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Overexpression of VDUP1 mRNA sensitizes HeLa cells to paraquat

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Abstract

5-Bromodeoxyuridine (BrdU) induces or suppresses senescence-associated genes in any types of mammalian cells. From a cDNA library upregulated by BrdU in HeLa cells, we identified the gene encoding VDUP1 as a senescence-associated gene in normal human fibroblasts. To address a role of VDUP1 in senescence, we established HeLa cell clones, V7 and V27, which express its mRNA in a doxycycline-dependent manner. Although their growth in liquid culture was moderately retarded, colony formation on semi-solid medium was strongly inhibited by overexpression of the mRNA. We also examined susceptibility of these clones to various reagents. Consequently, colony formation in liquid culture was strongly inhibited by paraquat in these clones. Their superoxide dismutase activity was normal.

Keywords: VDUP1; Cellular senescence; Active oxygen; Paraquat; SOD

Normal human fibroblasts enter replicative senescence under normal culture conditions [1]. Treatment with various agents induces premature senescence in normal human cells. To date, particular genes termed senescence-associated genes are shown to have induced or suppressed changes in various systems of cellular senescence using normal human fibroblasts [2], SV40-transformed human fibroblast [3], rat embryonic fibroblasts expressing conditional SV40 T antigen [4], fibroblasts of patients of Werner syndrome [5] and progeria syndrome [6], endothelial cells [7], and mitochondria in senescent human cells [8]. Recently, DNA arrays were introduced to examine expression of the senescence-associated genes in replicative and premature senescence [9].

Recently, we have found that BrdU induces a senescence-like phenomenon in any types of mammalian cells [10]. BrdU suppresses or induces expression of genes involved in cellular differentiation [11–16]. To obtain an insight into a molecular basis for the action of BrdU, we made a catalog of genes which are induced by BrdU in HeLa cells [17]. The majority of such genes are upregulated in senescent normal human fibroblasts [17].

Among the genes in the above catalog, we characterized the VDUP1 gene because this gene is shown to be highly induced in senescent normal human fibroblasts. VDUP1 is originally reported as a gene upregulated by vitamin D_3 in HL-60 cells [18], but its function is not known. Recently, it has been reported that the reduced form of thioredoxin (TRX) binds to VDUP1 in vivo and in vitro [19]. TRX is shown to have an important role in signal transduction in apoptosis [20]. TRX can reduce a disulfide bond formed by oxidative stress, and participates in the regulation of cellular redox state [21].

In this report, we constructed HeLa cell clones that express VDUP1 mRNA in a doxycycline (Dox)-dependent manner, and assessed a role of VDUP1 in cellular senescence.

Materials and methods

Cell culture. HeLa S3 cells were cultured in plastic petri dishes containing ES medium supplemented with 5% fetal bovine serum at 37 °C under 5% CO_2 and 95% humidity [10]. Semi-solid ES medium

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was solidified with 0.35% (w/v) agarose in plastic petri dishes as described previously [22].

Plasmids. Plasmid encoding VDUP1 in a Dox-dependent manner was constructed as follows. Full-length VDUP1 cDNA was synthesized by polymerase chain reaction (PCR) from HeLa cDNA. The cDNA was cloned into pGEM-T easy vector (Promega), sequenced, and subcloned into pUHD10-3 (a generous gift of Dr. H. Bujard of Universitat Heidelberg, Germany) [23]. Plasmid pUHG 172-1neo [24] encoding neomycin *N*-acethyltrasferase and the rTetR-VP16 fusion protein was provided by Dr. H. Bujard.

Plasmids containing a full-length cDNA of the wild-type or a mutated form of TRX (kindly provided by Dr. J. Yodoi of Kyoto University, Japan) were digested with a restriction endonuclease to yield the two types of cDNA fragments. These fragments were cloned into an expression vector pYN3215 (provided by Dr. H. Miyazawa of RIKEN, Japan) that contains the hygromycin B resistant gene cassette.

Plasmid transfection. Plasmids were precipitated with calcium phosphate and transfected to cells as described previously [25]. After incubation at 35 °C in 3% CO_2 and 95% humidity for 12 h, the cells were replated to isolate transfectant clones.

Northern blot analysis. Total RNA samples were prepared according to the acid guanidinium thiocyanate–phenol–chloroform method [26]. The samples (20 μg) were subjected to electrophoresis through 1% formaldehyde–agarose gel and transferred to nylon membrane (Biodyne B, Pall). The membrane was hybridized with a probe labeled with [α-^{32}P]dCTP using labeling kit (Amersham), washed, and exposed to an X-ray film as described previously [17].

