Enhanced expression of the urokinase-type plasminogen activator gene and reduced colony formation in soft agar by ectopic expression of PU.1 in HT1080 human fibrosarcoma cells

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Summary To investigate the cellular biological function of PU.1, a member of the Ets family of transcription factors, a vector capable of expressing the protein was transfected into HT1080 human fibrosarcoma cells. Exogenous expression of PU.1 in HT1080 cells reduced colony-forming efficiency but stimulated cell migration in soft agar, although it did not affect cell growth in adherent culture. Expression of the urokinase-type plasminogen activator (uPA) mRNA, which is known to be correlated with cell migration and invasion, was enhanced in PU.1 transfectants compared with mock transfectants. Run-on analysis demonstrated that uPA transcription was unaffected by PU.1, suggesting that this enhancement mainly occurs at a post-transcriptional level. On the other hand, treatment of HT1080 cells with the synthetic glucocorticoid dexamethasone (DEX; 10−7 M) significantly reduced uPA gene expression at a transcriptional level. Furthermore, DEX inhibited cell migration in soft agar without affecting cell growth. These negative effects of DEX on uPA expression and cell migration were alleviated by the expression of PU.1 in HT1080 cells, whereas expression of the N-ras oncogene, which is responsible for maintenance of the transformed phenotypes in HT1080 cells, was unaffected by PU.1 expression or DEX treatment in the cells. Our results suggest that expression of PU.1 can stimulate uPA gene expression at the post-transcriptional level, which may subsequently lead to activation of cell motility and/or reduced cell–cell adhesion, but reduces anchorage-independent growth of HT1080 cells.

Keywords: PU.1 gene; urokinase-type plasminogen activator; cell migration; cell growth

Materials and Methods

Plasmid construction

A 1.2-kb mouse PU.1 full-length cDNA (Klemsz et al, 1990), kindly donated by Dr D. Kabat, was cloned into an eukaryotic expression vector, pLRNL (Hung et al, 1988), downstream of the MuLV promoter.

Cell culture and transfection

Two subclones, NM-1 and cl-2, were isolated from a human fibrosarcoma cell line, HT1080, purchased from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were transfected with the PU.1 expression vector using Lipofectin reagent (Gibco/BRL) and transfectants were selected in the medium containing G418 (1 mg ml−1).

Western blot analysis

Proteins were electrophoresed in a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred electrophoretically onto a nylon membrane (Immobilon; Millipore). The blot was probed with anti-PU.1 polyclonal antibody (Santa Cruz). Chemiluminescent signals of the immunoreactive proteins were visualized on an X-ray film using the ECL kit (Amersham).

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Northern urokinase (1992) An of P-iodonitrotetrazolium DNA duplicated A2.5 DMEM with A Soft agar. Controls Cells PU.1 protein levels Doubling time (h) Colony formation (%)<sup>a</sup> HT1080 NM-1 - 15.0 47.7 ± 6.7 Vector - 16.0 35.5 ± 5.7 PU.1 transfecants NMPU.1-7 ++ 15.0 17.1 ± 1.4 NMPU.1-9-1 + 15.0 22.5 ± 4.5 NMPU.1-11-L +++ 15.0 17.7 ± 3.3 NMPU.1-26 ± 15.0 20.7 ± 6.3

*2.5 × 10<sup>3</sup> cells per plate were seeded. Colony formation was scored into duplicated dishes 3 weeks after the seeding.

Cell growth analysis

A total of 1 × 10<sup>6</sup> cells were cultured in 5 ml of culture medium in 6-cm plastic dishes. Cell numbers were counted every 24 h and doubling times were calculated by means of cell numbers from triplicated samples in the exponentially growing stage of cells.

Soft agar colony-formation assay

A total of 2.5 × 10<sup>3</sup> cells were plated in 0.3% Noble agar (Difco) in DMEM with 10% FBS in the presence or absence of 10<sup>-7</sup> M of DEX. After 3 weeks of incubation, cells were stained with P-iodonitrotetrazolium violet (1 mg ml<sup>-1</sup>) and colonies containing more than 64 cells were counted under a microscope. Morphology of colonies 4 days after cell seeding in soft agar was observed under a phase-contrast microscope at 200 × magnification.

DNA probes

An EcoRI fragment of human N-ras cDNA (Taparowsky et al., 1983) was derived from the Japanese Collection of Research Biosources-Gene (Tokyo, Japan). A Psrl fragment of human urokinase cDNA (Veder et al., 1984) was derived from the American Type Culture Collection (ATCC; Rockville, MD, USA).

