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NOTES

Increased Secretion of Type β Transforming Growth Factor Accompanies Viral Transformation of Cells

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Cells transformed by Harvey or Moloney sarcoma virus secrete at least 40 times as much type β transforming growth factor as their respective untransformed control cells. The transformed cells bind only 20 to 50% as much type β transforming growth factor as the control cells, suggesting that transformation causes down-regulation of the type β transforming growth factor receptor.

Phenotypic transformation of normal rat kidney (NRK) cells, measured by the acquisition of anchorage independence and the resultant ability to grow in soft agar, requires the combined action of three distinct polypeptide growth factors: type α and β transforming growth factors (TGFs) (1, 2, 24) and platelet-derived growth factor (PDGF) (4). It has been shown that certain virally transformed cells, but not the nontransformed control cells, secrete type α TGFs (8, 9, 20, 30). A similar correlation has recently been made for PDGF production by a variety of transformed cells (6). A coordinate increase in the secretion of type β TGFs after viral transformation has not yet been demonstrated. We now report that Harvey sarcoma virus-transformed NIH-3T3 mouse cells and Moloney sarcoma virus-transformed NRK cells secrete at least 40 times as much type β TGF as their respective untransformed control cells. In addition, we found that the receptor-binding activity for type β TGF in transformed cells is only 20 to 50% that of controls; these results suggest that the secretion of high levels of type β TGF by the transformed cells causes down-regulation of the type β TGF receptors, as has been previously been reported for type α TGF (8, 9, 20, 30) and PDGF (6) and their corresponding receptors after transformation.

Though both classes of TGFs share a common nomenclature, the recent purification to homogeniety of both type α (15–18) and type β (5, 11, 23) TGFs demonstrates that these two subsets of TGF activity differ markedly in their chemical structure. Type α TGFs, which include epidermal growth factor (EGF) and which bind to the EGF receptor (9, 13, 17, 19, 24) have an approximate molecular weight of 5,000 to 6,000 and consist of a single peptide chain with three disulfide bonds in homologous positions to those of EGF (15, 16). Type β TGFs, which bind to a unique receptor (C. A. Frolik, L. M. Wakefield, D. M. Smith, and M. B. Sporn, J. Biol. Chem., in press), have a molecular weight of 25,000 and are composed of two apparently identical polypeptide chains cross-linked by disulfide bonds (5, 11, 23). Type α (13, 17–19, 30) and β (5, 7, 11, 21, 23, 24) TGFs have been isolated from both neoplastic and nonneoplastic cells and tissues. To investigate whether aberrant expression of type β TGFs might be associated with viral transformation of cells, we compared the levels of type β TGF in the conditioned medium (CM) of two populations of NRK cells, one a control set and the other three passages after transformation by Moloney sarcoma virus (Mo-NRK cells). Likewise, we also compared type β TGF secretion in a parent nonneoplastic NIH-3T3 cell line with that of a cloned line transformed by Harvey sarcoma virus (Ha-NIH-3T3 cells).

Untransformed NIH-3T3 cells and virally transformed Ha-NIH-3T3 cells were seeded at a cell density of 2×10^6 and 1×10^6 cells per flask, respectively, in ten 150-cm^2 plastic tissue culture flasks (Costar, Cambridge, Mass.) containing 40 ml of Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) and were incubated in humidified 5% CO_2–95% air at 37°C. After 2 days, when cells were nearly confluent, monolayers were washed gently three times every 2 h with serum-free medium and then further incubated with serum-free medium. The first 24-h collection was discarded to avoid the last traces of serum contamination; media collected from the cells from 24 to 48 h were used for analysis. At both the beginning and the end of the collection period, two flasks were trypsinized and counted in a Coulter counter to determine cell number; viability was determined by using trypan blue exclusion. The number of viable NIH-3T3 cells was 39 and 18% of the original value (29×10^6 cells per flask) at 24 and 48 h, respectively, whereas the number of Ha-NIH-3T3 cells was 125 and 120% of the original cell number (28.8×10^6 cells per flask) at 24 and 48 h, respectively. For the Mo-NRK cells, a flask of NRK clone 49F cells was transformed by using a Moloney leukemia sarcoma virus complex. After three passages, the cells exposed to virus exhibited a transformed morphology and were seeded at a density of 10^5 cells per flask into four 75-cm^2 flasks; control NRK clone 49 cells were seeded at twice the cell density (4×10^5 cells per flask in five 150-cm^2 flasks). After 5 days, when the cells were confluent, serum-free CM was collected as described above, except that CM collections from 24 to 48 h and 48 to 120 h were pooled for analysis. The number of viable NRK cells was 72, 53, and 47% of the original value (8×10^6 cells per flask) at 24, 48, and 120 h, respectively, whereas the number of viable Mo-NRK cells was 110, 112, and 101% of the original cell number (14×10^6 cells per flask)

