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Early Induction of Ribonucleotide Reductase Gene Expression by Transforming Growth Factor β_1 in Malignant H-*ras* Transformed Cell Lines*

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Previous investigations have indicated that the suppression of proliferation by transforming growth factor (TGF) β_1 is often lost upon cellular transformation, and that proliferation of some tumors is stimulated by TGF-β. The present study provides the first observation of a link between TGF-β_1 regulation of this process and alterations in the expression of ribonucleotide reductase, a highly controlled rate-limiting step in DNA synthesis. A series of radiation and T24-H-*ras*-transformed mouse 10T½ cell lines exhibiting increasing malignant potential was evaluated for TGF-β_1 induced alterations in ribonucleotide reductase M1 and M2 gene expression. Early increases in M1 and/or M2 message and protein levels were observed only in malignant cell lines. The TGF-β_1 induced changes in M1 and/or M2 gene expression occurred prior to any detectable changes in the rates of DNA synthesis, supporting the novel concept that ribonucleotide reductase gene expression can be elevated by TGF-β_1 without altering the proportion of cells in S phase. T24-H-*ras*-transformed 10T½ cells were transfected with a plasmid containing the coding region of TGF-β_1 under the control of a zinc-sensitive metallothionein promoter. When these cells were cultured in the presence of zinc, a large induction of TGF-β_1 message was observed within 1 h. Both M1 and M2 genes were also induced, with increased mRNA levels appearing 2 h after zinc treatment, or 1 h after TGF-β_1 message levels were clearly elevated. In total, the data suggests a mechanism of autocrine stimulation of malignant cells by TGF-β_1, in which early alterations in the regulation of ribonucleotide reductase may play an important role.

Many biological and biochemical changes occur during malignant progression (1, 2), and these appear to be controlled through modification in key regulatory genes, most notably oncogenes (3–6) which are important in diverse cellular functions, including proliferation, cell-cell communication, and motility (3, 4). These functions are regulated by growth factors (7) and it is not surprising that some oncogenes encode proteins that are involved in growth factor signalling (8–10). Not only can cell proliferation be regulated through these mitogenic stimuli, but negative growth regulators such as the TGF-β[1] family appear to be important inhibitors in most tissue types (11). The growth inhibitory effects of TGF-β are lost in many but not all transformed epithelial cell lines, and it has been suggested that cells must overcome this growth inhibition to undergo transformation (11). A number of mechanisms to explain growth suppression by TGF-β have been proposed, including alterations in epidermal growth factor binding and response (12), inhibition of transcription of the growth related gene c-*myc* (13), inhibition of phosphorylation of the retinoblastoma gene product RB (14), and a decrease in phosphorylation and histone H1 kinase activity of the $p34^{cdc2}$ protein kinase (15). We have reported, that while TGF-β inhibited DNA synthesis in the 10T½ pulmonary fibroblast cell line, H-*ras* transformed 10T½ cell lines demonstrating a highly malignant phenotype, exhibited stimulation of DNA synthesis following exposure to TGF-β_1 (16). We have proposed that this dramatically altered response to TGF-β following malignant transformation by *ras* combined with an elevated rate of secretion of activated TGF-β_1 by these cells (17), suggests a role for TGF-β autocrine stimulation of cell proliferation in malignancy. This hypothesis predicts that in the presence of TGF-β, metastatic cell populations will exhibit significant alterations in expression of important genes involved in the coordination of DNA synthesis and cell proliferation. In this study, we test this idea by investigating the highly regulated expression of two genes that code for a rate-limiting enzyme of DNA synthesis, ribonucleotide reductase (18, 19).

