SIDEROPHORE-LIKE GROWTH FACTOR SYNTHESIZED BY SV40-TRANSFORMED CELLS ADAPTED TO PICOLINIC ACID STIMULATES DNA SYNTHESIS IN CULTURED CELLS

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1. Introduction

Medium conditioned by contact with SVT2Pi3 cells, a mutant of SV40-transformed BALB/3T3 adapted to grow in picolinic acid, contains a highly specific iron-binding ligand. The material, termed siderophore-like growth factor (SGF), was also produced by SVT2Pi3-A cells, a subline of SVT2Pi3 cells that grow in the absence of serum. Experiments have indicated that SGF is an ionophoropeptide of mol. wt ~1600. At a physiologically relevant concentration, SGF stimulates Fe^{3+} uptake and DNA synthesis in the mutant cells. These observations provide evidence for the existence of mammalian siderophores which may be important in the initiation of DNA synthesis in transformed cells.

The results of previous studies indicate that iron depletion may be the primary cause of the growth arrest induced by picolinic acid in cultured cells [1–3]. To investigate the mechanism of action of picolinic acid, mutant cell lines of SV40-transformed BALB/3T3 cells adapted to grow in picolinic acid have been isolated [4–6]. Here we report the finding of a siderophore-like growth factor in ultrafiltrates of medium conditioned by contact with the mutant cell lines. The discovery of SGF provides the first direct evidence for the existence of mammalian siderophores which may have an important role in cell proliferation.

2. Materials and methods

2.1. *Materials*

$^{59}Fe^{3+}$, $^{54}Mn^{2+}$, $^{57}Co^{2+}$ and $^{65}Zn^{2+}$ were obtained from ICN or New England Nuclear. [^{3}H]Picolinic acid (spec. act. 455 mCi/mmol) was custom made by Amersham/Searle. Sephadex was purchased from Pharmacia. Other materials were obtained from sources described [1–3].

2.2. *Cell culture*

Unless otherwise specified, cells were grown in Dulbecco-Vogt modified Eagle's medium (DEM) containing 10% calf serum (v/v) as in [4].

2.3. *Selection of mutant cells resistant to picolinic acid and adaptation of the mutant cells to grow in serum-free media*

The methods for isolation of cells resistant to picolinic acid have been described [4]. The cell line adapted to grow in 3 mM picolinic acid have been designated SVT2Pi3 [6]. This adaptation could be maintained by incubation in picolinic acid at suboptimal growth inhibitory concentrations (e.g., 1.5 mM). SVT2Pi3-A, is a subline of SVT2Pi3 cells that multiplies in the absence of serum [6].

2.4. *Factor purification*

To prepare 5.4 liters conditioned media, 8×10^8 SVT2Pi3 cells were planted in 120 tissue culture flasks (75 cm^2) in media containing 10% calf serum plus 1.5 mM picolinic acid. The medium from the cultures was collected, pooled, centrifuged at 4°C to sediment any cells, and stored frozen at −20°C until processing. Similarly, 1.8 liters conditioned media were collected from SVT2Pi3 cells grown in media with 10% calf serum without picolinic acid. An equal amount of media conditioned by SVT2Pi3-A cells grown in

serum-free media without picolinic acid were subjected to fractionation. The media was passed in succession through Amicon UM10, UM2 and UM05 filters in an ultrafiltration cell. The same concentration factor was used for all experiments (× 30). All operations were performed under sterile conditions at 4°C.

Ultrafiltrates were extensively tested during and after processing for bacterial and fungal contamination with negative results. Quality of ultrafiltrates was determined by polyacrylamide electrophoresis. Each of the ultrafiltrates (mol. wt: > 10 000; 10 000–1000; 1000–500; 500–0) was tested for radioactive ion-binding activity. For this purpose, centrifuged (30 000 × *g*/30 min), pH adjusted (7.0), ultrafiltrate samples were incubated with radioactive ions.

Samples were then chromatographed in Sephadex G-15, G-25, G-50 and G-75. The columns were equilibrated with a variety of buffers or deionized H_2O. The material which was retarded by the UM2 filter contained SGF activity. This ultrafiltrate was diluted 20 times with deionized water in the ultrafiltration cell and was concentrated again (× 20). Samples from this ultrafiltrate were further purified by treatment with activated charcoal and tested for ion-binding activity as indicated above.

