Loss of Differentiation Control in Transformed 3T3 T Proadipocytes

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ABSTRACT

Nontransformed 3T3 T mesenchymal/proadipocyte stem cells can be readily induced to differentiate, yet previous work has shown that 3T3 T cells that are spontaneously or virally transformed not only lose their normal growth control mechanisms but also lose the ability to differentiate. Loss of growth control can be due to autocrine mechanisms in some transformed cells, but the mechanisms involved in disrupting differentiation control are poorly understood. Our goal is to further define the growth and differentiation defects that arise in neoplastically transformed cells and the mechanisms underlying those defects. For example, exogenous transforming growth factor β and tumor necrosis factor, both of which are secreted aberrantly by some tumor cells, are known inhibitors of differentiation. In this study we transformed 3T3 T cells in vitro with chemical or UV irradiation treatment in order to determine if the acquisition of the transformed phenotype after these treatments is also associated with loss of differentiation control as it is with spontaneously or virally transformed cells. Four chemical and two UV-treated 3T3 T cell lines were isolated from type III foci and all have been found to be tumorigenic in syngeneic animals and to have lost the ability to differentiate. Relative to the parental cell line the differentiation abilities of the transformed clones ranged from 0 to less than 5%. In this regard, we also analyzed the normal and aberrant expression of three growth factors and differentiation inhibitors in transformed cells. Both transforming growth factor α and β were found to be expressed in nontransformed 3T3 T cells as determined by Northern blot analyses. In addition, both were found to be down-regulated during differentiation of 3T3 T cells. Transformed/differentiation-defective 3T3 T cells expressed varied levels of transforming growth factor α and β. Three of the transformed clones expressed particularly high levels of transforming growth factor α. Low levels of tumor necrosis factor expression were found in the normal cells and the transformed cells appeared to express tumor necrosis factor at similar levels. In contrast, none of the transformed cells expressed any of the differentiation-specific genes tested (lipoprotein lipase, glycerol-3-phosphate dehydrogenase, etc.). Even a transformed clone which could undergo growth arrest but not morphological differentiation expressed no differentiation-specific genes. Together, these data suggest that neoplastic transformation in general disrupts differentiation control. The aberrant expression of growth or differentiation-inhibiting factors may be involved in the loss of differentiation control in some transformed cells, but other mechanisms appear to be involved as well.

INTRODUCTION

Clinical observations and experimental studies have provided abundant evidence that the development of cancer is a multistep process. Carcinogenesis studies performed both in vivo and in vitro have demonstrated that this multistep process involves the development of defects in a variety of growth and differentiation regulatory mechanisms in the affected cell (1–9). The control of cellular differentiation and proliferation in normal cells is mediated in a complex fashion by a host of both positive and negative signals. In some cases, the autonomy of cancer cell growth is known to be caused, at least in part, by the aberrant production and response to growth factors, i.e., autocrine stimulation of proliferation (10–13). However, the loss of differentiation control manifested in most tumor cells and cells transformed in vitro can not be due solely to aberrant growth factor expression. For example, transforming growth factor β and cachectin/TNF, both of which are known to be anomalously secreted by some tumor cells, are potent inhibitors of distinct steps of adipocyte differentiation of 3T3 T cells as well as other proadipocytes (14–20). This suggests that the development of some cancers and their inability to differentiate properly may involve the aberrant production and response to differentiation-inhibiting factors as well as to growth factors. The fact that many metastatic and dysplastic disease states which are associated with aberrant differentiation also represent preneoplastic lesions further supports this conclusion (21).

In the 3T3 T system, it has been demonstrated that the expression of defects in the coupling of cellular differentiation and proliferation is of etiological significance in the initiation of carcinogenesis and in complete carcinogenesis (22–30). Furthermore, it has been demonstrated that during neoplastic transformation the loss of differentiation control mechanisms occurs prior to the loss of proliferation control mechanisms. For example, an initiating dose of a carcinogen can induce stable and heritable defects in the ability of 3T3 T stem cells to undergo either predifferentiation, reversible differentiation, or terminal differentiation; however, the same cells maintain intact proliferation control mechanisms (22).