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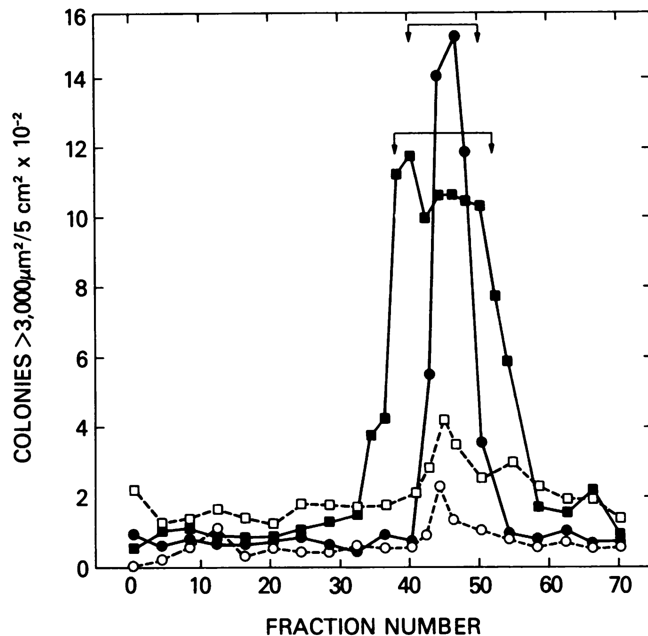

FIG. 1. HPLC purification of type β TGF from CM. Lyophilized CM samples were each dissolved in 5 ml of 0.1% trifluoroacetic acid and loaded onto a Waters μBondapak C18 column equilibrated with 20% acetonitrile–0.1% trifluoroacetic acid. The amounts of samples initially reconstituted to a volume equivalent to 1 ml per 10^7 cells were as follows: 24- to 48-h CM from NIH-3T3 (2.7 ml) and Ha-NIH-3T3 (4 ml) cells and 24- to 120-h CM from NRK (1 ml) and Mo-NRK (2.8 ml) cells. The gradient consisted of 20 to 40% acetonitrile–0.1% trifluoroacetic acid for 100 min, followed by a 15-min linear gradient to 60% and a final step to 80% at a flow rate of 0.8 ml/min. Samples, 300 μl for NIH-3T3 (○) and Ha-NIH-3T3 (●) and 250 μl for NRK (□) and Mo-NRK (■), of 1.6-ml fractions were used for soft-agar assay (23) in the presence of 0.8 nM EGF. Arrows indicate the region pooled.

at 24, 48, and 120 h, respectively. CM from each cell type was centrifuged at 100,000 × g for 1 h to remove any cellular debris and further processed as described (9). Lyophilized CM was reconstituted for assay in 4 mM HCl containing 1 mg of bovine serum albumin per ml by using 1 ml per 10^7 viable cells.