Ribonucleotide reductase is responsible for the *de novo* conversion of ribonucleotides to deoxyribonucleotides (18, 19), essential for the synthesis of DNA. The reaction is rate-limiting for DNA synthesis, and therefore the enzyme plays an important role in the regulation of cell division (18, 19). In mammalian cells the active enzyme contains two dissimilar components often called M1 and M2. Protein M1 is a dimer with a molecular weight of 170,000 and possesses complex substrate and effector-binding sites (19, 20). Protein M2 is also a dimer, has a molecular weight of 88,000, and contains non-heme iron and a unique tyrosyl-free radical needed for activity (19, 21). Interestingly, the activity of ribonucleotide reductase correlates more closely with DNA synthesis than any other biosynthetic activity (2), and in actively growing cells, S phase appears to be dependent upon synthesis of the M2 component, which is rate-limiting for ribonucleotide reductase activity (19, 22). Alterations in ribonucleotide reductase can have profound effects upon the biological properties of cells (18). For example, altered ribonucleotide reductase

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[1] The abbreviation used is: TGF, transforming growth factor.

TABLE I

Biological properties of the various mouse 10T½-derived cell lines

Data were summarized from previously reported results (5, 16, 17).

Cell line	Transformation	Experimental metastases[a]			TGF-β_1 proliferation response
		Tumor frequency subcutaneously[b]	Lung nodules ± S.E.	Degree of malignancy	
10T½		0/12	0	Normal	Inhibited
MDS.R5	Radiation	5/5	0	Benign	Inhibited
NR3	T24-H-*ras*	6/8	0.1 ± 0.1	Benign	No effect
NR4	T24-H-*ras*	10/10	2.0 ± 0.5	Low	Stimulation
C1	T24-H-*ras*	13/13	14 ± 5	Intermediate	Stimulation
C3	T24-H-*ras*	11/11	121 ± 20	High	Stimulation

[a] Injections were performed with 3×10^5 cells except for the MDS.R5 line, where 10^6 cells were injected.

[b] Tumorigenicity was determined by the frequency of mice with tumors following subcutaneous injections of 3×10^5 cells.

has been defined as a mutator locus in mammalian cells (23), the enzyme may be involved in certain immunodeficiency diseases in man (24), some studies have suggested that the activity is tumor progression-linked (2), and it appears to be important in critical early events in a mechanism of tumor promotion (25).

EXPERIMENTAL PROCEDURES

Cell Lines—Isolation and characterization of the mouse 10T½ cell lines used in this study have been described (5, 16, 17). In brief, cells were transfected with plasmid pAL8A, which contains T24-H-*ras* and the neo^R gene. After transfection, cell lines were isolated either as foci at confluence (C1 and C3) or by G418 resistance (NR3 and NR4). The cell line called MDS.R5 is a clone of radiation transformed 10T½ cells. These cell lines were routinely maintained at 37 °C in culture medium containing α-minimal essential medium (Flow Laboratories) supplemented with antibiotics and 10% (v/v) fetal bovine serum (Gibco). A summary of the malignant properties of these cell lines, and their response to TGF-β_1 in DNA synthesis assays is presented in Table I. It is important to note that the parental 10T½ cell line is not tumorigenic, the MDS.R5 and NR3 lines form benign tumors, and the remainder of the H-*ras* transfected lines were increasingly malignant.

Analysis of M1 and M2 Gene Expression and Protein Levels—To determine the effects of TGF-β_1 on the expression of M1 and M2 genes, medium containing 10% fetal bovine serum was removed and replaced with defined medium containing α-minimal essential medium supplemented with insulin (2.0 μg/ml) and transferrin (4.0 μg/ml), as we have previously reported (16). The doubling times of the cell lines used in this study varied between 18 and 24 h in defined medium. At various times (1, 2, and 4 h) porcine TGF-β_1 (R & D Systems) was added at 10 ng/ml to culture plates (150 mm) containing $2.5–3.2 \times 10^6$ cells. Cells were removed with 0.3% trypsin (Difco Laboratories) solution, RNA was prepared, and gene expression was determined by Northern blotting as we have described (26). A *Nco*I (Boehringer Mannheim) generated fragment of clone 65 (M1) or a *Pst*I (Boehringer Mannheim) fragment of clone 10 (M2) was used in these experiments (21, 25, 26). The levels of M1 and M2 proteins were determined by Western blot analysis (26, 27). Anti-M1 mouse monoclonal antibody, AD203, or anti-M2 rat monoclonal antibody JB4, was used in these investigations as we have previously reported (26, 27).