To date, only spontaneously or virally transformed 3T3 T stem cells have been fully characterized in terms of defects in growth and differentiation (2, 8, 29, 30). Therefore, it is not known if 3T3 T stem cells that are transformed by different kinds of carcinogens develop the same kinds of defects in the control of differentiation and proliferation. In other words, does neoplastic transformation in general cause the loss of both differentiation and growth control mechanisms? In this regard, it has also been demonstrated in other proadipocyte cell lines that transformation by different SV40 or polyoma T antigens (1, 3, 31) or by v-src or c-myc (5, 32, 33) induces different defects in the coupling of proliferation and differentiation. However, no one has examined the expression and regulation, or lack thereof, of differentiation-specific genes or growth and differentiation modulators such as TGF-α, TGF-β, or TNF.

In our studies we are trying to dissect the mechanisms by which the coupling of cellular proliferation and differentiation is abrogated in neoplastically transformed cells. One of our goals is to determine if loss of differentiation control is a common characteristic of transformed cells, regardless of the type of carcinogenic agent causing the transformation. Here we report that both UV irradiation and the chemical 4-nitroquinoline-oxide can induce tumorigenicity and loss of differentiation control of 3T3 T cells. Some clones express high levels of TGF-α transcripts but only minor changes in TGF-β have been found in growing clones. TNF levels are extremely low in normal 3T3 T cells and do not appear to increase in the transformed clones. However, TGF-β and TNF protein levels have not been determined. None of these costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: TNF, tumor necrosis factor; DMEM, Dulbecco’s modified Eagle’s medium; SD5, sodium dodecyl sulfate; PBS, fetal bovine serum; TGF-α, transforming growth factor α; TGF-β, transforming growth factor β; NTD, terminal differentiation state; 4NQO, 4-nitroquinoline-oxide; HP, human plasma; GPD, glycerol-3-phosphate dehydrogenase; IRGT, insulin-regulated glucose transporter; LPL, lipoprotein lipase; v-src, avian sarcoma virus; MTAg, middle T-antigen.
of the transformed clones were found to express any of the differentiation genes that were examined. All of the new transformed clones have lost the ability to undergo morphological differentiation as well as the ability to express any of the differentiation-specific genes that were tested.

MATERIALS AND METHODS

Cells and Tissue Culture. The BALB/c 3T3 T mesenchymal (proadipocyte) stem cell line was used in these studies. It was originally subcloned from clone A31 3T3 cells because of its propensity to differentiate into adipocytes (34). It has, however, subsequently been shown that 3T3 T cells are mesenchymal stem cells that can be induced to differentiate into other cell types, including macrophages (26, 35). Stock cultures of these cells are grown at 37°C in a humidified atmosphere of 5% CO₂. The stock culture medium consists of antibiotic-free DMEM containing 4500 mg/liter glucose and 10% heat-inactivated fetal bovine serum. Stock cultures are maintained at low density by passage when less than 70% confluent. To ensure that cultures were free of mycoplasma contamination, the cells are periodically assayed by the method of Chen (36). New stock cultures were initiated every 2–3 months from stocks stored in liquid nitrogen. Various clones of 3T3 T cells were maintained in the same manner.

Other Cell Lines. The other cell lines used have been previously described. They include MCA3T3, a chemically transformed 3T3 fibroblast (not 3T3 T), and three spontaneously transformed 3T3 T cell lines, T2, T3, and T8 (30). The T2, T3, and T8 clones were derived by nonmutagenic methods and are considered to be spontaneously transformed. The NTD clones (NTD-4, NTD-5, NTD-7, and NTD-11) are nontransformed clones of 3T3 T cells which can undergo the early phase of differentiation but remain in the nonterminal differentiation state (NTD) and do not lose their proliferative capacity (28). RAW 264.7 cells were used as a positive control for the expression of TNF (37).

