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A β-Type Transforming Growth Factor, Present in Conditioned Cell Culture Medium Independent of Cell Transformation, May Derive From Serum

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An alpha-type transforming growth factor (TGF_α) is produced at high levels by rat embryo cells transformed by the Snyder-Theilen strain of feline sarcoma virus (FeSV). Addition of 2 ng mouse epidermal growth factor (mEGF) during purification identified the presence of a second, EGF-dependent growth factor of the TGF beta type (TGF_β) in this conditioned medium. This factor had an approximate Mr of 12,000 and eluted at 37% acetonitrile during high performance liquid chromatography. This extracellular type of TGF_β activity also was present in conditioned medium of rat cells after infection with a transformation defective strain of Abelson leukemia virus, and hence expression of this growth factor activity was independent of cell transformation. Moreover, the presence of an EGF-dependent, 12,000 Mr clonogenic activity in extracts of bovine serum alone suggests serum as an origin for the B-type transforming growth factor initially observed in conditioned medium of Snyder-Theilen FeSV transformed cells. This does not, however, preclude the possibility that TGF_β is also secreted by the transformed rat embryo cells themselves.

Key words: peptides, serum, fibroblasts, colony formation, growth factor

The complexity of the detection system that operationally defines transforming growth factors (TGFs), based on anchorage-independent growth of otherwise normal cells in soft agar medium, has been emphasized recently [1]. Two distinct classes of TGFs, designated α and β, have been described, each with different biochemical properties and each with its own receptor [2]. Whereas TGF_α was associated with human tumors [3] and with retrovirus transformed cells of several species [4–7],

Abbreviations used: NRK, normal rat kidney; EGF, epidermal growth factor; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagles's medium; HPLC, high performance liquid chromatography.

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TGF_β was found in normal tissues of bovine kidney [8], human placenta [9], and in particularly high concentration in human platelets [10]. TGF_αs are related functionally to EGF and, unlike TGF_βs, compete with EGF for binding to [4] and stimulate tyrosine phosphorylation of well-defined membrane receptors [11]. To obtain very large colonies (> 9000 sq μm, 0.1 mm in diameter, or more than 50–100 cells) in soft agar, either TGF_α or EGF must be present with TGF_β. Without their combined synergistic action, TGF_α and TGF_β alone are relatively ineffective in promoting anchorage-independent cell growth in NRK cells.

During the stepwise isolation of a TGF_α obtained from the conditioned medium of Fisher rat embryo cells transformed by the Snyder-Theilan strain of feline sarcoma virus (ST-FeSV FRE CL 10) [12], it became evident that, paradoxically, increased purification resulted in diminished colony size in soft agar assays. In view of the requirement for the combined presence of TGF_α and TGF_β to effect maximal clonogenicity, the possibility was examined that progressive steps in isolation removed a second growth factor necessary for optimum colony growth in soft agar. Accordingly, exogenous mouse epidermal growth factor (mEGF) was added to the various fractions during the purification process. This revealed a previously unrecognized growth factor of the TGF_β type in that 1) a peak of clonogenic activity was detectable only after EGF addition, 2) the peak fractions potentiated by EGF failed to compete with EGF for binding to EGF membrane receptors, and 3) the clonogenic activity eluted during high performance liquid chromatography as a hydrophobic molecule [8–10]. Recently, this TGF_β was described and characterized in ST-FeSV transformed rat embryo cells by Massagué [13]. Accordingly, conditioned medium was evaluated from transformation-defective Abelson-MuLV infected rat cells, as well as bovine serum alone, for this EGF-dependent growth factor activity. Unexpectedly, the β-type TGF factor was present independent of cell transformation, and a factor similar in size and biological activity was detected in serum extracts. The identification of this TGF_β-like growth factor in conditioned medium of both transformed and nontransformed cells and its possible origin from serum is the subject of this letter.