2.5. *Biological assay of active fractions*

Fractions from gel filtration were tested on SVT2Pi3 cells for their ability to stimulate ion uptake and DNA synthesis. Cells were planted at approx. 10^5 cells/35 mm dish in DEM containing 0.5% calf serum. After 76 h, the media was replaced by new media containing 0.25% calf serum. Within 5 min of this medium change, equal aliquots from individual or pooled column fraction were added to the cultures. For determination of DNA synthesis, immediately after sample addition, 5 μl DEM containing 2 μCi [*methyl*-^{3}H]thymidine (New England Nuclear) were added to each dish. After 24 h, 48 h and 72 h incubation, incorporation of [^{3}H]thymidine into acid insoluble material was measured as in [1]. All biological assays reported here (ionic uptake and DNA synthesis) were reproduced in 6 separate experiments each.

2.6. *Other procedures*

The column fractions were assayed for quantitative ninhydrin as in [7]. Polyacrylamide electrophoresis was performed exactly as in [8]. Uptake of radioactive ions into total and acid-insoluble cell material was performed as indicated [3]. Atomic absorption spectrometry was done with a Perkin-Elmer atomic absorption spectrophotometer (Model 370).

3. Results

3.1. *In vitro characterization of SGF*

SGF was assayed in vitro by its capacity to carry $^{59}Fe^{3+}$ through gel filtration columns. SGF activity was found in the ultrafiltrate of mol. wt 10 000–1000 (fig.1a) and was absent in ultrafiltrates mol. wt: > 10 000; 1000–500 or 500–0. SGF was present in conditioned media of picolinic acid-treated cells. The factor was undetectable by atomic absorption spectrometry and just detectable by radioactive techniques in media from SVT2Pi3 cells grown in the absence of picolinic acid. The possibility that Fe-binding ligands present in calf serum contribute to some binding in vitro cannot be excluded by these studies, However, SGF is clearly produced by the cells because it was found in serum-free conditioned media (fig.1a–c). The factor did not bind $^{65}Zn^{2+}$ and no significant binding to $^{54}Mn^{2+}$ was observed. However, the factor clearly showed $^{57}Co^{2+}$-binding activity. The factor did not adsorb to activated charcoal (fig.1a). Fractions D and E (fig.1c) are ninhydrin positive (negative ninhydrin reaction prior to hydrolysis). No ninhydrin positive materials were detected before or after hydrolysis in Fractions D and E of control conditioned media. SGF was dialyzable through standard membranes (Spectraphore 2000). In gel filtration experiments through Sephadex G-25 SGF activity eluted just in front of bacitracin (mol. wt 1411). Thus, these experiments indicate that SGF is a peptide with a mol. wt 1500–1700.

3.2. *Stimulation of Fe^{3+} accumulation in SVT2Pi3 cells by SGF*

As shown in fig.1, the relationship between Fe^{3+} binding in vitro, Fe^{3+} uptake and DNA synthesis was investigated. Experiments clearly showed that exactly the same fractions that bind $^{59}Fe^{3+}$ in vitro promote $^{59}Fe^{3+}$ accumulation in vivo (fig.1a,b). Significant differences in $^{59}Fe^{3+}$ uptake with respect to control

could be found as early as 1 min of addition of the factor. Although SGF binds $^{57}Co^{2+}$ in vitro, it did not stimulate accumulation of $^{57}Co^{2+}$ in vivo. $^{59}Fe^{2+}$ and ^{54}Mn uptake remained unchanged by SGF.

3.3. *Stimulation of DNA synthesis by SGF*

Figure 1c shows that both fractions D, E stimulated DNA synthesis while other column fractions were ineffective. These results were confirmed and quantitized by autoradiography and flow microfluorometric analysis (data not shown).

