Elevated Growth Factor Levels in Transformed Mouse Embryo Cells Treated with N,N-Dimethylformamide

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ABSTRACT

The transformed mouse embryo fibroblast cell line AKR-MCA, produces several transforming growth factor (TGF) activities which can be identified in cell extracts and serum-free conditioned medium. Treatment of these transformed cells with 1% N,N-dimethylformamide resulted in a more normal phenotype and an increased level of TGFs in cell extracts and conditioned medium. In addition, an 11-fold increase in an epidermal growth factor receptor-competing activity was observed in cell extracts and conditioned medium compared to control untreated cells. Fractionation of the conditioned medium on Bio-Gel P-100 showed that the same size classes of TGFs were present in N,N-dimethylformamide-treated as well as untreated cells. The increased EGF-receptor-competing activity was eluted in two peaks at M, 6500 and M, 4000. The M, 6500 peak did not coelute with a TGF peak in the N,N-dimethylformamide-treated cells.

INTRODUCTION

TGFs are heat- and acid-stable polypeptides which have the ability to reversibly induce a transformed phenotype in nontransformed cells. These factors were originally described in conditioned medium from murine sarcoma virus-transformed mouse fibroblasts and similar peptides were found in other types of neoplastic cells and in embryonic mice and rats. Recently, Anzano et al. and Massague have shown that TGF preparations from virally transformed human and rat cells contain 2 types of factors necessary for transformation; TGF-α (M, 6000), which competes with EGF for its receptor, and TGF-β, which does not complete for the EGF receptor. The NH2-terminal sequence of human, mouse, and rat TGF-α is homologous to mouse EGF (12–14).

TGF-β has also been isolated from virally transformed rat fibroblasts, human platelets, human placenta, and bovine kidney. TGF-β is a 2-chain molecule (M, 23,000 to 25,000) composed of two M, 13,000 polypeptide chains held together by disulfide bonds. TGF-β does not interact with the EGF receptor and requires TGF-α or EGF for the induction of large colonies in soft agar (2). When NRK cells are used as target cells, TGF-β causes colony formation with AKR-2B cells in the absence of exogenously added EGF.

Previous work from our laboratory has shown that treatment of AKR-MCA cells with DMF caused these cells to assume a more differentiated phenotype with respect to cell morphology, membrane antigens, and phosphoproteins. In addition, DMF treatment eliminated the ability of the malignant cells to grow with anchorage independence and restored normal growth controls such that EGF could induce mitogenesis in these cells, and they no longer responded to nutrient replenishment. The AKR-MCA cells produced at least 2 size classes of TGF activity which have been associated with anchorage-independent growth. Others have shown that cultures of embryonal carcinoma cells which had been induced to differentiate by retinoic acid responded to the exogenous addition of sarcoma growth factor (a TGF-α), while undifferentiated parental cells produced TGFs of their own and would not respond to exogenous growth factor. This suggested that differentiation agents such as retinoic acid and DMF might interfere with the production or response of the cells to growth factors. Therefore, in this study, we compare the growth factors present in conditioned medium and cell extracts of AKR-MCA cells grown in the presence or absence of DMF. The levels of TGFs and an EGF-receptor competing activity in the cells and in the conditioned medium were higher if the cells were grown in the presence of 1% DMF.

MATERIALS AND METHODS

Collection of Conditioned Medium. AKR-MCA cells (from Dr. H. L. Moses, Mayo Clinic, Rochester, MN) were seeded in 75-cm² flasks (Coming) at 180,000 cells/flask in McCoy's Medium 5A supplemented with 10% FBS (Grand Island Biological Co.) and antibiotics (streptomycin/penicillin). At confluence, the cells were washed with serum-free medium and then maintained in serum-free medium for 5 days. At this time, the conditioned medium (from 15 flasks) was collected and dialyzed (Spectrapor 3 tubing; M, 3500 cutoff) versus 1% acetic acid; aliquots were taken for assay, and the remainder was used for gel filtration chromatography. The remaining cells were extracted as described below. For the DMF treatment, cells were seeded at 250,000 cells/flask and maintained in McCoy's Medium 5A as above containing 1% DMF. The serum-free medium used also contained 1% DMF. The conditioned medium from DMF-treated cells (30 flasks) was collected and processed as described above. Protein concentrations were determined by a dye-binding assay (Bio-Rad) using BSA as a standard.

Extraction of Cells. Intracellular material was obtained from the cells by the acid/ethanol procedure of Roberts et al. (20). After removal of the conditioned medium, the cells were frozen and then thawed. The cells were extracted in the flasks by adding 10 ml of HCl/ethanol/H₂O (0.04/2/1) at 4°. The solution was stirred overnight at 4° and centrifuged at 15,000 × g for 20 min. The supernatant was adjusted to pH 5.3. ethanol (2 volumes) and diethyl ether (4 volumes) were added, and the protein was precipitated at −20° for 48 hr. Following centrifugation at 10,000 × g for 15 min, the precipitate was dried in a vacuum, resuspended in 1 ml acetic acid, and dialyzed extensively against 1% acetic acid.

