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PDGF-LIKE GROWTH FACTOR INDUCES EGF-POTENTIATED PHENOTYPIC TRANSFORMATION OF NORMAL RAT KIDNEY CELLS IN THE ABSENCE OF TGFβ

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SUMMARY. Using a growth factor defined assay for anchorage-independent growth (van Zoelen, E.J.J., van Oostwaard, Th.M.J., van der Saag, P.T. and de Laat, S.W. (1985) J. Cell. Physiol. 123, 151- 160), we have studied the ability of polypeptide growth factors produced by Neuro-2A neuroblastoma cells to induce anchorage-independent growth of normal rat kidney cells. Neuro-2A cells produce and secrete a PDGF-like growth factor in addition to TGFβ, which can be fully separated from each other by means of reverse-phase HPLC. Using a new, very sensitive technique for detection of TGFβ in growth factor samples based on its additional ability to act as a growth inhibitory factor, it is shown that the PDGF-like growth factor does not contain any detectable TGFβ. Still this neuroblastoma derived PDGF-like growth factor is able to induce anchorage-independent growth of NRK cells, particularly in the additional presence of EGF. It is concluded that under growth factor defined assay conditions TGFβ is not essential for phenotypic transformation of NRK cells.

Transforming growth factors (TGFs[2]) have the ability to reversibly induce phenotypic transformation of non-transformed cells. Anchorage-independent proliferation of normal rat kidney (NRK) cells has been shown to require the combined action of TGFα and TGFβ, in which epidermal growth factor (EGF) can substitute for TGFα (1). Studies on the exact role of polypeptide growth factors in this process have always been hampered, however, by the additional presence of growth factors from serum in the assay. Platelet-derived growth factor (PDGF) for example, is unable to induce anchorage-independent proliferation of NRK cells under serum-containing assay conditions, nor is it able to potentiate the activity of TGFβ (2). When plasma is used in the assay media instead of serum, however, PDGF strongly potentiates the combined activity of

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[2]Abbreviations: TGF, transforming growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; NRK, normal rat kidney; SH-FCS, growth factor-inactivated fetal calf serum; dThd, thymidine.

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EGF and TGFβ (3). Recently, methods have become available to culture NRK cells under growth factor-defined (4) or serum-free (5) conditions. Using such media, the exact growth factor requirements for anchorage-independent proliferation can be determined (6,7).

Mouse neuroblastoma Neuro-2A cells have been shown to secrete polypeptide growth factors which induce anchorage-independent proliferation of NRK cells (8,9). The major mitogen secreted by these cells is a PDGF-like growth factor, which can be separated from other growth factor activities by means of reverse--phase HPLC (9). Using a serum-containing assay system it was shown that this PDGF-like growth factor induces anchorage-independent proliferation of NRK cells, which is strongly potentiated by EGF (9). Since under these conditions TGFβ from serum (3) may interfere with this process, we have now tested the ability of Neuro-2A secreted polypeptide growth factors to induce anchorage--independent proliferation of NRK cells under growth factor-defined conditions.

MATERIALS AND METHODS

Neuroblastoma derived TGFs – Serum-free medium conditioned by mouse neuroblastoma Neuro-2A cells during exponential growth was collected, lyophilized, extracted with 1 M acetic acid and run on a Bio-Gel P-100 column as described (8). Active fractions containing both mitogenic activity and the ability to induce phenotypic transformation (induction of soft agar growth) of NRK cells were pooled (apparent molecular mass of 15,000 – 20,000) and run on a Hi-Pore C_{18} reverse-phase HPLC column (250 by 4.6 mm; RP318; Bio-Rad) using a linear acetonitrile gradient of 0.1% per minute in 10 mM TFA (9). At a flow rate of 1 ml/min, 2 ml fractions were collected in 2 ml 2 M HAc containing 0.01% bovine serum albumin (BSA).

TGFβ growth inhibition assay – Mink lung CC164 cells were plated at 5000 cells/1.8 cm^2 in 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum. After attachment TGFβ, lyophilized column fractions or other growth factors to be tested were added in 0.1 ml binding buffer (DMEM containing 50 mM BES and 0.1% BSA, pH 6.8). After 48 hours 0.5 μCi [^{3}H]--thymidine (dThd; 47 Ci/mmol; ICN) was added, and after another 16 hours of incubation, incorporation of the dThd into cellular DNA was determined.