Carcinogenesis and Tumorigenicity Studies. Previous studies with 4NQO and UV irradiation used mass cultures to determine if type III foci would form or if immediate changes in differentiation response occurred. Clones of transformed cells were not isolated in those previous studies (22, 27). Treatment with carcinogens and isolation of clones were performed as previously described (22, 27, 38). Briefly, experimentally growing cultures of 3T3 T cells were treated with 4NQO at a concentration of 100 ng/ml for 24 h. Cell transformation was determined initially by the focus formation assay (22, 27, 38). Type III foci were isolated, expanded in culture, and then tested for tumorigenicity in syngeneic mice. One × 10⁵ cells were injected s.c. into young adult male BALB/c mice. Tumor presence was determined by palpation. 3T3 T cells were used as a negative control for tumor formation and MCA3T3 cells were used as a positive control. To obtain clones of cells that had been transformed with a physical carcinogen, growing 3T3 T cells were subjected to UV irradiation as previously described (22, 27). UV irradiation was with a GTE 15T8 lamp that supplied predominantly 254-nm irradiation. Exponentially growing cultures were irradiated at 270 erg/mm².

Induction of Adipocyte Differentiation. Platelet-poor HP was routinely used to induce the differentiation of 3T3 T cells at low cell density (35). 3T3 T cells were first plated at 1.5 × 10⁵ cells/cm² in 6-well cluster plates in DMEM containing 10% FBS. Large flasks (150 cm²) were used in experiments when mRNA was to be isolated. After attachment (4–24 h), the cells were refed with differentiation-inducing medium (25% HP in DMEM containing biotin and glycerol-3-phosphate dehydrogenase (23, 24, 35)). In this low cell density differentiation system, differentiation usually begins on days 4–6 and reaches a maximum by days 8–12 depending on the particular lot of plasma. Proliferation was assayed either by counting cells in situ in tissue culture dishes using an inverted phase microscope equipped with an ocular grid that has been calibrated with a linear micrometer or by trypsinization and counting in a hemacytometer. At least 10 grid fields were counted at random for each group and the cell density was determined by averaging duplicate wells and was expressed as cells/cm² (15).

Bacteriological Plate Differentiation Assay. Stock cultures of cells growing in DMEM/10% FBS were rinsed twice in phosphate-buffered saline and were removed from the flask by incubation in 0.1% EDTA in phosphate-buffered saline (pH 7.4) at 37°C for 30 min or until the cells rounded up or detached from the surface. Any remaining attached cells were mechanically dislodged and all of the cells were sedimented by centrifugation at 500 × g for 5 min and resuspended in heparinized DMEM with 25% HP. Cell suspensions were plated onto LAB-TEK 100-mm bacteriological Petri dishes at a density of 1–5 × 10⁵ cells/cm². Differentiation was induced by culturing cells in DMEM containing 25% HP described above. Because this technique inhibits impedes proliferation it may provide the proper environment to determine if a transformed cell can indeed differentiate (28, 29).

Northern Blot Analyses. RNA was harvested as previously described (16, 39). Briefly, cells were lysed with cold lysis buffer (10 mM Tris, pH 7.4–100 mM NaCl-2 mM EDTA-1% SDS-50 μg/ml proteinase K). The lysate was sheared and incubated for 0.5–3 h at 37°C. The lysate was adjusted to 400 mM NaCl and shaken overnight at room temperature in the presence of 0.1–0.3 g of oligodeoxynucleotidyl acid cellulose. The cellulose was washed, and then the polyadenylated RNA was eluted and quantitated using UV spectrophotometry. One to 10 μg of polyadenylated RNA was separated on 1.3% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to Nytran (Schleicher and Schuell) membranes by capillary transfer or electrobolt. The membranes were UV irradiated for 30 s using a stratalinker (Strategene) and dried at 65°C. Hybridization probes were prepared in the presence of [α-32P]dCTP (Amersham) using the random primer method (40) or [α-32P]dCTP for riboprobes. Hybridizations were carried out at 43°C (65°C for riboprobes) in a solution containing 50% formamide, 250 μg/ml denatured calf thymus DNA, 0.1% SDS, 1× Denhardt’s solution, 5× sodium saline-citrate, and 50 μg/ml polyadenyllic acid. The membranes were then washed for 1.5 h with 3 changes of wash solution (0.1× standard saline-citrate, 0.1% SDS). The blots were then exposed to X-ray film at −80°C with 2 intensifying screens.