Northern blot analysis

Total RNA (10 μg) extracted from cells was denatured and electrophoresed in 1.2% agarose gels containing 0.66 m formalde-hyde. Hybridization was performed as described (Kondoh et al., 1992) using cDNA probes labelled with [32P] α-dCTP by the method of Feinberg and Vogelstein (1983). Specific hybridization was visualized by film autoradiography and band intensity was measured by a densitometer (GS-700, Bio-Rad).

Nuclear run-on assay

A nuclear run-on assay was performed as described (Kondoh et al., 1991). Filters were blotted with 5 μg of Psrl fragment of human urokinase cDNA and 5 μg of BamHI/EcoRI fragment of human cardiac actin DNA (Gunning et al., 1984). Specific hybridization was visualized and band intensity was measured by the BAS-2000 image analysing system (Fuji Film, Tokyo, Japan).

RESULTS

Expression of PU.1 protein in PU.1-transfected HT1080 cells

To examine cell biological function of PU.1, we transfected a pLRNL expression vector containing full-length mouse PU.1 cDNA into a clone (NM-1) of PU.1-negative human fibrosarcoma HT1080 cells. Four independent PU.1-expressing HT1080 NM-1 clones were obtained and designated NMPU.1-1, NMPU.1-8-1, NMPU.1-11-L and NMPU.1-26 respectively. Western blot analysis using polyclonal antibody against PU.1 revealed that NMPU.1-7 and NMPU.1-11-L cells expressed higher and the highest levels of the protein, whereas NMPU.1-8-1 and NMPU.1-26 cells expressed moderate and the lowest levels of the protein respectively. No PU.1 protein was observed in parental HT1080 NM-1 cells (Figure 1).

Cell biological features of PU.1-transfected HT1080 cells

We first examined the effect of ectopic expression of PU.1 on growth properties of HT1080 NM-1 cells. As shown in Table 1, we found no substantial effect of PU.1 on the doubling time for proliferation of NM-1 cells grown in the adherent culture. However,
when we examined growth of the cells in soft agar, we found a significant reduction in colony formation in PU.1-transfected cells compared with the control cells of parental and mock-transfected NM-1 cells. The degree of reduction of colony-forming ability in the transfectants seemed to be inversely correlated with the degree of the levels of PU.1 protein in these cells (Table 1). Morphological examination of soft-agar colonies at the early stage under a microscope demonstrated that all the PU.1 transfectants exhibited colonies with cells sparsely distributed. By contrast, parental cells and mock transfectants exhibited colonies with very limited cell spreading in soft agar (Figure 2), suggesting that expression of PU.1 may increase migration of cells in soft agar. Representative morphological appearances of the parental cells and a PU.1 transfectant (NMPU.1-7) grown in soft agar are shown in Figure 2A and B respectively. Other PU.1 transfectants also exhibited similar growing patterns to NMPU.1-7 (data not shown). In spite of the difference in growth patterns in soft agar, no particular morphological alterations were observed between PU.1 transfectants and the control cells grown in plastic culture plates (data not shown).

Expression of the uPA and N-ras genes in PU.1-transfected HT1080 cells

It has been reported that the activated N-ras gene is crucial for keeping the transformed phenotypes of HT1080 cells (Hall et al. 1983; Paterson et al. 1987) and that the elevated levels of expression of urokinase-type plasminogen activator (uPA) are associated with cell migration and malignant transformation in several types of tumour cells (Kirchheimer and Remold, 1989; Chambers et al., 1995). Therefore, we examined whether PU.1 could affect expression of these genes in HT1080 NM-1 cells. As shown in Figure 3A, NMPU.1-7 and NMPU.1-11-L cells expressed higher levels of PU.1 mRNA transcribed from the viral long terminal repeat (LTR) in the expression vector (a 2-kb transcript and a 4-kb through-reading transcript), NMPU.1-8-1 cells moderate level, and NMPU.1-26 lower level. No expression of the PU.1 gene was observed in parental and mock-transfected NM-1 cells. The levels of PU.1 mRNA were almost consistent with the protein levels detected by Western blot analysis shown in Figure 1. Expression of the N-ras oncogene was not markedly affected by ectopic expression of PU.1 in NM-1 cells. However, the steady-state levels of uPA mRNA were significantly enhanced in all of the PU.1 transfectants compared with parental NM-1 cells and a mock transfectant (compare lanes 1 and 3 with lanes 5, 7, 9 and 11 in Figure 3A). This was also true in another series of transfectants (LRPU.1-6, LRPU.1-9, LRPU.1-14 and LRPU.1-15) established by transfecting the PU.1 expression vector into another HT1080 subclone, cl-2 (compare lanes 1 and 3 with lanes 5, 7, 9 and 11 in Figure 3B).