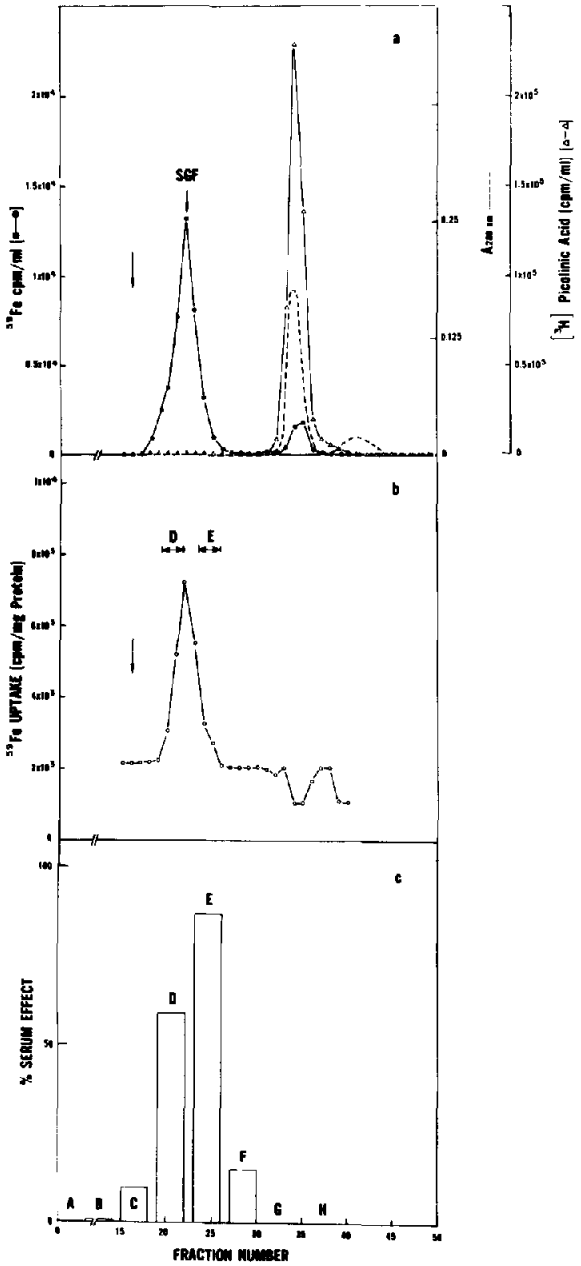

Fig.1. In vitro binding of $^{59}Fe^{3+}$ and in vivo stimulation of $^{59}Fe^{3+}$ uptake and [^{3}H]thymidine incorporation by fractions from a Sephadex G-25 column (0.9 × 60 cm). Conditioned media in which SVT2Pi3-A cells had grown for 48 h was processed as described in the text.

(a) In vitro binding of $^{59}Fe^{3+}$. A sample of ultrafiltrate (mol. wt 10 000–1000) adjusted to pH 7.0, was incubated with $^{59}FeCl_3$ for 16 h. The sample was then applied to a Sephadex G-25 column. Water was the eluant. The elution pattern of [^{3}H]picolinic acid from a separate experiment is superimposed for comparison with the elution point of SGF.

(b) Uptake of $^{59}Fe^{3+}$. A sample from ultrafiltrate (a) was chromatographed, without isotope added, in a separate column. Fractions were lyopholized, reconstituted with deionized water and aliquots were mixed with $^{59}Fe^{3+}$. $^{59}Fe^{3+}$ uptake was tested on SVT2Pi3 cells. Values represent total $^{59}Fe^{3+}$ uptake at 8 h. Each point is the average from 2 cultures.

(c) DNA synthesis. A sample of ultrafiltrate was chromatographed as in (b). Fractions were arbitrarily divided (A–H), pooled lyophilized and reconstituted with deionized H_2O. DNA synthesis in SVT2Pi3 cells was measured after 72 h of continuous exposure to the isotope and ng quantities of fraction material (e.g., fraction E, represents 366 ng/ml tissue culture media, maximal amount of material added to the cells in this experiment). Results are expressed as a percentage stimulation (1.63×10^5 cpm/mg protein) produced by 0.25% calf serum. Each point is the average from 3 cultures. Standard deviation did not exceed 8% of the mean.

4. Discussion

The in vitro and in vivo specificity of SGF for iron precisely correlates with the ability to stimulate DNA synthesis (fig.1a–c). These findings indicate that the same factor regulates iron uptake and DNA synthesis. Iron has been recognized as an essential element in the control of DNA synthesis in cultured cells [3,9,10]. Therefore, SGF may be the first example of a mammalian-siderophore growth factor which plays an important role in the control of DNA synthesis in the mutant cell lines.

Siderophores have been clearly demonstrated in microorganisms and the presence of such compounds in plants have been reported (reviewed in [11]). Plasma of pregnant women or humans with iron-deficient anemia contains low mol. wt iron-binding ligands which have not been characterized [12]. Small mol. wt biological ion carriers are thought to exist or have been isolated [13]. An electrogenic K^+/Ca^{2+} ionophore from beef-heart mitochondria was characterized as a neutral peptide of mol. wt 1600 [14]. Peptides have been shown to have the ability to bind metal ions [15]. Furthermore, low mol. wt synthetic peptides have the capacity of forming ion channels in artificial membranes [16]. These studies, considered in conjunction with the present results, suggest that SGF is an iron-ionophore peptide released by the cells to the culture media.

The present studies suggest that cellular or extracellular chelates, like SGF, may have a relevant function as components of the transformed cell micronutrient transport system(s) for Fe and other trace metal ions. In addition, the findings presented here support the hypothesis [6] that neoplastic cells produce chelating agents, like SGF, which may be important determinants of uncontrolled growth and invasiveness of transformed cells.

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