Differentiation-specific Probes. Clone 1 is a 0.75-kilobase fragment of mouse DNA inserted into the EcoRI site of PMBL9. The fragment was removed using EcoRI and random hexamer labeled. The probe hybridizes to a 4.5-kilobase band which is greatly induced by adipogenesis (41) and was a gift from Gordon Rongold. GPD is a 0.3-kilobase fragment of mouse GPD inserted into the PstI site of pGEM4Z. The fragment was removed with PstI and random hexamer labeled. It hybridizes to a 3.5-kilobase band which is greatly induced during adipogenesis (42, 43) and was a gift from Bruce Spiegelman. IRGT (muscle- and adipose-specific) is a 2.085-kilobase insert in the EcoRI site of Bluescript sk−. It was linearized with BsrHI and the antisense riboprobe was generated with T7. It hybridizes to a 2.8-kilobase band which is greatly induced during adipogenesis (44) and was a gift from Rob Piper and David James. LPL is a 1.43-kilobase mouse LPL fragment inserted into the EcoRI site of pGEM2. The insert was removed with EcoRI and random hexamer labeled. The probe selects for a 3.4-kilobase fragment which is greatly induced during adipogenesis (45) and was a gift from Mike Schotz.

Growth Factor/Differentiation Inhibitor Probes. TGF-α is a 1.4-kilobase fragment of human TGF-α inserted into the EcoRI site of SP65. The plasmid was linearized with HindIII and a riboprobe was generated with SP6. This probe hybridizes to a 5.1-kilobase band. This probe was a gift from Rik Derynck. TGF-β is a 1.25-base pair fragment of mouse TGF-β inserted into the polylinker of pGEM4Z. The plasmid was linearized with EcoRI and a riboprobe was made using T7 RNA polymerase. The probe hybridizes to a 2.8-kilobase band and was a gift from Rik Derynck. TGF-β is a 1.1-kilobase insert in pGEM4Z and a riboprobe can be generated using BamHI to linearize and SP6 RNA polymerase. The probe hybridizes to a 2-kilobase band and was a gift from Genentech.

Other Probes. 1B15 (cyclophilin) is a 0.68-kilobase fragment of mouse cyclophilin inserted into the polylinker of pSP65. The insert was removed with a double cut of BamHI and PstI and random hexamer labeled. The probe can also be linearized with PstI for transcription with SP6 RNA polymerase. It hybridizes to a putative constitutive band at 0.8 kilobase, although in 3T3 T cells it appears to be regulated similar to β-actin during growth arrest and differentiation (16, 39). This probe was a gift from Jim Douglass.
RESULTS

Transformation and Tumorigenicity Studies of 3T3 T Stem Cells. It has been shown in several adipocyte systems that both spontaneously transformed and virally transformed cells lose their ability to differentiate (1-3, 5, 8, 28-31, 46). To determine if disruption of differentiation control is a general phenomenon associated with transformation, 3T3 T cells were transformed with two kinds of carcinogenic treatments that were different than those previously used. Some cultures were treated with the chemical carcinogen 4NQO and others with the physical carcinogen UV irradiation. Four different clones of 4NQO treated cells were successfully isolated from type III foci (Table 1) and were designated 4NQO-1, 4NQO-2, 4NQO-3, and 4NQO-4. Two clones of UV treated cells were isolated (UV-1 and UV-3) from type III foci (Table 1). Type III focus formation is one criterion for determining neoplastic transformation, but the best test is tumor formation in a syngeneic host. Therefore, these six clones were tested for their ability to form tumors when injected s.c. into BALB/c mice. All of the clones were found to be tumorigenic in syngeneic mice (Table 1). Every animal developed a tumor that was given an injection of a 4NQO cell line. The two UV cell lines each formed tumors in four of the six animals given injections. No tumors formed in animals given injections of parental 3T3 T cells, even after four months. The positive control cell line, MCA3T3 (not a derivative of 3T3 T) induced tumor formation in 2 weeks. Of the new cell lines, the time of first tumor appearance was 3 or 4 weeks for the 4NQO cell lines and 6 weeks for the UV cell lines. These data demonstrate by two different criteria that these new clones are indeed transformed/tumorigenic.