Fisher rat embryo cells transformed by different isolates of feline sarcoma virus each release TGF_αs into cell culture medium that are functionally and biochemically similar [12]. As shown in Figure 1A for medium conditioned by ST-FeSV FRE CL 10 cells, the major peak of EGF-competing radioreceptor activity was found to elute at around 10,000 Mr (fractions 68–77) on a P-30 Bio-Gel column. An additional minor peak of EGF-competing activity eluted in the 20,000 Mr range (fractions 38–46). Moreover, identical peaks in approximate proportion to their EGF-competing activity are seen when these column fractions are examined for their ability to promote clonal growth of NRK cells in soft agar medium (Fig. 1B, filled circles). Addition of exogenous mouse EGF (2 ng per fraction) to each fraction revealed a new EGF-dependent clonogenic activity without EGF-competing activity in fractions 58–66, corresponding to the 12,000 Mr range (Fig. 1B, open circles). This previously unrecognized clonogenic activity was also heat- and acid-stable and required intact disulfide bonds for activity (data not shown). Consequently, conditioned medium from ST FeSV FRE CL 10 cells contain a TGF_β-like polypeptide as well as the reported TGF_α type [12], which confirms the work of Massagué [13,14].

To examine whether TGF_β-like clonogenic activity was related to the transformed phenotype, conditioned medium from transformation-defective Abelson-MuLV infected rat cells [6] was evaluated. Interestingly, the EGF-dependent clonogenic

activity was present in the absence of the viral-induced transformed phenotype (Fig. 1C). The demonstration of this TGF_β-like activity in conditioned medium of rat cells nonproductively infected with transformation-defective Abelson-MuLV mutants, which do not secrete TGF_αs [6], indicates its presence to be independent of TGF_α production. Because platelets contain a tenfold higher concentration of TGF_β [10] than other tissues [8,9] and because serum itself contains a platelet-derived transforming growth factor [15,16] 250 ml of fetal calf serum was acid-ethanol extracted, equivalent to what would be present in 2.5 liters of Dulbecco's MEM used for cell culture growth of ST-FeSV FRE CL 10 cells or transformation-defective Abelson-MuLV mutant cells. After P-30 Bio-Gel chromotography, a peak of soft agar activity was detected at approximately 12,000 Mr only after an addition of 2 ng exogenous EGF (Fig. 1D).

Complete separation of the TGF_α and TGF_β from conditioned medium of ST-FeSV FRE CL 10 cells was achieved by high performance liquid chromatography on C_{18} in a Bondapak column using a linear elution gradient of acetonitrile in 0.1% trifluoroacetic acid (Fig. 2). The two clonogenic activities present in fraction pool 58–77 from the P-30 Bio-Gel chromatography shown in Figure 1 elute differently in reverse phase HPLC. The EGF-competing activity (Fig. 2A) elutes much earlier (approximately 20% acetonitrile) than the clonogenic activity potentiated by addition of EGF (approximately 37%) (Fig. 2B, filled circles). This latter activity did not compete with EGF for binding to A431 membrane receptors and stimulated colony formation in soft agar only in the presence of exogenously added EGF (or TGF_α). Thus, by apparent molecular weight (12,000 Mr), HPLC elution (37% acetonitrite), biologic behavior (clonogenicity promoted by very low nanogram amounts of EGF or TGF_α), and its insensitivity to heat, acid, and heparin, the factor resembles TGF_β present in neoplastic [2] and normal [17] cells.

These results confirm recent reports [13,14,18,19] and extend them in that this TGF_β-like factor also is present in conditioned medium of cells infected with transformation-defective virus. Consistent with our observation, an EGF-dependent NRK colony-stimulating activity produced by nontransformed (AKR-2B) cells has been reported previously [19]. Moreover, our preliminary evidence indicates its presence in serum alone. Consequently, because conditioned medium of transformation-defective mutants of rat embryo cells and even serum itself apparently contain this type of EGF-dependent growth factor, expression of this β-type TGF is independent of TGF_α production and the transformed phenotype and may derive from serum itself.

