Early Induction of Ribonucleotide Reductase Gene Expression by Transforming Growth Factor \( \beta_1 \) in Malignant H-\textit{ras} Transformed Cell Lines*

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Previous investigations have indicated that the suppression of proliferation by transforming growth factor (TGF) \( \beta_1 \) is often lost upon cellular transformation, and that proliferation of some tumors is stimulated by TGF-\( \beta \). The present study provides the first observation of a link between TGF-\( \beta \) 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-\textit{ras}-transformed mouse 10T\( ^{1/2} \) cell lines exhibiting increasing malignant potential was evaluated for TGF-\( \beta \), 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-\( \beta_1 \) 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-\( \beta_1 \), without altering the proportion of cells in S phase. T24-H-\textit{ras}-transformed 10T\( ^{1/2} \) cells were transfected with a plasmid containing the coding region of TGF-\( \beta_1 \), under the control of a zinc-sensitive metallothionein promoter. When these cells were cultured in the presence of zinc, a large induction of TGF-\( \beta_1 \) 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-\( \beta_1 \) message levels were clearly elevated. In total, the data suggests a mechanism of autocrine stimulation of malignant cells by TGF-\( \beta_1 \), 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-\( \beta \) family appear to be important inhibitors in most tissue types (11). The growth inhibitory effects of TGF-\( \beta \) 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-\( \beta \) 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 p54\textsuperscript{ras} protein kinase (15). We have reported, that while TGF-\( \beta \) inhibited DNA synthesis in the 10T\( ^{1/2} \) pulmonary fibroblast cell line, H-\textit{ras} transformed 10T\( ^{1/2} \) cell lines demonstrating a highly malignant phenotype, exhibited stimulation of DNA synthesis following exposure to TGF-\( \beta_1 \) (16). We have proposed that this dramatically altered response to TGF-\( \beta \) following malignant transformation by \textit{ras} combined with an elevated rate of secretion of activated TGF-\( \beta_1 \) by these cells (17), suggests a role for TGF-\( \beta \) autocrine stimulation of cell proliferation in malignancy. This hypothesis predicts that in the presence of TGF-\( \beta_1 \), 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 \textit{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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¶ The abbreviation used is: TGF, transforming growth factor.
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 10T% 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 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 10TY2 cells. These cell lines were routinely maintained at 37 °C in culture 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 supplemented with 10% fetal bovine serum (Gibco). A summary of the malignant properties of these cell lines, and their response to TGF-β1 in DNA synthesis assays is presented in Table I. It is important to note that the parental 10T% 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 metastatic.

**Analysis of M1 and M2 Gene Expression and Protein Levels**—To determine the effects of TGF-β1 on the expression of M1 and M2 genes, medium containing 10% fetal bovine serum was removed and the intermediate and highly metastatic lines (C1 and C3) showed significant elevations in both M1 and M2 gene expression, the component which is rate-limiting for ribonucleotide reduction in actively growing cells (18, 19, 22). 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, JC2, was used in these investigations as we have previously reported (26, 27).

**RESULTS AND DISCUSSION**

TGF-β1 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 10T% 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-β1 treatment, and the magnitude of these changes correlated with their malignant potential. The poorly 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-β1-induced gene expression. Densitometric analysis of Northern blots 1 h after TGF-β1 exposure gave estimates of approximately 3- and 6-fold elevations in M1 mRNA levels in NR4, 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-β1 and ribonucleotide reductase genes, C1 cells were co-transfected by lipofection (28), with a plasmid (pPK9A) containing the coding region of TGF-β1, under the control of a zinc-sensitive metallothionein promoter and the hygromycin resistance pY3 marker. The pPK9A plasmid contains the coding region of porcine TGF-β1 cDNA inserted between the metallothionein promoter and human growth hormone polyadenylation sequence. The TGF-β1 sequence also contains mutations previously described by Brunner et al. (29), which change the cysteines at 223 and 225 in the

![Fig. 1. Northern blot analysis of M1 mRNA (M1) or M2 mRNA (M2) levels following exposure to 10 ng/ml of TGF-β1 for 1, 2, and 4 h. Increased M1 and M2 mRNA levels were noted with 1 h of TGF-β1 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.](image)

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polypeptide to serines, resulting in the secretion of active mature TGF-β. 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 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 colony exhibiting TGF-β induction in the presence of zinc (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-β1 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-β1 treatment, demonstrating a direct relationship between TGF-β1 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-β1. 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-β1 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-β1 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 [3H]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-β1 occurred prior to any detectable changes in the rates of DNA synthesis. These observations are consistent with previous investigations when TGF-β1 effects on DNA synthesis in the cell lines examined in this study have been described (16, 17). These studies have shown that TGF-β1 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-β1 treatment (data not shown). These results support the novel concept that TGF-β1 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, or through autocrine TGF-β 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 TGF-β, in which early alterations in gene expression.

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

REFERENCES