Loss of Differentiation Ability in Transformed 3T3 T Cells. Each of the new clones were subjected to differentiation-inducing medium. Each cell line was tested 3-8 times and normal 3T3 T cells were used as the control for each experiment. All six of the new transformed/tumorigenic clones had lost the ability to undergo morphological differentiation (Fig. 1). Their abilities to differentiate relative to the parental cell line were UV-1 (1.4%), UV-3 (1.6%), 4NQO-1 (4.6), 4NQO-2 (2.6), 4NQO-3 (0), and 4NQO-4 (2). The differentiation enhancer AD47439 (23) was also tested on three of the transformed cell lines (UV-3, 4NQO-2, and 4NQO-3) and the presence of this drug did not induce differentiation (data not shown).

It is interesting to note that the 4NQO-2 cell line, although incapable of undergoing differentiation, can still undergo growth arrest at low cell density when treated with differentiation inducing medium (data not shown). Typically, low cell density refers to approximately $5 \times 10^3$ to $1 \times 10^4$ cells/cm$^2$. Confluent density is usually $5 \times 10^4$ cells/cm$^2$. All of the new cell lines, other than 4NQO-2, merely slowed their growth when the medium was switched from growth medium (10% FBS) to the growth factor-deficient differentiation-inducing medium (25% HP) and proceeded to confluency despite the lack of exogenous growth factors.

Growth Inhibitory Bacteriological Plate Microenvironment for Assaying Differentiation. This assay was used as a supplemental assay for testing the differentiation ability of transformed clones. This technique has certain advantages for testing transformed cells (26, 28, 29). Because bacteriological plates are not treated for cell growth, cell proliferation is inhibited or impeded. This characteristic is important because some of the transformed lines express high levels of TGF-α mRNA (see below) and tumors derived from 3T3 T cells can make growth factor activity (47). If these new transformed clones are also producing differentiation-inhibiting activity, this activity would be masked in the routine differentiation assays because the cells used in the assay would be stimulated to proliferate. Therefore, this assay utilizing growth-inhibitory nontissue culture plates can be used to test for the presence of autocrine differentiation inhibiting factors without the interference of autocrine growth factors.

We tested several of the new tumorigenic clones for their ability to differentiate in this growth restrictive assay. Of the three clones tested (UV-1, UV-3, and 4NQO-1), none could differentiate in this assay (Fig. 2). In fact, in this assay the analysis (counting random fields of cells) yielded 0% differentiation for each of these three transformed clones tested. However, thorough scanning of each dish resulted in the observation of at least one fat cell.

Expression of Differentiation-specific Genes. Although none of the transformed clones could undergo morphological differentiation, the possibility that aberrant expression of differentiation genes could occur was tested. Many new genes are turned on during normal adipocyte differentiation and it is not known if any of them can be turned on independently of the others or if in a transformed cell some might be turned on independently of morphological differentiation. In addition, many transformed cells are known to express a variety of genes in an anomalous fashion. Therefore, several differentiation genes were examined in various transformed clones. The differentiation-specific genes LPL, GP D, and IRGT were screened for their expression in the clones during various growth, arrest, and differentiation states. In growing normal 3T3 T cells differentiation-specific genes are expressed at undetectable or very low levels and expression increases during differentiation (16, 39). The differentiation-specific genes were found to be expressed at very low or undetectable levels in growing transformed cells (Fig. 3, A and B). Fig. 3A demonstrates the lack of LPL (top) and GDP (bottom) expression in various clones relative to differentiated 3T3 T cells (Lane 1). Fig. 3B compares the expression of IRGT in differentiated 3T3 T cells (Lane 9) to various differentiation-defective clones. IRGT was only expressed at high levels in normal differentiated 3T3 T cells. C/EBP and clone 1 expression were also analyzed in some of the clones and the results were
similar to that of the other differentiation-specific genes. We have found no differentiation genes to be induced in the transformed cells under any condition, including long-term culture in the presence of differentiation-inducing medium. More than 10 ten clones of differentiation-defective cells have been analyzed for differentiation-specific gene expression.