RESULTS AND DISCUSSION

TGF-β_1 effects on the expression of M1 or M2 genes in the various cell lines shown in Table I are presented in Fig. 1. Induction of ribonucleotide reductase gene expression was observed, but only in malignant cell lines. For example, no induction of M1 or M2 gene expression was observed in parental 10T½ cells or in benign tumors, MDS.R5 and NR3. However, the metastatic cell lines, NR4, C1 and C3 exhibited obvious alterations in M1 and/or M2 gene expression following only 1 h of TGF-β_1 treatment, and the magnitude of these changes correlated with their malignant potential. The poorly

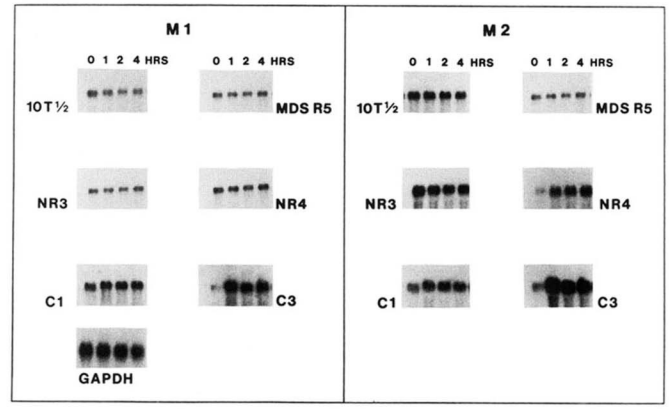

FIG. 1. **Northern blot analysis of M1 mRNA (M1) or M2 mRNA (M2) levels following exposure to 10 ng/ml of TGF-β_1 for 1, 2, and 4 h.** Increased M1 and M2 mRNA levels were noted with 1 h of TGF-β treatment in lines C1 and C3 and M2 was elevated in the NR4 line. RNA loading was determined with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA (25) for each of the blots. The observations shown above were not due to differences in RNA loading. An example of a M1 RNA loading control is provided for the C1 cell line.

metastatic line, NR4, demonstrated a clear elevation in M2 gene expression, the component which is rate-limiting for ribonucleotide reduction in actively growing cells (18, 19, 22). The intermediate and highly metastatic lines (C1 and C3) showed significant elevations in both M1 and M2 gene expression, with the most malignant C3 cell line exhibiting the largest changes in TGF-β_1-induced gene expression. Densitometric analysis of Northern blots 1 h after TGF-β_1 exposure gave estimates of approximately 3- and 6-fold elevations in M1 mRNA levels in C1 and C3 cells, respectively, and about 4-, 3.5-, and 7-fold increases in M2 message in NR4, C1, and C3 cells, respectively.

To directly investigate the relationship between the expression of TGF-β_1 and ribonucleotide reductase genes, C1 cells were co-transfected by lipofection (28), with a plasmid (pPK9A) containing the coding region of TGF-β_1 under the control of a zinc-sensitive metallothionein promoter and the hygromycin resistance pY3 marker.[2] The pPK9A plasmid contains the coding region of porcine TGF-β_1 cDNA inserted between the metallothionein promoter and human growth hormone polyadenylation sequence. The TGF-β_1 sequence also contains mutations previously described by Brunner *et al.* (29), which change the cysteines at 223 and 225 in the

[2] S. K. Samuel, R. A. R. Hurta, P. Kondaiah, N. Khalil, E. A. Turley, J. A. Wright, and A. H. Greenberg, submitted for publication.