Assay of superoxide dismutase (SOD) activity. SOD activity was measured with a diagnosis kit (Wako Pure Chemical Industries, Japan) that measures decrease in color of difolmazan, a reduced form of nitroblue tetrazolium, formed with superoxide anion generated by the action of xantine oxidase on xantine. Briefly, cells were harvested by tripsinization, suspended in 50 mM Tris–HCl buffer (pH 7.5), disrupted by sonication, and centrifuged. The resulting supernatant was used for the assay according to the protocol of the supplier. SOD activity was calculated as follows: $(OD_{blank} - OD_{sample})/OD_{blank}/10$ μg protein/20 min $\times 100$. Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories).

Results

Isolation of clones that conditionally overexpress VDUP1 mRNA

To observe an effect of overexpression of VDUP1, we constructed HeLa cell clones that conditionally express VDUP1 mRNA. For this purpose, we employed the reverse tetracycline-dependent expression system [27]. First, a plasmid encoding the transactivator protein rTetR-VP16 was transfected to HeLa cells, and a clone termed T1 that stably expresses the fusion protein was isolated. Second, a plasmid encoding VDUP1 under the control of rTetR-VP16 and Dox was co-transfected to T1 cells with plasmid pGKpuro encoding puromycin *N*-acethyltrasferase [28]. Several transfectant clones were selected by resistance to puromycin, and used for further characterization. By northern blot analysis, we chose two independent clones named V7 and V27. In the absence of Dox, V27 did not express a detectable level of exogenous VDUP1 mRNA whereas V7 expressed a low level of the mRNA. Upon addition of Dox, these clones immediately and efficiently induced VDUP1 mRNA attaining saturated levels of the mRNA within one day (Fig. 1). Protein levels for VDUP1 in these clones were not exactly determined due to lack of specific antibody.

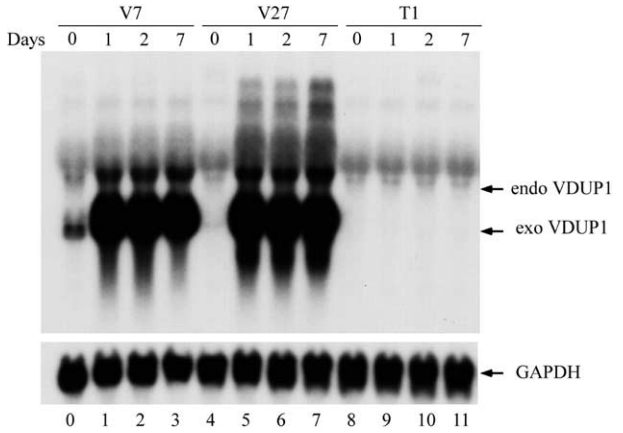

Fig. 1. Northern blot analysis of VDUP1 mRNA. Total RNA samples were prepared from T1, V7, and V27 cells at intervals after addition of Dox as indicated, and subjected to Northern blot analysis as described in Materials and Methods. Arrows indicate endogenous and exogenous species of VDUP1 mRNA, and GAPDH mRNA.

Growth in liquid and on semi-solid medium

We examined growth of T1, V7, and V27 in the presence of Dox (Fig. 2). Overexpression of VDUP1 mRNA slightly retarded growth rates of V7 and V27. The doubling times for V7 and V27 became 1.4 and 1.5 times longer, respectively, by addition of Dox. Dox had no effect on T1. V7 and V27 cells did not show a significant morphological change or induction of senescence-associated β-galactosidase (data not shown).

Then we examined their colony-forming ability on semi-solid agarose medium. Cells were plated on surface of the medium containing 0, 0.5, and 1 μg/ml Dox and allowed to form colonies (Fig. 3). In the absence of Dox, T1, V7, and V27 formed colonies normally. When Dox was added to the medium, colony formation was inhibited dose-dependently in V7 and V27. T1 formed colonies normally under any conditions.

Sensitivities to drugs

Since colony formation under aerobic conditions was inhibited in V7 and V27, we examined their sensitivities to various reagents capable of giving oxidative stress to cells. We used superoxide generators, paraquat and plumbagin [29,30], hydrogen peroxide, a generator of hydroxyl radical, and mitomycin C, a DNA damaging agent [31,32]. When cells were assayed in liquid medium containing Dox, paraquat significantly inhibited colony formation dose-dependently in V7 and V27 (Fig. 4).