It had been shown previously that anchorage-independent growth of NRK cells assayed in 10% serum, which provides the necessary PDGF (4), and optimal levels of type α TGFs, represented here by EGF, are a function of the concentration of type β TGF added (1). Thus, to obtain an estimate of the relative levels of type β TGFs in the unfractionated CM of either Mo-NRK or Ha-NIH-3T3 cells (compared with CM from their respective control cells), samples of CM were assayed for the induction of colony formation of NRK cells in the presence of 0.8 nM EGF. Both types of sarcoma virus-transformed cells appeared to have ca. 30 to 40 times as much type β TGF activity as the control cells (data not shown). To verify that the assayed type β TGF-dependent colony formation of the unfractionated CM was actually due to type β TGF, CM samples from the two transformed cell types and their respective control cells were subjected to further purification by reverse-phase high-pressure liquid chromatography (HPLC) on a Waters μBondapak C18 column by using an acetonitrile gradient containing 0.1% trifluoroacetic acid as described previously (1, 2). Type β TGF-dependent colony-forming activity was eluted at 37 to 40% acetonitrile (Fig. 1), in agreement with our earlier reports (1, 2, 11, 23). After this HPLC step, which separates the type α and β TGFs, the pooled type β TGF fractions showed no colony-forming activity when assayed in the absence of added EGF (data not shown). Dose-response curves of the pooled type β TGF fractions from Ha-NIH-3T3 and Mo-NRK cells assayed in the presence of 0.8 nM EGF showed that the transformed cells released 40 times more type β TGF than their respective controls (Fig. 2). To confirm that this activity represented type β TGF, a sample of the pooled HPLC fractions from the Ha-NIH-3T3 CM (Fig. 1) was lyophilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence of reducing agent (14). After electrophoresis, the gels were sliced, extracted, and assayed as previously described (23). Assay in the presence of 0.8 nM EGF gave a single peak of colony-forming activity which migrated with an estimated molecular weight of 25,000 (Fig. 2, inset).

For final confirmation, it was shown that Ha-NIH-3T3 CM type β TGF purified on HPLC competed with ^{125}I-labeled human platelet type β TGF for binding to A549 human lung carcinoma cells (Fig. 3). The 50% effective dose of reconstituted Ha-NIH-3T3 cell CM (106 μl/ml) was equivalent to 76 pM of homogeneous human platelet type β TGF; no competition was observed with CM of control NIH-3T3 cells. Thus, the type β TGFs secreted by the transformed cells have been shown to have four properties characteristic of type β TGFs: (i) the EGF dependence of the colony-forming activity assayed on NRK cells (1, 22, 24), (ii) the elution pattern during HPLC (1, 2; Fig. 1), (iii) the apparent molecular weight of 25,000 on sodium dodecyl sulfate-polyacrylamide gels (5, 11, 23; Fig. 2), and (iv) the ability to compete for binding with ^{125}I-labeled human platelet type β TGFs (Frolik et al., in press; Fig. 3).

Since it had previously been shown that virally transformed cells that secrete type α TGFs (8, 9, 13, 20, 27, 30) or PDGF (6) have an accompanying loss of assayable cell surface receptors for that ligand, we next compared the binding of ^{125}I-labeled type β TGF to cell surface receptors of the virally transformed cells and their untransformed counterparts. The binding of the radiolabeled type β TGF to the Ha-NIH-3T3 and Mo-NRK cells is only 20 and 50%, respectively, of the binding to the control cells (Fig. 4A and B). Scatchard analysis (25) of the binding to the Ha-NIH-3T3 cells and the untransformed NIH-3T3 cells shows K_ds of 27 and 68 pM and receptor numbers of 18,000 and 83,000 per cell, respectively (Fig. 4a). Scatchard analysis of the binding of type β TGF to Mo-NRK and untransformed NRK cells results in curvilinear plots (Fig. 4b). Nonetheless, it can be seen that the number of type β TGF receptors in Mo-NRK cells is less than in the control NRK cells. Detailed analysis of the properties of the type β TGF receptor (Frolik et al., in press) suggests that only the high-affinity receptors of the NRK cells are important for the biological activity of type β TGF.