Cell cultures and materials – Growth factor-inactivated fetal calf serum (SH-FCS) was prepared as described (4). NRK cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium, supplemented with 30 nM Na_2SeO_3, 10 μg/ml transferrin, 0.2% BSA, 10% SH-FCS, 5 μg/ml insulin and 25 ng/ml EGF (receptor grade; Collaborative Research). The soft agar growth assay under growth factor defined conditions was performed as described (4, 6). The mitogenesis assay on quiescent Swiss 3T3 fibroblasts (cumulative incorporation of [^{3}H]-dThd between 8 and 24 hours after stimulation) was performed as described (8). Pure TGFβ was a generous gift from Drs. R.K. Assoian and M.B. Sporn (National Institutes of Health, Bethesda, MD) and pure human PDGF from Dr. C.H. Heldin (University of Uppsala, Sweden).

RESULTS AND DISCUSSION

Mouse neuroblastoma Neuro-2A cells produce and secrete two mitogenic polypeptide growth factors, which can be fully separated by means of reverse-phase HPLC (8,9). Both factors are able to induce phenotypic transformation of NRK cells in serum-containing media, and therefore they have been designated (9) neuroblastoma-derived transforming growth factors (ND-TGFs). The more hydrophilic activity ND-TGFI (see fig. 1) has strong mitogenic activity for Swiss 3T3 cells (fig. 1A), and corresponds to a PDGF-like growth factor. This is

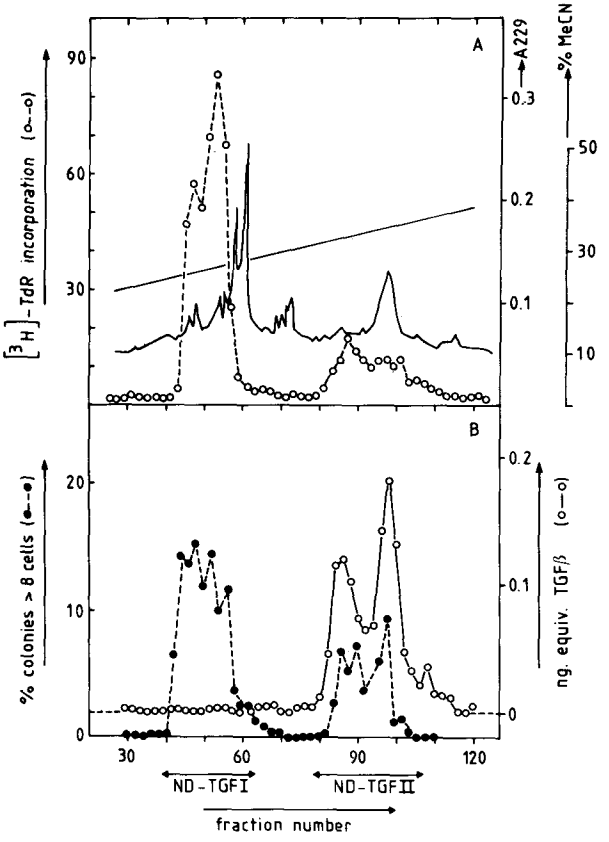

Figure 1. Identification of neuroblastoma-derived transforming growth factors (ND-TGFs) after reverse-phase HPLC column elution. Fractions were assayed for A: protein content (A_{229}) and mitogenic activity (o - - - o), and B: soft agar growth inducing activity under growth factor defined conditions in the additional presence of EGF (● - - - ●) and TGFβ content (o ——— o). TGFβ content was determined from growth inhibitory activity towards CCl64 cells, calibrated as shown in fig. 2. Fraction numbers 40-60 have been designated ND-TGFI, fraction numbers 80-105 ND-TGFII.

From the 4 ml fractions, 0.4 ml was tested for mitogenic activity on quiescent Swiss 3T3 cells (incorporation of [^{3}H]-dThd), 2 ml for induction of NRK soft agar growth in the SH-FCS containing growth factor defined assay in the presence of 2 ng/ml EGF and 0.25 ml for growth inhibition towards CCl64 cells.

demonstrated by the fact that the activity can be immunoprecipitated quantitatively by anti-PDGF antibodies (for further characterization of ND-TGFI, see ref. 9). The more hydrophobic ND-TGFII has properties very similar to TGFβ, as will be demonstrated in the present study.

