 72 h incubation at 37°C. The ordinate gives the fold increase in cell number compared to SFM = 1.0. The source of conditioned media is indicated here and in the text. Also shown are the effect of 10% FBS, or a combination of PDGF (5 ng/ml) and EGF (20 ng/ml) (P+E) (see Materials and Methods). Bars are given for means with standard deviations.

to have much less growth-stimulating activity on R^- cells. The BRL-3A cell line has been previously used for the characterization of other novel growth factors (Dulak & Temin 1973a,b, Dulak & Shing 1977, Nissley *et al.* 1977, Straus, Coppock & Pang 1981), and we therefore chose the conditioned medium of this cell line to investigate the growth stimulating activity on R^- cells.

Growth stimulation of diluted BRL-3A-CM on R^- cells

We carried out preliminary experiments to determine the concentration-dependence of the growth stimulating activity. As Figure 2 shows, the stimulation of R^- cells to grow (here after 72 h incubation at 37°C) increased with increasing concentrations of BRL-3A CM. The same experiment was repeated by measuring, instead of cell number, the fraction of cells entering DNA synthesis, as measured by autoradiography with [^{3} H]-thymidine. The results (not shown) confirmed the concentration dependence of the stimulating activity in the CM, suggesting that some factor(s) exist in the BRL-3A CM which enable the R^- cells to enter S phase and eventually to divide.

Growth or DNA synthesis stimulation of BRL-3A-CM on R^- cells is reproducible

To confirm the preliminary data described above, several independent experiments were carried out for both growth promotion and DNA synthesis stimulation of R^- cells by the BRL-3A-CM (Figures 3 & 4). In Figure 3, we determined the increase in cell number of R^- cells, stimulated with either 10% FBS or CM from BRL-3A cells. In six independent experi-

Figure 2. Growth stimulation of R^- cells by diluted BRL-3A conditioned medium (CM). R^- cells were prepared as described in Materials and Methods and Figure 1, and then incubated in SFM supplemented with either 10% FBS or decreasing concentrations of CM from BRL-3A cells. The abscissa gives the ratios of CM to SFM. Cell counting was done on triplicate cultures at time 0 and after 72 h incubation at 37°C. The ordinate gives the cell number $\times 10^4$ per cm^2 .

ments the CM stimulated the proliferation of R⁻ cells, albeit always at a lower level than serum. In Figure 4, DNA synthesis, rather than cell number, was measured, using [³H]-thymidine autoradiography (see Materials and Methods). In seven separate experiments, the CM from BRL-3A cells was found to stimulate DNA synthesis in R⁻ cells. Indeed, the level of stimulation is almost the same as that of 10% FBS, indicating that the growth stimulatory activity in the CM is more effective in inducing DNA synthesis than cell division (see Discussion). These findings also show that the growth or DNA synthesis stimulating activity of BRL-3A-CM on R⁻ cells is reproducible. Because DNA synthesis seems to be a more sensitive indicator of CM activity than cell growth, in subsequent experiments with the CM of BRL-3A cells, we used DNA synthesis as the regular assay for growth stimulating activity.

The stimulating activity is repeatedly secreted into the conditioned medium

In order to collect large amounts of CM for subsequent studies, we asked whether the growth stimulating activity could be collected repeatedly from the same plate of BRL-3A cells. Different rounds of collection of BRL-3A conditioned medium, designated as CMr-1, 2, 3, 6, 7, 8 in Figure 5, were tested for their ability to stimulate DNA synthesis in R⁻ cells. Even the eighth round (CMr-8) of collection of CM from the same plates of BRL-3A cells could stimulate DNA synthesis in R⁻ cells, although a slight decrease in activity could be perceived after repeated harvesting from the same plate.

Figure 3. Growth stimulation of R⁻ cells by BRL-3A-CM is reproducible. R⁻ cells were plated in triplicate at a density of $5 \times 10^3/\text{cm}^2$ overnight at 37°C, then washed with Hank's solution three times, and treated as indicated below. Cell counting was done on triplicate cultures at time 0 (horizontally striped bar) and after 72 h incubation at 37°C with SFM (closed bar), 10% FBS (striped bar), or BRL-3A conditioned medium (open bar). The ordinate gives the cell number $\times 10^4$ per cm^2 . Data from six independent experiments are given.

Figure 4. Stimulation of DNA synthesis by BRL-3A-CM of R^- cells. R^- cells were plated and made quiescent as described in Materials and Methods. They were then left unstimulated (SFM, closed bar) or treated with either 10% FBS (striped bar) or BRL-3A conditioned medium (open bar). The percentage of nuclei labelled by [^{3} H]-thymidine in a 24-h period is shown on the ordinate. Data from seven independent experiments are given.