Assay of the unfractionated CM from all cells for type α TGF-dependent colony formation (done by assay of the NRK indicator cells in the presence of 10 pM type β TGF) showed type α TGF activity in the CM of the transformed cells but not of the control cells, in agreement with the results of previous investigations (8, 9, 20, 30). Therefore, we examined whether the binding of ^{125}I-labeled EGF (12) to cell surface receptors of these cells would also be reduced. Analysis showed a K_d of 2.2 nM and 8,000 receptors per cell for the NIH-3T3 cells; the Ha-NIH-3T3 cells bound no radiolabeled EGF (Fig. 4C). In contrast, the mixed popula-

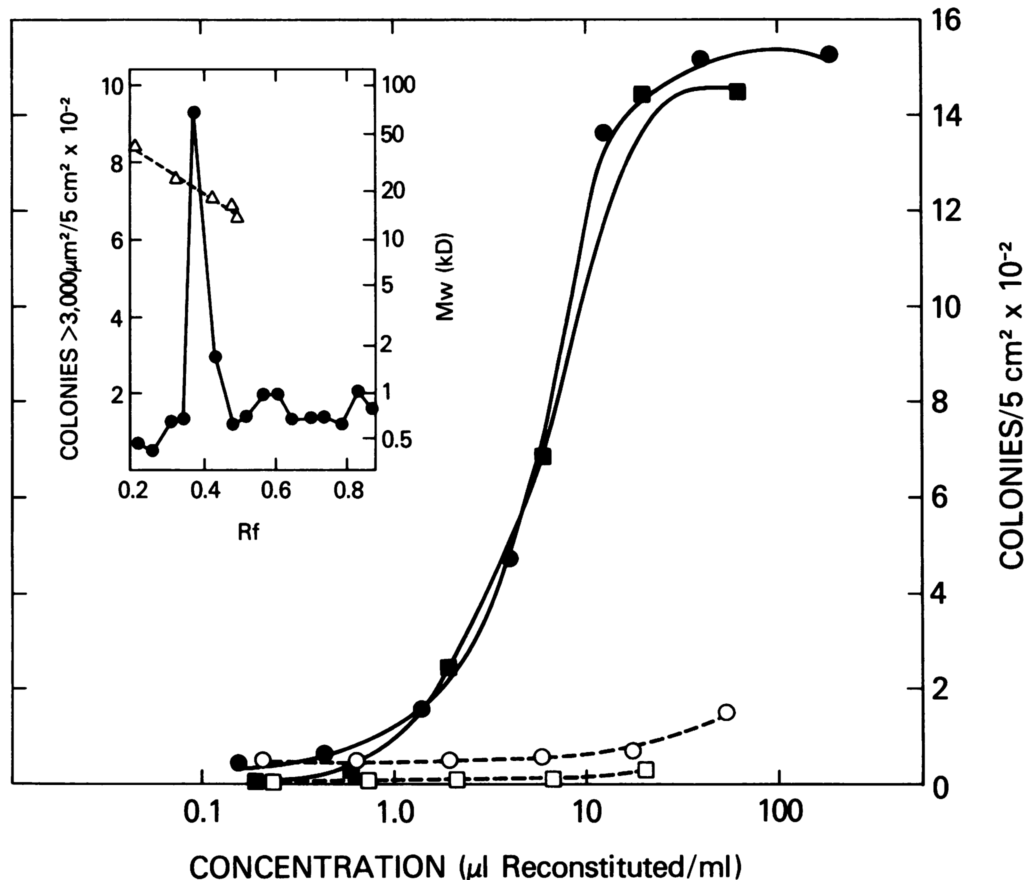

FIG. 2. Dose-response curves of type β TGF activity from CM of untransformed and virally transformed cells after HPLC purification. Type β TGF fractions (see arrows, Fig. 1) were pooled, lyophilized, and reconstituted in 1 ml per 10^7 cells of 4 mM HCl containing 1 mg of bovine serum albumin per ml. Soft-agar colony-forming activity in the presence of 0.8 nM EGF was determined as described in Table 1, footnote *a*. Each point is an average count per 5 cm^2 of duplicate plates for colonies of >6,000 μm^2 for NIH-3T3 (○) and Ha-NIH-3T3 (●) cells and for colonies of >3,000 μm^2 for NRK cells (□) and Mo-NRK (■) cells. The inset shows sodium dodecyl sulfate polyacrylamide gel electrophoresis of a sample from Ha-NIH-3T3 CM after HPLC. The sample (6% of the total pool) was dissolved in 50 μl of sample buffer without reducing agent and applied to a 15% discontinuous gel according to the method of Laemmli (14). Type β TGF activity was extracted from the gel as described (23) and assayed for colony-forming activity (●) in the presence of 0.8 nM EGF. ^{14}C-labeled molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Md.) were ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), and cytochrome *c* (12,300). R_f is the mobility of ^{14}C-labeled protein standards (△) relative to the bromphenol blue dye marker.