Figure 1B shows that, when assayed in the presence of growth factor-inactivated serum, both ND-TGFs are able to induce NRK soft agar growth in the additional presence of EGF according to an elution pattern which is virtually coincident with that of mitogenic activity. This strongly indicates that the mitogens in ND-TGF are directly involved in the induction of phenotypic transformation. In order to investigate the column eluens for the presence of the modulator TGFβ, we have made use of the fact that TGFβ acts as a bifunctional regulator of cellular growth (10, 11). From the work of Holley and coworkers it is known that CC164 mink lung cells are particularly sensitive to the growth inhibitory activity of a factor secreted by BSC-1 cells, now known to be closely related or identical to TGFβ (10). Figure 2 shows a dose-response curve for growth inhibition of CC164 cells by TGFβ in comparison with its ability to induce NRK soft agar growth in the additional presence of EGF. Clearly the growth inhibition assay (half-maximal effect at 70 pg TGFβ) is much more sensitive towards TGFβ than the soft agar growth assay (half-maximal effect at 300

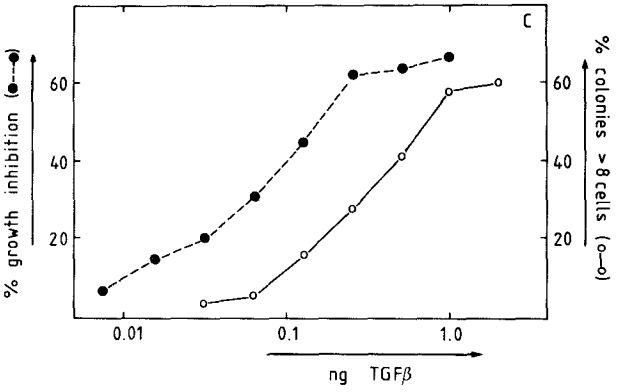

Figure 2. Dose-response curves for TGFβ induced growth inhibition of CC164 cells (● - - - ●) and induction of NRK soft agar growth in the presence of 2 ng/ml EGF (o ——— o).
Optimal concentrations of TGFβ reach a 70% reduction in [^{3}H]-dThd incorporation by CC164 cells. Dose-response curve for induction of NRK soft agar growth by TGFβ was assayed in calf serum containing medium in the additional presence of 1 ng EGF per 0.5 ml preincubation medium, as described (8). Colonies larger than 8 cells were scored after 14 days of incubation.

pg TGFβ). This demonstrates that TGFβ can be identified and quantified in column fractions if CCl64 growth inhibitory activity is present besides EGF—potentiated soft agar growth inducing activity. The detection limit of the inhibition assay (20 pg TGFβ) makes this assay even more sensitive than a competition assay with ^{125}I–TGFβ for TGFβ receptor binding (detection limit approximately 50 pg TGFβ; ref. 12–14). In spite of a higher specifity, the binding assay also suffers from the drawback that cells with a sufficiently high number of TGFβ receptors are not available, making quantification very difficult. Defined polypeptide growth factors like insulin and PDGF do not significantly affect TGFβ induced growth inhibition of CCl64 cells; only EGF tends to antagonize the effect of TGFβ to a certain extent (data not shown). TGFβ can therefore be identified and quantified by means of the CCl64 growth inhibition assay without interference from other polypeptide growth factors in a test sample, particularly if no EGF or EGF–like growth factor is present. Retinoic acid is also able to inhibit CCl64 proliferation, up to a maximum of approximately 30% inhibition (not shown). Together these data demonstrate that the two modulating agents for phenotypic transformation of NRK cells, TGFβ and retinoic acid (6) can be identified and quantified by this method.

Figure 1B shows that the column elution profile of ND–TGFI contains EGF—potentiated soft agar growth inducing activity, but is completely devoid of growth inhibitory activity on CCl64 cells. The presence of TGFβ in these column fractions could not have been obscured by other growth factors, since addition of 0.2 ng/ml TGFβ in the assay together with the ND–TGFI fractions resulted in 50% growth inhibition in all cases, exactly as would be expected according to fig. 2 (data not shown). Furthermore the ND–TGFs do not contain EGF–competing activity (8). In contrast, strong inhibitory activity is observed in ND–TGFII, which coincides exactly with EGF–potentiated soft agar growth inducing activity. Figure 3A shows in a dose–response curve that ND–TGFII has relatively poor mitogenic activity on Swiss 3T3 cells, very similar to the effects of pure TGFβ on this cell line (6). Furthermore, ND–TGFII induces NRK soft agar growth only in the additional presence of EGF (see Figure 3C, D), similar to TGFβ (6).