Bio-Gel Chromatography. The conditioned medium was fractionated on Bio-Gel P-100 (Bio-Rad; 1.5 x 30 cm) in 1 ml acetic acid at a flow rate of 5 ml/hr. The sample (16 mg protein from untreated cells or 6.4 mg protein from DMF-treated cells) was loaded onto the column, and the elution was monitored for absorbance at 280 nm. 0.5-ml fractions were collected every 20 min in 1-ml aliquots. The fraction containing TGF was collected, lyophilized, and redissolved in water for further analysis.

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protein from DMF-treated cells) was applied in 1 ml of 1 M acetic acid, and 1 ml fractions were collected. Aliquots (0.5 ml for TGF or 0.2 ml for EGF-receptor competing activity) of every other fraction were lyophilized for assay of activities. Bovine serum albumin, cytochrome c, and insulin were used to calibrate the column. All activity profiles have been corrected for the different protein loads.

**Soft Agarose Assay.** Underlayers of 0.4 ml of 0.8% agarose (Sea Plaque; FMC Corporation, Marine Colloids Division, Rockland, ME) growth medium were plated in 9-mm culture plates and allowed to solidify at room temperature. Each pair of these were overlaid with a single cell suspension (0.4 ml of 2.0 x 10⁸ AKR-2B cells in 0.4% agarose in McCoy's Medium 5A supplemented with 10% FBS containing aliquots of cell extracts, conditioned medium, or column fractions. The plates were then incubated at 37° (5% CO₂ in a humidified incubator) and examined for growth on Days 14 to 21. Colonies (10 or more cells) were scored on an inverted microscope. One unit of TGF activity is the amount of protein (in a 1-ml assay) which will give 50% maximum colony formation.

**EGF Competition Assay.** A membrane fraction from human placenta was obtained as described by Hirata and Orth (8). Membranes were washed 3 times with 10 mM Tris, pH 7.4, and resuspended to a final membrane protein concentration of 200 μg/ml in 10 mM Tris, pH 7.4, containing BSA (1 mg/ml). The assay mixture (0.3 ml final volume) contained 20 μg membrane protein, 125I-labeled mouse EGF (3 ng/ml), and sample or standard mouse EGF and was incubated for 1 hr at 4°. The membranes were collected by centrifugation, and the pellets were counted in a Beckman γ-counter. Data are expressed as ng-equivalents of standard mouse EGF. Column fractions to be tested for EGF competition activity were lyophilized and dissolved in 10 mM Tris, pH 7.4, BSA (1 mg/ml) for assay. Mouse EGF was isolated by the method of Savage and Cohen (21) and 125I-labeled by the chloramine-T method (1, 9) to a specific activity of 50 to 70 cpm/pg.

### RESULTS

**Growth Factors in Conditioned Medium.** AKR-MCA cells have been shown to secrete TGF activities into tissue culture medium (24). AKR-MCA cells were grown in the absence or presence of 1% DMF, and the conditioned medium (after dialysis versus acetic acid and concentration by lyophilization) was examined for the presence of TGF activities. Two fibroblast-like cell lines (NRK and AKR-2B) were used as indicator cells. Conditioned medium collected from AKR-MCA cells grown in the presence of 1% DMF showed increased TGF activity upon both indicator cell lines compared to conditioned medium from untreated cells (Table 1). The TGF activity measured using NRK cells showed a higher increase (40-fold) than did the TGF activity measured using AKR-2B cells (4-fold).

The conditioned medium was also examined for activity which competed for binding with EGF to its receptor on human placenta membranes. As reported by Tucker et al. (24), conditioned medium from untreated AKR-MCA cells contained a low level of this activity. This EGF-receptor competing activity was 11-fold higher in conditioned medium from cells treated with 1% DMF. Therefore, treatment of the transformed AKR-MCA cell line with 1% DMF resulted in an increased level of growth factors secreted into the medium.

**Intracellular Growth Factors.** The levels of intracellular growth factors were examined to determine whether the higher levels of growth factors present in the conditioned medium from DMF-treated AKR-MCA cells were due to an increase in the synthesis or secretion of these factors. Table 2 shows that the intracellular levels of TGFs and the EGF-receptor competing activity were elevated in AKR-MCA cells grown in the presence of DMF. As noted by others (24), the Bio-Gel profile of intracellular TGFs was similar to that obtained from conditioned medium. The high levels of intracellular TGF activities suggest that AKR-MCA cells grown in 1% DMF show an increased synthesis (or decreased degradation) of growth factors which is reflected by increased levels of extracellular factors.

**Gel Filtration Chromatography.** Growth factors from DMF-treated and untreated cells were compared by chromatography of conditioned media on Bio-Gel P-100 in 1 M acetic acid. The protein profiles of the 2 conditioned media samples were similar, except that the conditioned medium from untreated cells contained fewer low-molecular-weight proteins (Chart 1A). The protein profile, as well as the following activity profiles, was corrected for the different amounts of protein loaded on the column.