Effect of glucocorticoid on uPA expression and cell migration in HT1080 cells

It is certainly evident that synthetic glucocorticoids such as dexamethasone (DEX) act as suppressors of uPA at the transcriptional
level in HT1080 cells (Medcalf et al., 1986). Furthermore, transcrip-
tional interference between PU.1 and steroid/vitamin receptor
family members has been reported in vitro using the chloram-
phenicol acetyltransferase (CAT) assay (Gauthier et al., 1993).
Therefore, we examined whether expression of PU.1 interferes
with the effect of DEX on uPA expression in vivo in HT1080 cells.
As shown in Figure 3A and B, treatment of HT1080 cells and mock
transfectants with $10^{-7}$ M of DEX greatly reduced uPA mRNA
levels in these cells. Although a decrease in the level of uPA tran-
scripts was also observed in PU.1-transfected cells after treatment
with DEX, the levels were still higher than those in parental and
mock-transfected control cells without DEX treatment.
Densitometric analysis revealed that the levels were reduced to
80%, 62%, 73% and 40% in NMPU.1-7, NMPU.1-8-1, NMPU.1-
11-L and NMPU.1-26 cells, whereas they were reduced to 40% and
50% in parental and mock-transfected NM-1 cells respectively
(Figure 3A). In the same context, DEX reduced the levels of uPA
mRNA to 60%, 33%, 55% and 87% in LRPU.1-6, LRPU.1-9,
LRPU.1-14 and LRPU.1-15 cells, but to less than 10% in control
c1-2 cells, respectively (Figure 3B). In contrast, DEX did not affect
N-ras gene expression in these PU.1-expressing cells as well as the
control cells. In HT1080 cells, DEX seemed to increase slightly
rather than decrease expression of the transfected PU.1 gene. These
results indicate that DEX specifically reduced uPA expression in
HT1080 cells but the degree of reduction was alleviated by expres-
sion of the PU.1 gene.

The effect of DEX on growth properties of PU.1 transfectants
was then examined. Neither cell growth in adherent culture nor
colonization in soft agar of HT1080 cells was inhibited even
in the presence of DEX (data not shown). Microscopic examina-
tion of the colonies in soft agar showed that parental and mock-
transfected HT1080 NM-1 cells formed tight and more compact
confines without cell spreading in soft agar in the presence of
DEX compared with the colonies in the absence of DEX (compare
A with C in Figure 2). On the other hand, PU.1 transfectants failed
to form compact colonies, although cell spreading was much
reduced in the presence of DEX (compare C with D, and B with D
in Figure 2). These results suggest that DEX reduces cell motility
and/or enhances cell-cell adhesion and that ectopic expression
of PU.1 alleviates the effect of DEX in HT1080 cells.

**Transcriptional and post-transcriptional control of the
uPA gene by ectopic expression of PU.1 and DEX
treatment in HT1080 cells**

In order to examine whether the effects of PU.1 and DEX on uPA
expression occurred at the transcriptional level, a nuclear run-on
assay was performed using nuclei isolated from PU.1-transfected
and mock-transfected cells in the presence or absence of DEX.
LRPU.1-15 was chosen as a representative PU.1 transfectant
because of the highest expression of uPA mRNA among the
isolated clones. A mock-transfected clone was used as a control. As
shown in Figure 4, the levels of uPA transcription were very similar
in both cells, although the accumulation of uPA mRNA was signif-
icantly higher in LRPU.1-15, compared with mock-transfected
cells. The levels of the actin transcription, examined as a control
gene, were almost the same between control and LRPU.1-15 cells.
Northern blot analysis demonstrated that levels of actin mRNA
were also unchanged between the two (data not shown). On the
other hand, the relative level of uPA transcription in mock-trans-
fected cells, as judged in comparison with the level of actin tran-
scription, was significantly reduced to 25% by DEX treatment,
whereas in LRPU.1-15 cells it was merely reduced to 88%. The actin
transcription was not reduced by DEX in both cells. These
results suggest that the transcription rate of the uPA gene was unaf-
fected by PU.1 in the absence of DEX and that increase in steady-
state level of uPA mRNA by PU.1 is mainly accounted for by
post-transcriptional control. Meanwhile, transcription of the uPA
gene was markedly reduced by DEX treatment. The negative effect
of DEX on the uPA transcription was, however, significantly allevi-
ated by expression of PU.1 in HT1080 cells.