tion of Mo-NRK cells showed only slightly reduced binding relative to NRK cells (Fig. 4D); however, a transformed clone of the Moloney sarcoma virus-transformed NRK cells (49F-T1) previously isolated (10) bound no radiolabeled EGF (Fig. 4D). These differences in the binding of EGF to the Mo-NRK cells, a mixed population of cells a few passages after transformation with virus, and to the cloned 49F-T1 cells are in agreement with the observations of Todaro et al. (28), who reported progressive loss of EGF binding to cells after viral transformation. Again, Scatchard analysis of the binding to the NRK cells yields curvilinear plots, but it appears that the high-affinity component of the EGF binding has been lost in the Mo-NRK cells (Fig. 4d).

The reduced binding of EGF and type β TGF to the virally transformed cells (Fig. 4) is consistent with the increased secretion by these cells of type α (8, 9, 13, 15–20) and β TGFs (Fig. 1 and 2). The smaller surface area of transformed cells relative to the control cell population is unlikely to be a contributing factor to the observed reduction of cellular binding: previous studies (29) comparing the binding of multiplication-stimulating activity and EGF in NRK cells and their sarcoma virus transformants showed that binding of the former ligand was unchanged, whereas binding of the latter was reduced to 10% that of control levels, concommitant with the increase in type α TGF secretion after transformation. Although other mechanisms might contribute, our data suggest that the reduced binding results from a combination of occupation of cell surface receptors by the secreted ligands and ligand-induced receptor down-regulation (6, 8, 9, 20, 28). Reduction of assayable EGF and PDGF receptor binding has proven a consistent marker of cells secreting type α TGFs (9, 27) or PDGF (6), and exogenous type β TGF has recently been shown to induce down-regulation of its own receptors (Frolik et al., in press). Collectively, these data suggest an autocrine mechanism of action (26) of these peptide growth factors to maintain the phenotype of the sarcoma virus-transformed cells. Calculation of the concentration of type β TGF in the original CM in which the cells were growing shows concentrations of 62 and 0.23 pM for the transformed Mo-NRK cells and their untransformed controls, respectively, and concentrations of 160 and 0.97 pM for the transformed Ha-NIH-3T3 cells and their untransformed parent cells, respectively (Table 1). Although the amount of type β TGF secreted per cell was only 40-fold