The profile of TGF activity acting on NRK indicator cells was similar to that obtained by Tucker et al. (24) and showed 2 major activity peaks of approximately M, 30,000 and 12,000 and a minor peak at M, 6000 (Chart 1B). As expected from the activity measurements made on the conditioned medium, there was more activity from the conditioned medium derived from DMF-treated cells. However, all the size classes of TGF activity were present in conditioned medium from cells grown in the presence or absence of 1% DMF (Chart 1B).

The same results were obtained using AKR-2B cells as the indicator of TGF activity (Chart 1C). The major activity peaks (M, 35,000 and 15,000) were present in both preparations, and there was more activity from the conditioned medium from DMF-treated AKR-MCA cells. The elution profile for the EGF-receptor competing activity is shown in Chart 1D. The amount of this activity in conditioned medium from cells grown in the absence of DMF was very low and eluted at approximately M, 6000. The activity derived from DMF-treated cells was much greater and showed 2 activity peaks at M, 6500 and M, 4000. The M, 4000 activity correlates with the lowest-molecular-weight TGF peaks (versus NRK cells), while the M, 6500 activity does not directly correspond to a TGF activity peak.

### DISCUSSION

In this study, we have shown that growing the transformed AKR-MCA cell line in the presence of 1% DMF resulted in an

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**Table 1**

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<th>Growth factor activity in conditioned medium</th>
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<td>Conditioned medium from AKR-MCA cells was assayed for TGF activity using NRK or AKR-2B cells and for EGF-receptor competing activity as described in &quot;Materials and Methods.&quot;</td>
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<tr>
<td>TGF activity (units/mg protein) &amp; EGF-competing activity (ng/mg protein)</td>
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<tr>
<td>NRK &amp; AKR-2B</td>
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**Table 2**

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<th>Growth factor activity in cell extracts</th>
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<tr>
<td>Acid/ethanol extracts were assayed for TGF activity using NRK cells and for EGF-receptor competing activity as described in &quot;Materials and Methods.&quot;</td>
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<tr>
<td>TGF activity (units/mg protein) &amp; EGF-competing activity (ng/mg protein)</td>
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<td>NRK &amp; AKR-2B</td>
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increase in both the intracellular and extracellular levels of growth factor activities. The profiles of TGF activities obtained using NRK or AKR-2B cells as indicators of activity were similar to those previously reported by Moses et al. (15, 24). These investigators found that most of the TGF activity from AKR-MCA cells was TGF-β (as evidenced by EGF synergism of colony formation by NRK cells in soft agarose and the lack of EGF receptor competing activity coeluting from Bio-Gel with NRK activity) and was eluted from Bio-Gel in 2 peaks at M, 24,000 and M, 13,000, with a relatively minor peak at M, 5000. Similar size classes of TGF activities were observed for AKR-MCA cells in this study and, while DMF treatment increased both intracellular and extracellular levels of growth factors, the qualitative nature of the size classes was not altered. Bio-Gel chromatography yields only a partial purification of α and β TGFs and may not completely resolve the 2 activities from each other (3, 4, 7, 18). Since AKR-MCA cells produce little TGF-α (15, 24), the increased EGF-receptor competing activity could represent an increase in TGF-α levels due to DMF treatment. Neither the M, 6500 nor the M, 4000 peaks had mitogenic activity, and the 2 peaks eluted from reverse-phase HPLC at acetonitrile concentrations different from those of TGF-α or EGF. Therefore, DMF treatment of AKR-MCA cells does not increase the levels of TGF-α. Additionally, there may be DMF-induced alterations in the levels of other factors that are not identified by the NRK and AKR-2B indicator systems. We are currently growing preparative quantities of cells and conditioned medium to permit the further investigation of these growth factors.

Previous work from our laboratory has shown that DMF treatment reverses the expression of several transformation associated properties of AKR-MCA cells, including growth factor independence and the ability to grow with anchorage independence (5). These biological properties have been associated with an autocrine function of TGFs (22, 24). Consequently, one possible explanation for the effects of DMF on AKR-MCA cells was the inhibition of the synthesis and/or secretion of TGFs. However, the results of this study show that, rather than inhibiting the synthesis and secretion of TGFs, DMF treatment of AKR-MCA cells leads to increased production of growth factors. Furthermore, the results indicate that high concentrations of the factors are not, of themselves, sufficient to maintain the transformed phenotype.

Several investigators have shown that normal cells secrete TGFs but do not demonstrate transformed properties, such as the ability to grow with anchorage independence (15, 16, 19, 24, 25). Moses and his colleagues have suggested that this may be due to a higher sensitivity of transformed cells for TGFs (24). The results of this study are consistent with the induction of a decreased sensitivity to TGFs in AKR-MCA cells by DMF, as
REFERENCES


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