Growth and DNA synthesis stimulating activity is larger than 10 kDa

Ultrafiltration through the YM10 membrane disks were initially used to separate the CM into two fractions, i.e. larger than 10 kDa and smaller than 10 kDa. The CM was also concentrated about 30 times during this procedure. When tested for activity, the volumes of the fraction were adjusted so that their effects could be compared on the same activity/volume basis. In three independent experiments, the stimulating activity was found in the fraction larger than 10 kDa, albeit the stimulation was lower than that observed for the original CM or for 10% FBS (Figure 6a). The fraction smaller than 10 kDa was found to have negligible growth promoting effect on R^- cells (Figure 6a). Again, the differences were clearer when the fractions were tested for their ability to stimulate DNA synthesis (Figure 6b). In fact, in this assay, the fraction larger than 10 kDa was as active as 10% FBS in stimulating DNA synthesis, while the fraction lower than 10 kDa was essentially inactive. In addition, a series of concentrations (0, 0.25, 0.5, 1.25, 2.5, 5.0, 7.5 and 10 μ g/ml) of partially purified protein from the fraction larger than 10 kDa were tested for their ability to stimulate DNA synthesis in R^- cells. The results (Figure 7), clearly show a concentration dependent activity in this fraction, consistent with the data shown in Figure 2 for the unfractionated CM. Therefore, the growth and DNA synthesis stimulating activity in BRL-3A CM is larger than 10 kDa.

The DNA synthesis stimulating activity is heat-labile and proteinase K-sensitive

To test other characteristics of the CM activity, R^- cells were made quiescent, and then treated with either 10% FBS, or BRL-3A CM, or CM pre-heated at 65°C, 85°C, and 100°C for 30 min, or left unstimulated (SFM). The results shown in Figure 8, panel a, indicate that

the DNA synthesis stimulating activity is heat-sensitive. This activity can also be abrogated by proteinase K and the effect could be counteracted by addition of a proteinase K inhibitor (Figure 8b). The results from these experiments suggest that the DNA synthesis stimulating activity in BRL-3A CM is associated with a polypeptide larger than 10 kDa.

DISCUSSION

R⁻ cells are fibroblasts generated from mouse embryos with a targeted disruption of the IGF-IR genes (Baker *et al.* 1993, Liu *et al.* 1993) by the same protocol used to generate 3T3 cells. They have been extensively characterized in previous publications: (1) they grow in 10% serum, albeit at a reduced rate (Sell *et al.* 1993); (2) they do not grow in SFM supplemented by purified growth factors (Sell *et al.* 1993, 1994); and (3) they are refractory to transformation by a variety of cellular and viral oncogenes (Sell *et al.* 1993, 1994, Coppola *et al.* 1994, DeAngelis *et al.* 1995, Morriane *et al.* 1995, Miura *et al.* 1995, Steller *et al.* 1996, Valentinis *et al.* 1994, 1997), that readily transform other 3T3 cell lines. The growth deficits of R⁻ cells are promptly corrected by the stable transfection of a plasmid expressing the human IGF-IR cDNA.

The fact that R⁻ cells are totally unresponsive to known, available growth factors, and yet grow in 10% FBS, suggests that serum must contain another growth factor, which has not yet been identified, and that can stimulate the growth of cells devoid of IGF-IRs. Identification

Figure 5. The DNA synthesis stimulating activity of CM from BRL-3A cells can be repeatedly collected. R⁻ cells were plated and made quiescent as described in Materials and Methods. They were then treated with either 10% FBS, or CM from different rounds of collection from the same plates of BRL-3A cells. CMr-1, 2, 3, 6, 7, 8, represents consecutive rounds of collection. The percentage of nuclei labelled by [³H]-thymidine in a 24-h period is shown on the ordinate. Control cells were left unstimulated in SFM.

Figure 6. The growth and DNA synthesis stimulating activity is larger than 10 kDa. (a) R⁻ cells were plated in triplicate at a density of $5 \times 10^3/\text{cm}^2$ overnight at 37°C, and treated as usual. Bars: day 0 (dotted bar), SFM (closed bar), 10% FBS (striped bar), BRL-3A conditioned medium (open bar), fraction larger than 10 kDa (horizontally striped bar), fraction smaller than 10 kDa (vertically striped bar). See Materials and Methods for the fraction of CM. Cell counting was done on triplicate cultures at time 0 and after 72 h incubation at 37°C. The ordinate gives the cell number expressed in $\times 10^4/\text{cm}^2$. Data from three independent experiments are given. (b) R⁻ cells were plated and made quiescent as described in Materials and Methods and in (a). The percentage of nuclei labelled by [³H]-thymidine in a 24-h period is shown on the ordinate. Bars: SFM (closed bars), 10% FBS (striped bar), BRL-3A conditioned medium (open bar), fraction larger than 10 kDa (horizontally striped bar), fraction smaller than 10 kDa (vertically striped bar). Data from four independent experiments are shown.