Expression of TGF-β during Normal Differentiation. Before testing the possibility that some of the transformed clones were producing aberrant levels of the differentiation-inhibitor TGF-β, normal expression, if any, had to be determined. Therefore, normal 3T3 T cells were examined for TGF-β expression during logarithmic growth and differentiation. The expression of TGF-β was also compared to that of a differentiation-specific gene, i.e., clone 1. It can be seen in growing 3T3 T cells (Fig. 4, Lane 1) that TGF-β is expressed (top), whereas the differentiation-specific gene clone 1 (bottom, upper band) is not. Once cell differentiation occurs the expression of the two genes changes. Fig. 4, Lane 2 represents differentiated cells. The differentiation-specific gene clone 1 is expressed at high levels in the differentiated cells (Fig. 4, Lane 2), whereas TGF-β expression has decreased in the differentiated cells relative to growing cells (Lane 1). The bottom panel was probed with clone 1 and 1B15 at the same time. The lower band of the bottom panel is 1B15. It is a lane loading marker and decreases during differentiation in a manner similar to actin (16, 39).

Expression of TGF-β in Transformed 3T3 T Cells. The Northern blot in Fig. 5 compares TGF-β expression in growing 3T3 T cells to the levels in eight transformed clones of 3T3 T cells. Some clones appear to express higher levels of TGF-β than control 3T3 T cells (Fig. 5, Lane 5), whereas at least one transformed clone (UV-3; Lane 6) appears to express lower levels. Of the transformed lines tested, only 4NQO-2 cells will undergo growth arrest at low density when subjected to differentiation-inducing medium. The point at which they are blocked in their ability to differentiate is the same point in the G1 phase of the cell cycle called GD (23, 24, 35). GD is followed by a reversible/nonterminal phase of differentiation called nonterminal dif-

Expression of TNF in Normal, Transformed, and Differentiation-defective Clones. The differentiation process of 3T3 T cells includes predifferentiation growth arrest at a specific point in the G1 phase of the cell cycle called GD (23, 24, 35). GD is followed by a reversible/nonterminal phase of differentiation called nonterminal dif-

4 Unpublished observations.
is known to be expressed at high levels in some transformed cells and was chosen for study in these initial experiments. However, normal expression levels needed to be determined prior to examining expression in the transformed 3T3 T cells. The differentiation-specific gene LPL and TGF-α were compared in confluent-arrested, growing, and differentiated 3T3 T cells. LPL expression was greatly elevated in differentiated cells (Fig. 7, top, Lane 3). Fig. 7 demonstrates that there is expression of TGF-α in normal growing 3T3 T cells (Lane 2) and that the level of expression is quite a bit lower in differentiated cells (Lane 3). In addition, the blot was probed with 1B15 to compare lane loading. Similar to actin, 1B15 (cyclophilin) expression was lower in confluent (arrested) cells and differentiated cells than in growing cells (16, 39).

Expression of TGF-α in Transformed 3T3 T Cells. The Northern blot in Fig. 8 compares TGF-α expression levels in 3T3 T cells to three transformed clones. It can be seen that the transformed clones 4NQO-3, 4NQO-4, and UV-1 (Fig. 8, Lanes 1–3) all express higher levels of TGF-α than normal 3T3 T cells (Lane 4). Other transformed 3T3 T cells were found to express somewhat different levels of TGF-α than normal 3T3 T cells, but those results vary and appear to be somewhat isolation dependent (data not shown). It is possible that the cell lines that have variable TGF-α levels are either extremely sensitive to cell density or to feeding schedules. None of the transformed clones appear to express lower levels of TGF-α than the normal 3T3 T cells. Preliminary data suggest that biologically active growth factor activity is being secreted by 4NQO-3 cells.