Previously, homogenous preparations of highly purified TGF_α of human melanoma origin were shown to cause efficient phenotypic transformation of NRK indicator cells in culture [20]. This observation probably can be explained on the basis of high concentrations of a TGF_β-like factor in serum lots used in assaying human TGF_α preparations for biological activity in the soft agar assay. It has been shown that platelet-derived growth factor (PDGF) levels in whole blood serum from normal humans and baboons far exceeds that found in their plasma alone [21]. Moreover, the concentration of $_\beta$TGF, like PDGF, is low in platelet-poor plasma [15]; similarly, the concentration may be elevated in whole blood serum by the clotting process. This may as well be true for other serum-derived growth factors. Development of a defined culture medium free of endogenous serum-derived transforming growth factors both to obtain conditioned cell culture medium and to use in soft agar assays will help greatly to resolve the dilemma of the origin of this TGF_β-like activity.

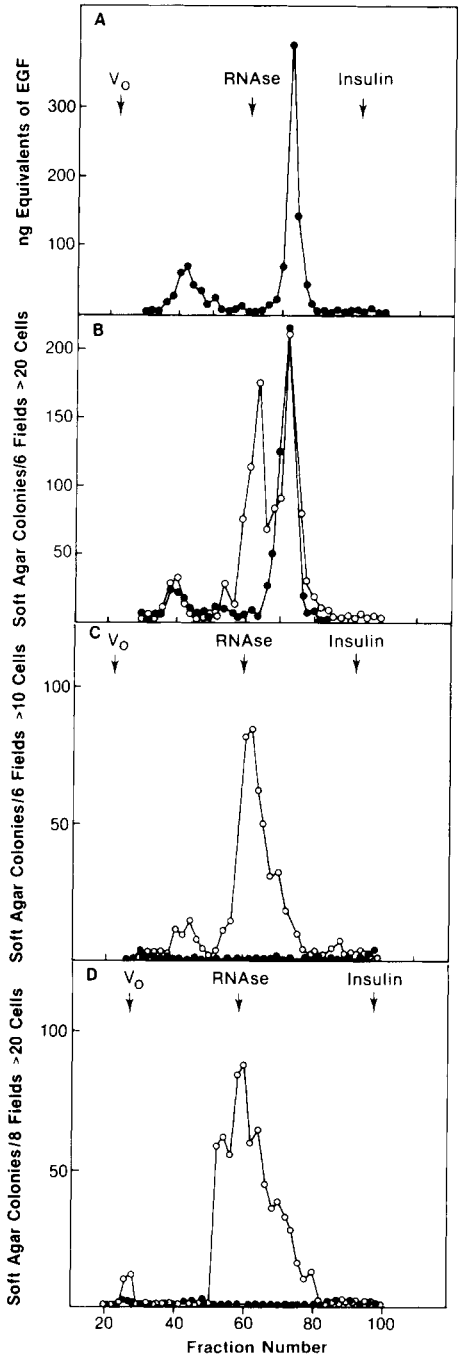

Fig. 1. Bio-Gel P-30 chromatography of acid-ethanol extracts of conditioned medium from ST-FeSV transformed rat embryo cells (A,B), transformation-defective Abelson-MuLV mutant rat embryo cells (C), and fetal calf serum (D). Radioreceptor assay (A) and soft agar assay (B,C,D) in the absence (●) and presence (○) of 2 ng EGF. Aliquots of 200 μl were assayed from 3.5 ml fractions. The growth conditions of ST-FeSV FRE CL 10, and transformation-defective Abelson-MuLV infected rat cells, has been described [12,6]. After confluent T-150 flasks of each cell line were rinsed with serum-free