polypeptide to serines, resulting in the secretion of active mature TGF-β_1. Colonies containing transfected DNA were identified in growth medium supplemented with 0.2 mg/ml hygromycin (30). The cell line called A20 was derived from a colony exhibiting TGF-β_1 induction in the presence of zinc and was used in the studies shown in Fig. 2. Prior to zinc addition we noticed that A20 cells contained an elevation in TGF-β_1 message of severalfold when compared to the non-transfected C1 population (data not shown), suggesting that a basal level of transcription from the recombinant TGF-β_1 may be occurring in the A20 line. When A20 cells were cultured in defined medium (16) containing zinc sulfate, a large induction of TGF-β_1 message was observed within 1 h (Fig. 2). Interestingly, both M1 and M2 genes were also induced, with increased message levels appearing 2 h after addition of zinc or 1 h after TGF-β_1 message levels were observed to be maximal. These results are consistent with observations presented in Fig. 1, in which ribonucleotide reductase gene expression in malignant cells was elevated following 1 h of TGF-β_1 treatment, demonstrating a direct relationship between TGF-β_1 and the expression of M1 and M2 genes.

Western blot analysis was performed to determine if the changes in ribonucleotide reductase message levels shown in Fig. 1 were also observed at the protein level. As shown in Fig. 3 an elevation in protein M1 was observed in C1 and C3, but not in 10T½ cells, following 1 h exposure to TGF-β_1. Furthermore, analysis of M2 protein showed an increase in the levels of this protein in C1, C3, and NR4 cells, but not in parental 10T½ cells (Fig. 3). Densitometric measurements indicated approximately 2- and 4-fold increases in M1 protein in C1 and C3 cells, respectively, and about 2-, 3-, and 5-fold elevations of M2 protein in NR4, C1, and C3 cells, respectively. These changes in protein levels are consistent with TGF-β_1 induction of M1 and/or M2 message (Fig. 1).

Although the changes observed in M1 and/or M2 gene expression occurred relatively quickly following TGF-β_1 treatment (within 1 h), making it unlikely that a block at S phase or an unusual movement of cells into S phase could account for the obvious growth factor modifications in ribonucleotide reductase, this point was addressed directly by measuring the incorporation of [^{3}H]thymidine (ICN Radiochemicals) into DNA as described previously (16). Fig. 4 shows that changes in M1 and/or M2 gene expression in metastatic cells by TGF-β_1 occurred prior to any detectable changes in the rates of

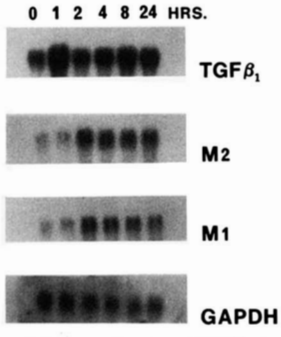

FIG. 2. **Northern blot analysis of TGF-β_1, M1, and M2 gene expression in the A20 cell line following exposure to 100 μM zinc sulfate for 1, 2, 4, 8, and 24 h.** The A20 cell line has been transfected with the pPK9A plasmid containing TGF-β_1 cDNA regulated by the zinc-sensitive metallothionein promoter. The TGF-β_1 probe used in these experiments was obtained by *Bgl*II digestion of the pPK9A plasmid. RNA loading was determined using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA (25). Control experiments showed that the zinc sulfate treatment used in these experiments did not induce M1 or M2 gene expression in C1 cells.