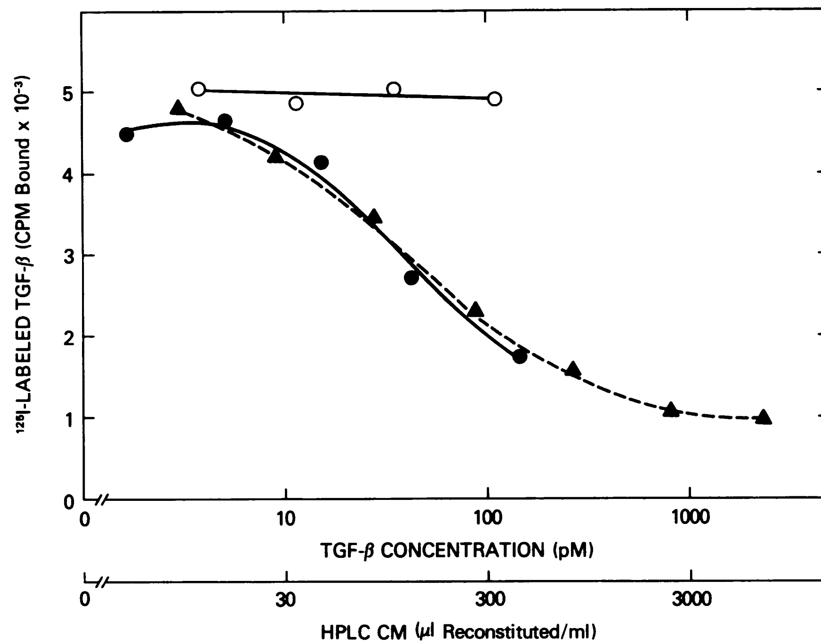

FIG. 3. Receptor binding competition of Ha-NIH-3T3 CM type β TGF with ^{125}I-labeled human platelet type β TGF. Homogenous human platelet type β TGF (5) was iodionated as described (Frolik et al., in press). A549 human lung carcinoma cells (10^5 cells in 1 ml of Dulbecco modified Eagle medium containing 10% calf serum) were plated in 24-well, 16-mm-diameter dishes (Costar). After 24 h, the medium was replaced with medium containing 1% calf serum to allow maximal expression of type β TGF receptors. The binding competition assay with ^{125}I-labeled platelet type β TGF (152 μCi/μg) was performed 24 h later as described (Frolik et al., in press). Values are the average of duplicate determinations of human platelet type β TGF (▲) and HPLC-purified type β TGF pool (Fig. 1) from NIH-3T3 (○) and Ha-NIH-3T3 (●) cells.