DISCUSSION

Current models suggest that cancer develops in association with the expression of defects in the control of both cellular differentiation and proliferation (5, 6, 9, 22, 28). Furthermore, the expression of defects in the control of differentiation may occur in the early stages of carcinogenesis. For example, it has been shown that either UV irradiation or chemical carcinogens, at doses that initiate but do not promote carcinogenesis, induce stable and heritable defects in stem cell differentiation without affecting the control of cell proliferation (22, 27) and it has been demonstrated that an initiating dose of carcinogen can induce specific defects in the ability of epidermal cells to undergo terminal differentiation (9). TGF-β and TNF, both of which are known to be anomalously secreted by some tumor cells, are potent inhibitors of distinct steps of differentiation in 3T3 T proadipocytes as well as other proadipocytes (14, 15, 17–19). TGF-β and TNF are classified as specific inhibitors of differentiation because they only affect differentiation and do not affect growth in this system. Growth factors such as serum, fibroblast growth factor, epidermal growth factor, etc., can inhibit differentiation indirectly by stimulating cell

![Fig. 7. Northern analysis of differentiation-specific and TGF-α gene expression during normal 3T3 T growth and differentiation. Polyadenylated RNA (2.5 µg/lane) was analyzed as described in "Materials and Methods." Normal confluent, growing, and differentiated cells were analyzed. The same blot was probed, stripped, and reprobed in order to examine the differentiation gene LPL (top), TGF-α (middle), and 1B15 expression (bottom). Lane 1, confluent-arrested cells; Lane 2, growing cells; Lane 3, differentiated cells.](http://cancerres.aacrjournals.org)
proliferation (49). These and other studies suggest that the development of most cancers involves both growth and differentiation defects and that the inability to differentiate properly may involve the aberrant production and response to differentiation-inhibiting factors as well as to growth factors.

Previously, only spontaneously and virally transformed 3T3 T cells had been characterized in terms of defects in growth and differentiation (2, 8, 28, 30). The current study demonstrates that 3T3 T preadipocytes that have been transformed chemically or by UV irradiation lose their ability to undergo morphological differentiation and the ability to express differentiation-specific genes. Therefore, the current data along with previous work with other types of carcinogens (described above) suggest that neoplastic transformation in general disrupts the control of cellular differentiation. It should be emphasized that subcloning itself does not cause loss of differentiation ability. Many normal subclones of 3T3 T cells have been isolated previously without affecting the differentiation capability relative to the parental line (26, 28, 29).

Other laboratories have also studied the effects of neoplastic transformation on differentiation control in preadipocytes. Various kinds of growth and differentiation defects have resulted. An inverse correlation between the ability to differentiate and the acquisition of the tumorigenic phenotype, was found in the 1246 preadipocyte cell line (46). It was found that transformed cells lost the ability to differentiate and that these transformed cells were secreting a growth stimulating activity that was insulin-like. In another study, 3T3 L1 preadipocytes transformed by v-src lost the ability to differentiate (5). Conditioned medium from the transformed cells contained a heat stable factor(s) which inhibited the differentiation of the parental 3T3 L1 preadipocytes. Growth factor activity was also detected in the conditioned medium. The latter study suggested that viral transformation of cells with the oncogene v-src inhibits differentiation via an extracellular intermediate; it was postulated that this differentiation-inhibiting activity might be TGF-β. Freytag et al. (32, 33) found that overexpression of c-myc blocks the ability of 3T3-L1 preadipocytes to differentiate. Cherington et al. (1) found that either polyoma MTAg or large T-antigen could inhibit the differentiation of 3T3-F442A preadipocytes. Of the various growth alterations induced by the polyoma T-antigens it was suggested that each could be due to the induction of endogenous growth factors. The authors postulated that the aberrant secretion of differentiation inhibitors such as TGF-β or cachectin/TNF could be the basis for the specific differentiation block elicited by T-antigens. They also explored the effects of SV40 large T-antigen (31). Their results demonstrated that the differentiation-inhibiting region of large T was different than that of the transforming region.