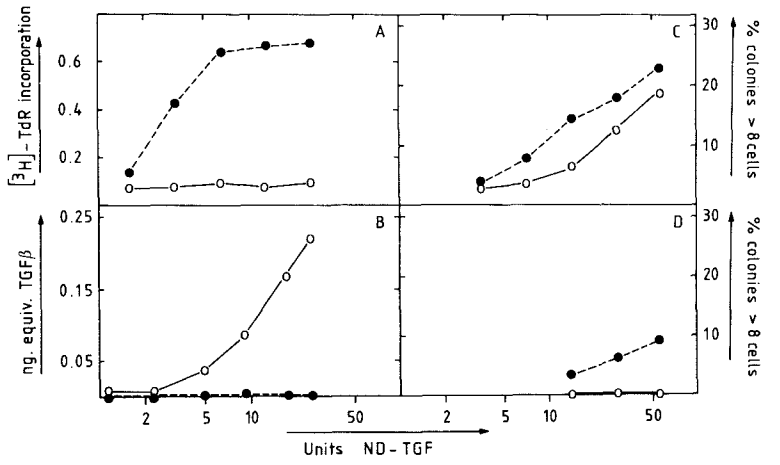

Figure 3. Dose-response curves for induction of mitogenic activity on quiescent 3T3 cells (A), quantification of TGFβ from growth inhibition analysis on CC164 cells (B) and induction of NRK soft agar growth under SH-FCS containing growth factor defined assay conditions in the presence (C) and absence (D) of 2 ng/ml EGF, induced by ND-TGFI (● - - - ●) and ND-TGFII (o ——— o). Amounts of ND-TGFI (pooled fractions 40-60) and ND-TGFII (pooled fractions 80-105) tested in the various assays have been expressed in units, indicating the volume (ml) of Neuro-2A conditioned medium from which the amount of material tested was originally obtained. The mitogenic activity of ND-TGFI, which results from the presence of a PDGF-like growth factor, corresponds to an activity per liter Neuro-2A conditioned medium similar to that of 450 ng PDGF. Based on previous data this indicates a 60% recovery of ND-TGF during the reverse-phase HPLC procedure (9).

In addition, ND-TGFII elutes from the reverse-phase HPLC column at similar acetonitrile concentrations as TGFβ (9). These data together provide strong evidence for the presence of TGFβ in ND-TGFII (quantified in Figure 3B).

Figure 3C, D show that ND-TGFI induces NRK soft agar growth which is strongly potentiated by the additional presence of EGF. Since no CC164 growth inhibitory activity is present in ND-TGFI, this potentiating effect of EGF is not mediated by either TGFβ or retinoic acid. The presence of a PDGF-like growth factor fully accounts for the mitogenic activity of ND-TGFI, and therefore our results strongly point towards a role of PDGF-like growth factors in phenotypic transformation of NRK cells. In various other studies TGF-like activities have been identified in conditioned media and cellular extracts, which differ in their properties from both TGFα and TGFβ (6, 15 and references therein). In the case of TGF-like activity secreted by mouse embryonal carcinoma cells (15), there is good evidence that this activity is related to the presence of a PDGF-like growth factor (16). Combined with the present obser-

vations, this could indicate that PDGF-like growth factors may play a much more general role in phenotypic tranformation of NRK cells than previously assumed. Rizzino et al. (7) have recently shown that PDGF by itself is able to induce NRK soft agar growth under serum-free conditions. To what extent these growth factor samples have been tested for the presence of TGFβ was not stated. We have confirmed, however, that PDGF is able to induce loss of density-dependent inhibition of growth of NRK cells under serum-free conditions, although only at high concentrations (E.J.J. van Zoelen; manuscript in preparation). Since PDGF from human platelets may be structurally different from PDGF-like growth factors secreted by tumor cells (17), the present data make it necessary that the properties of these two factors in inducing phenotypic transformation of NRK cells be compared.

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