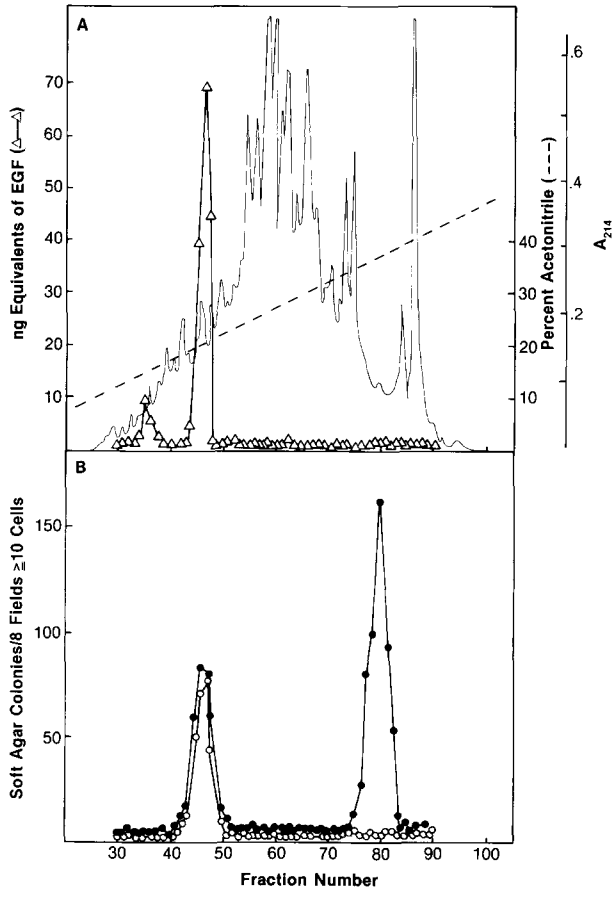

Fig. 2. HPLC of pool of fractions (58–77) from Bio-Gel P-30 chromatography (Fig. 1A,B). Aliquots of 200 μl from alternate fractions were lyophilized for radioreceptor assay (A) and soft agar assay (B) in the absence (○) and presence (●) of 2 ng EGF in each fraction. A Waters Associates (Milford, MA) C18 u-Bondapak® reverse phase column (3.9 × 300 mm) was employed on an Altex model 324 HPLC (Beckman Instruments, Palo Alto, CA). Gradient elution over a period of 2 hr at a flow rate of 1 ml/min and at room temperature, from 0 to 60% acetonitrile (Burdick and Jackson) in 0.05% trifluoroacetic acid (TFA) (Pierce Chemicals) adjusted to pH 2.5 with ammonium hydroxide, was used to develop the chromatogram after an initial isocratic elution for 5 min with 0.05% TFA. One ml fractions were collected. Duplicate aliquots (100 μl) were taken for EGF-competition assays, and 800 μl aliquots for soft agar colony assays as described below.

medium, three successive 24 hr harvests of conditioned media (2.5 liter total) were obtained for acid-ethanol extraction [6]. EGF-competing activity of lyophilized aliquots of individual fractions was determined as previously described [3]. Assays were performed in multi-well tissue culture plates (Linbro/Flow Labs, Hamden, Connecticut) using A431 human carcinoma cells seeded at 8×10^3 cells/well and HPLC-purified mouse EGF as the ^{125}I-labeled tracer and reference standard. Soft agar clonogenic assays were carried out in Dulbecco's modified Eagle's medium containing 10% calf serum as previously described [22]. A 0.5% agar base layer and 0.3% agar overlay containing the test sample were used. Normal rat kidney (NRK) cells, clone SA6, were seeded at 3×10^3 cells/35 mm dish, overlaid with additional medium containing 0.3% agar on day 5, and were incubated at 37° C in a humidified 5% CO_2 atmosphere and scored on the indicated day, usually day 14, for the number of colonies, 10 cells or larger or 20 cells or larger, as indicated per eight low power fields.

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