**DISCUSSION**

We found that exogenous expression of PU.1 in HT1080 human
fibrosarcoma cells increased cell migration in soft agar. In associa-
tion with these cell biological changes, expression of the uPA
mRNA, which encodes a serine protease correlated with cell
migration and invasion, was enhanced in PU.1-transfected
HT1080 cells compared with mock transfectants. The uPA gene
has a transcriptional enhancer region, which consists of binding
sites for Fos/Jun family transcription factors AP-1 and an Ets
family transcription factor PEA3, and this region has been
reported to be critical for activation of the gene in HT1080 cells
(Nerlov et al., 1991). In murine macrophages, Ets-2 activates the
uPA enhancer by binding to the PEA3 site, whereas other Ets
family transcription factors such as PU.1 and PEA3 do not activate
the enhancer (Stacey et al., 1995). To examine whether purified
PU.1 protein or that in a crude nuclear extract from LRPU.1-15
cells can bind to the PEA3 site, we performed an electrophoretic
mobility shift assay. However, PU.1 did not bind to this site (data
not shown), suggesting that PU.1 cannot directly affect the
enhancer region of the uPA gene through the PEA3 site within this
region. Furthermore, run-on analysis demonstrated that PU.1 did

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not affect uPA gene expression at the transcriptional level in HT1080 cells. Therefore, the accumulation of the uPA mRNA in PU.1-transfected cells may be due to the post-transcriptional mechanism(s), perhaps by increasing the uPA mRNA stability in the cells. There are several reports demonstrating post-transcriptional regulation of uPA gene; in rat fibroblasts uPA gene expression is post-transcriptionally suppressed compared with highly metastatic carcinoma cells (Henderson et al., 1991, 1992); uPA mRNA stability is specifically increased by a synergistic effect of Ca²⁺ and cAMP in renal epithelial cells (Altus et al., 1987, 1991; Zeigler et al., 1990); and cycloheximide, an inhibitor of protein synthesis, increases the uPA mRNA half-life from 70 min to >20 h (Altus et al., 1991). Therefore, there may be a protein(s) of short-half life, potentially attaching to the A+U-rich elements in the 3′- untranslated region of the uPA mRNA and responsible for specific degradation of the mRNA (Altus et al., 1991; Henderson and Kefford 1991). PU.1 may affect the level and/or function of such proteins.

There are several reports showing that glucocorticoid can lower uPA activity in a number of cell types (Littlefield et al. 1985; Medcalf et al. 1986). Consistent with previous reports by others (Medcalf et al. 1986), our results suggest that the suppression of the uPA gene expression by DEX occurs at the transcriptional level in HT1080 cells. Furthermore, we demonstrate that DEX antagonized the effect of PU.1 on morphology of colonies grown in soft agar: PU.1 stimulated cell migration, whereas DEX inhibited it. In parallel with this, PU.1 alleviated the negative effect of DEX on uPA expression at the transcriptional level in HT1080 cells. It has been reported that the glucocorticoid receptor (GR) interferes with the function of PU.1 and vice versa in human breast cancer cells (Gauthier et al., 1993). Similar mutual functional interference between Fl-l, another ETS family protein, and steroid hormone receptors was also reported recently (Darby et al., 1997). Therefore, our observation may be accounted for by such a functional interference, by which PU.1 blocks the transcriptional suppressing activity of GR in HT1080 cells.

We also found that PU.1 brought about negative effects upon the anchorage-independent growth of HT1080 cells: the PU.1 transfectants exhibited reduced colony formation in soft agar. Similar observations have been reported in some tumours: constitutive expression of Ets-1 reverts the tumorigenicity in human colon cancer cells (Suzuki et al., 1996) and stable expression of a trans-dominant mutant of PU.1 or Ets-2 also reverts Ras-transformed NIH/3T3 cells (Langer et al., 1992; Wasylyk et al., 1994). It is known that HT1080 cells harbour the activated N-ras oncogene, which is essential for maintaining the transformed phenotypes of the cells, and inhibition of N-ras gene expression rendered the cells less malignant (Hall et al., 1983; Paterson et al., 1987). However, PU.1-transfected HT1080 cells expressed similar levels of N-ras mRNA to the parental cells and mock transfectants (Figure 3A and B). As a member of the Ets family proteins is one of the nuclear targets of the ras signalling pathway to activate ras-responsive elements (Wasylyk et al., 1990; Galang et al., 1994), the ectopic expression of PU.1 may perturb endogenous Ets family proteins, which mediate the ras signals crucial for maintenance of the transformed phenotypes of HT1080 cells.

In conclusion, our results could provide an insight into a novel function of Ets-related transcription factor PU.1 on several transformed phenotypes provoked, in part, by the activated N-ras oncogene.

**ABBREVIATIONS**

DEX, dexamethasone; DMEM, Dulbecco’s modified Eagle minimum essential medium; DMSO, dimethylsulphoxide; GR, glucocorticoid receptor; MuLV, murine leukemia virus; LTR, long terminal repeat; SDS, sodium dodecyl sulphate; uPA, urokinase-type plasminogen activator.

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