Grimaldi et al. (3) tested the effects of polyoma T-antigens on the ob17 preadipocyte cell line. Transformation induced by the complete early region of polyoma virus yielded tumorigenic cells that could not differentiate. Transformation using vectors encoding only MTAg yielded tumorigenic cells that could not differentiate under normal conditions. However, MTAg transformed cells were able to differentiate when placed at low density in growth-restrictive conditions, i.e., serum-free defined medium. There was an inverse relationship between the potential of an MTAg-transformed clone to grow in low serum and its ability to differentiate. Their experiments demonstrated two points concerning neoplastic transformation and differentiation: (a) that, as in the case of cells transformed by the complete early region of polyoma, if a transformed cell cannot stop growing it cannot differentiate; and (b) as illustrated by the cells transformed by MTAg which, although tumorigenic and capable of growing to high cell densities in the presence of serum, do not grow in low serum concentrations and under special conditions can be induced to differentiate. This suggests that the coupling of proliferation and differentiation in MTAg transformed cells is defective and under normal conditions the cells will not differentiate because the cells continue to grow. However, when conditions that are very restrictive to growth are used the cells still have the capability to differentiate. This suggests that other transformed cells might be able to differentiate, if their growth could be inhibited. Our experiments utilizing the bacteriologic plate assay (2) were an effort to duplicate the type of results obtained by Grimaldi et al. (3) with MTAg. We were not successful with the three cell lines that were tested.

We also tested the hypothesis that the aberrant expression of growth factors and/or of specific differentiation inhibitors is involved in uncoupling proliferation and differentiation. It is known that various growth factors are expressed in normal cells. In general, growth factors are antagonistic to differentiation in the 3T3 T system due to the fact that they stimulate growth and do not allow the cells to undergo growth arrest and differentiate (23, 24, 35, 47, 49). Changes in expression of other proteins could be involved in these processes as well (50, 51). If a nontransformed cell, such as 3T3 T, is making a growth factor and secreting it, then this could cause an indirect inhibition of differentiation by stimulating growth. Therefore, in order for differentiation to occur the expression of the growth factor would need to be down-regulated. The same would be true for a differentiation-inhibiting substance such as TGF-β. The present study does indeed demonstrate that nontransformed 3T3 T cells express TGF-α and TGF-β and that both appear to be down-regulated during differentiation. They appear to be regulated in a reciprocal fashion to differentiation genes such as GPD and LPL. Altered regulation of either these types of processes could result in, or be involved in, neoplastic transformation. It has been established that tumors arising from transformed 3T3 T cells secrete at least one growth factor activity in high quantities (47).

We had hypothesized that the adipocyte differentiation inhibitors TGF-β and TNF might play a role in blocking differentiation of the transformed preadipocytes. Our analysis of TGF-β expression did reveal that several transformed clones have higher TGF-β transcript levels than the normal 3T3 T cells. We did not see large increases in TNF transcript levels; however, the studies have not been extended to characterization at the protein level. Previous work showed that nontransformed yet differentiation-defective clones of 3T3 T cells secrete terminal differentiation-inhibiting activity into the medium. The activity inhibited terminal differentiation in a manner similar to that caused by TNF but was not fully characterized to determine if it was indeed TNF (14). Here we examined the expression of TNF in transformed clones as well as the clones that are known to secrete TNF-like activity. Aberrant expression of TNF transcripts was not detected in any of the cells tested. This suggests two possibilities regarding TNF: (a) autocrine expression of TNF may not play a role in disrupting differentiation of transformed preadipocytes; or (b) aberrant regulation of TNF is at a posttranscriptional level. Further characterization of TNF RNA processing and TNF proteins levels are required to adequately address this question. The same is true for TGF-β.

Several conclusions can be drawn from the data presented here and from previous studies. First, regardless of the type of transforming agent, loss of the ability to differentiate is a common characteristic of
transformed proadipocytes. Second, a wide variety of growth and differentiation defects occur in the transformed cells. These defects include aberrant expression or regulation of at least several growth/differentiation factor regulatory genes and the loss of ability to express differentiation-specific genes. The data suggest that there are probably at least several different ways to disrupt differentiation and each of those yields the same ultimate result.

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REFERENCES

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