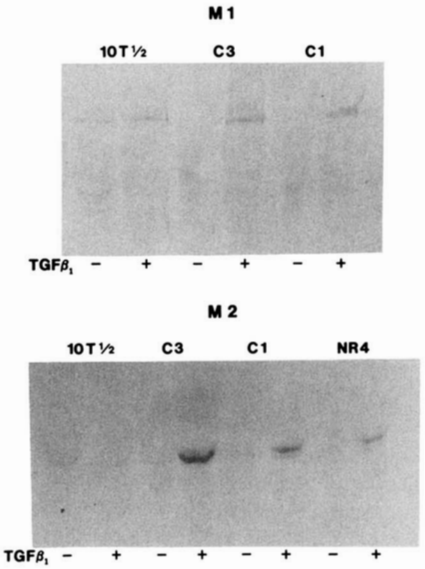

FIG. 3. **Western blot analysis of M1 protein (*M1*) or M2 protein (*M2*).** 120 μg of cell extract protein was loaded in each lane. Untreated cells (−) were compared to cells exposed to 10 ng/ml of TGF-β_1 for 1 h (+).

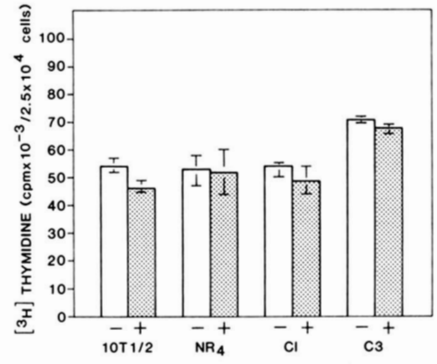

FIG. 4. **DNA synthesis assay during early induction of M1 and M2 gene expression by TGF-β_1.** Medium containing 10% fetal bovine serum was removed and replaced with the defined medium described under "Experimental Procedures," and cells were exposed to TGF-β_1 for 1 h, the time at which M1 and M2 gene expression was elevated. Cells were pulsed with [^{3}H]thymidine and incorporation of label into trichloroacetic acid-precipitable material was determined (16).

DNA synthesis. These observations are consistent with previous investigations where TGF-β_1 effects on DNA synthesis in the cell lines examined in this study have been described (16, 17). These studies have shown that TGF-β_1 treatment inhibits the synthesis of DNA in 10T½ and benign tumor cells, while stimulating DNA synthesis in the malignant NR4, C1, and C3 cell lines, with maximal effects occurring after about 42 h of growth factor exposure (16). No stimulation of DNA synthesis in malignant cell lines was observed until at least 18 to 24 h of TGF-β_1 treatment (data not shown). These results support the novel concept that TGF-β_1 induces ribonucleotide reductase gene expression without changing the proportion of cells in S phase.

There is evidence suggesting that the regulation of mammalian ribonucleotide reductase during the cell cycle occurs primarily at the protein level through the synthesis and breakdown of the M2 component, with relatively little change in protein M1 (19, 22). The present study demonstrates that significant alterations can occur in both M1 and M2 protein and message levels in mammalian cells, and interestingly, the

changes presented in this study occurred only in malignant H-*ras*-transformed cells, in response to exogenously added TGF-β_1 or through autocrine TGF-β_1 stimulation. Furthermore, these alterations to ribonucleotide reductase appeared to be uncoupled from the S phase of the cell cycle. The intracellular signals responsible for this novel regulation of ribonucleotide reductase are unknown. Indeed, very little is presently known about the regulatory signal pathways linking ribonucleotide reductase to DNA synthesis in the normal cell cycle. Of interest, the M2 message can be elevated by treating cells with the tumor promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (25). 12-*O*-Tetradecanoylphorbol-13-acetate response elements are recognized, for example, by the transcription factor AP1 (jun/fos) (31). This is a similar pathway to that utilized by TGF-β_1 for autoinduction (32), and for the regulation of transin/stromelysin gene expression (33). It would clearly be of interest to delineate the signal pathways that regulate DNA synthesis by TGF-β_1 through controlling the expression of the ribonucleotide reductase genes. The present study suggests that *ras*-transformed cells have uncoupled TGF-β_1 signaling and are now utilizing a novel pathway for stimulation of ribonucleotide reductase gene expression.

Collectively, the data presented in this study support a mechanism of autocrine stimulation of abnormal proliferation of malignant cells by TGF-β_1, in which early alterations in the regulation of the highly controlled ribonucleotide reductase are important.

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