Figure 7. DNA synthesis stimulating activity is concentration-dependent. R^- cells were plated and made quiescent as described in Materials and Methods. They were then treated with different concentrations (0, 0.25, 0.5, 1.25, 2.5, 5.0, 7.5 and 10 $\mu\text{g/ml}$) of partially purified proteins from the fraction of the CM larger than 10 kDa. The percentage of nuclei labelled by [^3H]-thymidine in triplicate cultures in a 24-h period is shown on the ordinate.

of growth factors in serum are notoriously difficult, and we thought that we could circumvent this problem by identifying a similar activity in the CM of certain cell lines. Although even this approach has also proved to be laborious, we would like to present here our data indicating that: (1) a growth stimulating activity for R^- cells can be identified in the CM of some, but not all, cell lines; and (2) a partial characterization of this activity indicates that it has the properties of a polypeptide larger than 10 kDa. This statement is supported by the finding that the activity is heat-labile, proteinase K-sensitive and can be fractionated by size.

This activity, for which we propose the temporary name of BRL-3A growth factor (BRL-GF), induces DNA synthesis more efficiently than cell growth. This is not unusual, and there are many reports in the literature indicating that certain stimuli can induce DNA synthesis without stimulating cell division, or with a much lower induction of cell division (DeAngelis *et al.* 1995, Valentinis *et al.* 1994, for a review Baserga 1985). In fact, many purified growth factors are as good as 10% serum in stimulating DNA synthesis, but rarely match serum (even in combination) in stimulating cell division. We have not tested BRL-GF for transformation, because colony formation in soft agar requires already 10% serum, even when the cells are completely transformed.

When BRL-3A cells are grown on a separate coverslip in the same dish as R^- cells, they can stimulate DNA synthesis in the latter cells (Valentinis *et al.* 1997), indicating that BRL-GF is secreted in substantial amounts and is a powerful growth factor. It has been further purified through various columns, and it probably is related to a molecule of about 75 kDa (unpublished data from our laboratory), but the last steps of purification have proved to be very difficult. However, it is not one of the known growth factors, for the reasons given

Figure 8. The DNA synthesis stimulating activity is heat labile and protease sensitive. (a) R^- cells were plated and made quiescent as described in Materials and Methods. They were then treated with 10% FBS (striped bar), BRL-3A conditioned medium (CM, open bar), CM pre-heated for 30 min at 65°C (dotted bar), 85°C (vertically striped bar), and 100°C (horizontally striped bar), respectively, or left unstimulated (SFM, closed bar). The percentage of nuclei labelled by [^{3} H]-thymidine in a 24-h period is shown on the ordinate. (b) R^- cells were plated and made quiescent as described in Materials and Methods. SFM (a) and 10% FBS (b) were included as controls. One ml of BRL-3A conditioned medium was pre-incubated at 37°C for 20 min without (c) or with (d) 100 μ l of proteinase K beads (PKB, 1 mg/ml) before mixing with 1 ml of SFM and adding to the quiescent R^- cells. PKB was also used to treat the CM along with PMSF (20 μ M) (e) to monitor its specific effect. The mean percentage of nuclei labelled by [^{3} H]-thymidine in duplicate in a 24-h period is shown on the ordinate.

above, and also because of its probable size. Again because of its probable size, we have directly tested the effect of hepatocyte growth factor (HGF) on R⁻ cells, but the results indicated that the HGF was unable to stimulate DNA synthesis in R⁻ cells and an anti-HGF antibody could not neutralize the DNA synthesis stimulating activity of partially purified protein from the BRL-3A CM (not shown).

Despite the preliminary nature of this report, we thought that it should be made known, because it makes two important points: (1) CM from BRL-3A cells (and serum) contains a polypeptide that does not have the characteristics of known growth factors, and is therefore in all probability a novel growth factor; and (2) because IGF-I is a required growth factor for many cell types in defined media (Baserga *et al.* 1997), R⁻ cells constitute a valuable resource in the search of growth factors that bypass the requirement for IGF-I.

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