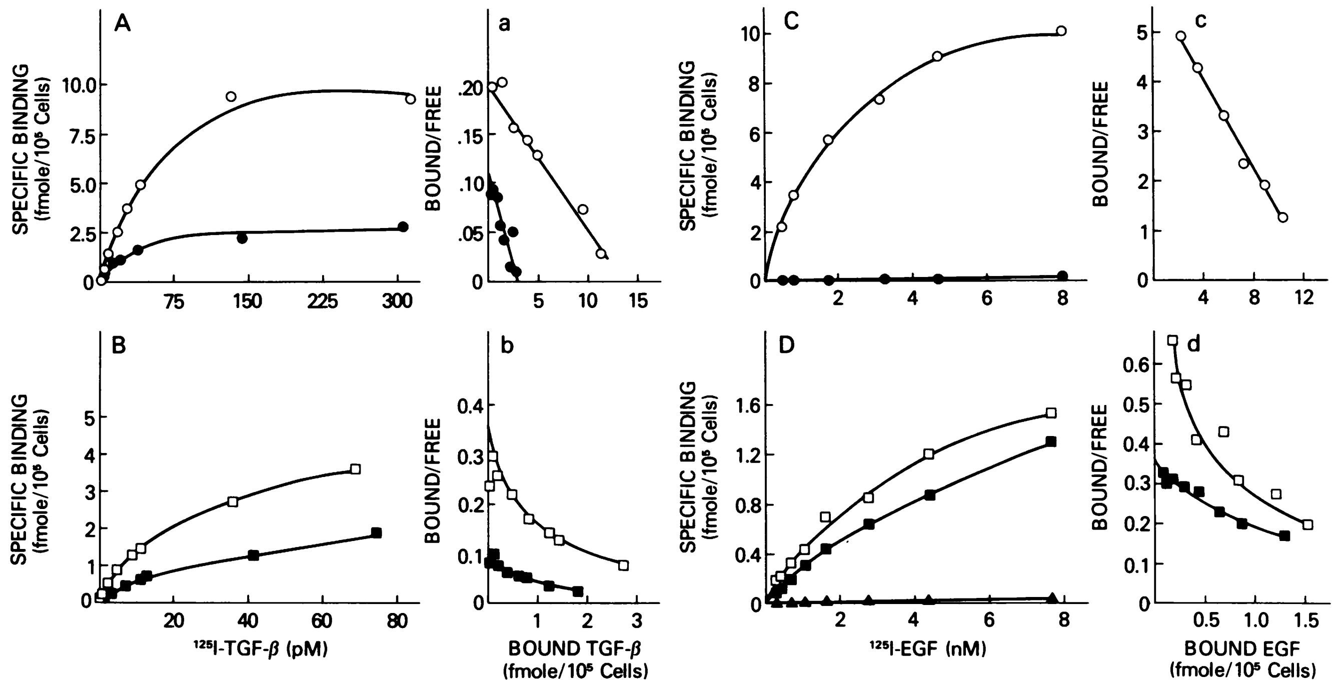

FIG. 4. Radioreceptor binding studies in virally transformed and untransformed cells. Human platelet type β TGF (5) and murine EGF (22) were iodinated by respective chloramine T procedures (12; Frolik et al., in press). Cells were plated in 24-well, 16-mm-diameter dishes at 10^5 cells in 1 ml of Dulbecco modified Eagle medium containing 10% calf serum and processed as described in the legend to Fig. 3. Comparative binding assays between control and transformed cell lines were done by using similar seeding densities and culture conditions. EGF binding was assayed 24 to 36 h after seeding as described (3). For the type β TGF binding assay, cells were changed to medium containing 1% calf serum 24 h after plating to allow maximal expression of type β TGF receptor, and the binding assay was performed 24 h later as described (Frolik et al., in press). Triplicate wells were counted to determine the actual cell number at the time of assay. Specific binding was determined by subtracting the nonspecific binding, i.e., the amount of radioactivity bound in the presence of either 500 ng of unlabeled type β TGF per ml or 600 ng of unlabeled EGF per ml. Values are the average of duplicate or triplicate determinations. (A) Binding of ^{125}I-labeled type β TGF (100 μCi/μg) to NIH-3T3 (○) and Ha-NIH-3T3 (●) cells. (B) Binding of ^{125}I-labeled type β TGF (53 μCi/μg) to NRK (□) and Mo-NRK (■) cells. (C) Binding of ^{125}I-labeled EGF (124 μCi/μg) to NIH-3T3 (○) and Ha-NIH-3T3 (●)cells. (D) Binding of ^{125}I-labeled EGF (287 μCi/μg) to NRK (□) and Mo-NRK (■) cells and to a Moloney sarcoma virus-transformed clone of NRK, 49F-T1 (▲). The Scatchard (25) plots of (A), (B), (C), and (D) are shown in (a), (b), (c), and (d), respectively.

TABLE 1. Levels of HPLC-purified type β TGF from CM of untransformed and transformed cells[a]

Cells	Cell no. ($\times 10^7$)	Vol of medium collected (ml)	Type β TGF		
			Total amt (pmol)	pmol/10^7 cells	Concn (pM) in serum-free culture medium
Untransformed NRK	1.9	400	0.09	0.047	0.23
Transformed Mo-NRK	5.6	160	10	1.8	62
Untransformed NIH-3T3	6.5	320	0.31	0.048	0.97
Transformed Ha-NIH-3T3	29	320	50	1.7	160

[a] Seedings of cells and collection of CM are described in the text. The amount of type β TGF was determined by a soft-agar colony-forming assay (23) and quantitated by comparison of dose-response curves with a pure-platelet type β TGF standard (5).

greater for transformed cells as compared with controls, the actual concentration in the medium was 160- to 270-fold greater. The concentration of type β TGF in the CM of the Mo-NRK cells is consistent with an autocrine mechanism of transformation, since the 50% effective dose for type β TGF-dependent colony formation of NRK cells has been shown to be ca. 2 to 4 pM (5, 11, 23). Taken together with previously published data for type α TGF (8, 9, 20, 30) and PDGF (6), the data presented here for type β TGF demonstrate that sarcoma virus-transformed cells secrete elevated levels of at least three different polypeptide growth factors shown to play a role in the transformation of cells (4).

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