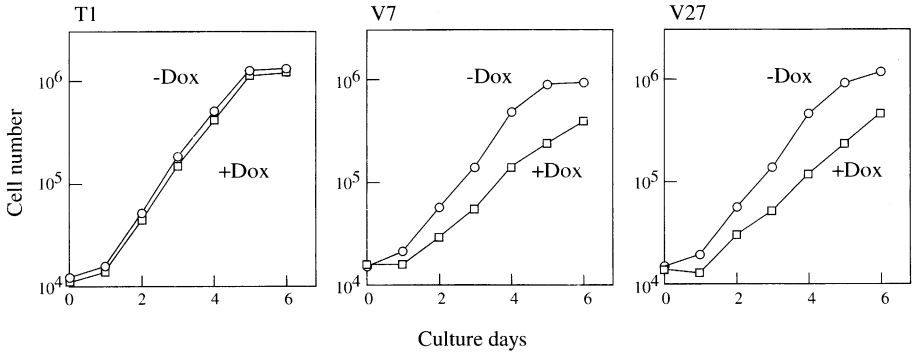

Fig. 2. Growth curves in liquid medium. T1, V7, and V27 cells were plated in 35 mm dishes at 1×10^4 cells per dish, and cultured in the absence (circles) and presence (squares) of 1 μM Dox. At intervals, the cells were collected and counted with a Coulter counter.

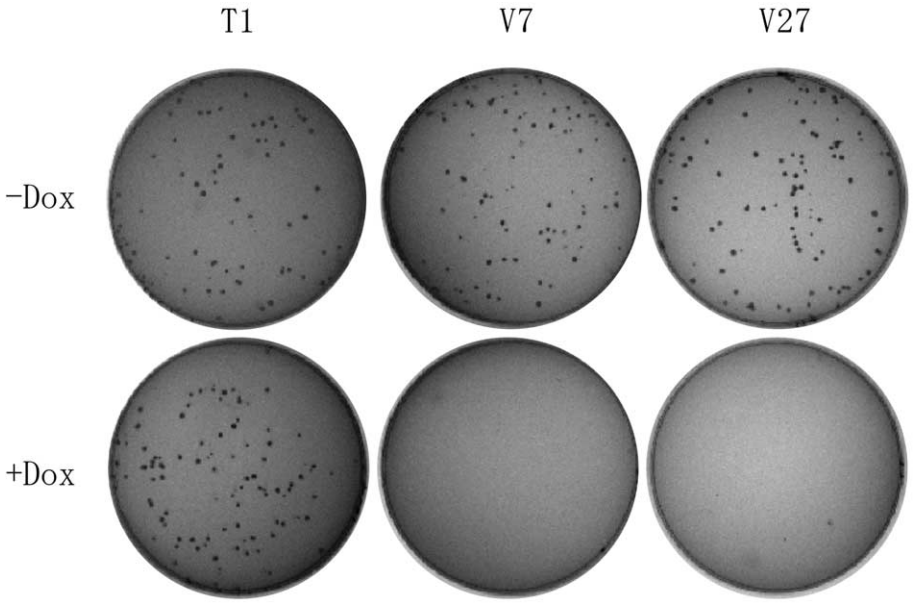

Fig. 3. Colony formation on semi-solid medium. T1, V7, and V27 cells were pretreated with 1 μg/ml Dox for 2 days and replated on surface of semi-solid agarose medium containing 1 μg/ml Dox. After incubation for 10 days, colonies were stained with Crystal Violet.

ID_{10} values, which reduce colony formation to 10% of controls, were 80, 20, and 20 μM, respectively, for T1, V7, and V27 in the presence of 1 μM Dox. In the absence of Dox, the values were 80 μM for all of the clones. Unexpectedly, the other reagents tested did not show a significant difference in their effects among T1, V7, and V27 (Fig. 4).

We examined SOD activity in T1 and V27 cultured in the presence of Dox because the activity was altered in cell mutants resistant to paraquat (A. Joguchi et al., manuscript in preparation). However, no difference was found in the activity among these clones when the cells were cultured in the presence and absence of Dox (Fig. 5).

Transfection of plasmids encoding TRX

VDUP1 is shown to bind to the reduced form of TRX and suppress its activity to reduce disulfide bonds of oxidized proteins [19]. Therefore, we investigated whether ectopic expression of TRX was able to rescue the toxicity of paraquat in V7 and V27. We transfected plasmid encoding the wild-type or a mutant form of TRX and hygromycin B phosphotransferase. After selection in medium containing hygromycin B, the cells were pooled from colonies and replated to assay sensitivity to paraquat. In another experiment, transfected cells were directly selected in medium containing hygromycin B and paraquat. However, no significant difference was observed in the sensitivity to paraquat in V7 and V27 under any assay conditions (data not shown).

Discussion

We searched for a role of VDUP1 in cellular senescence. The VDUP1 gene was first reported as a gene

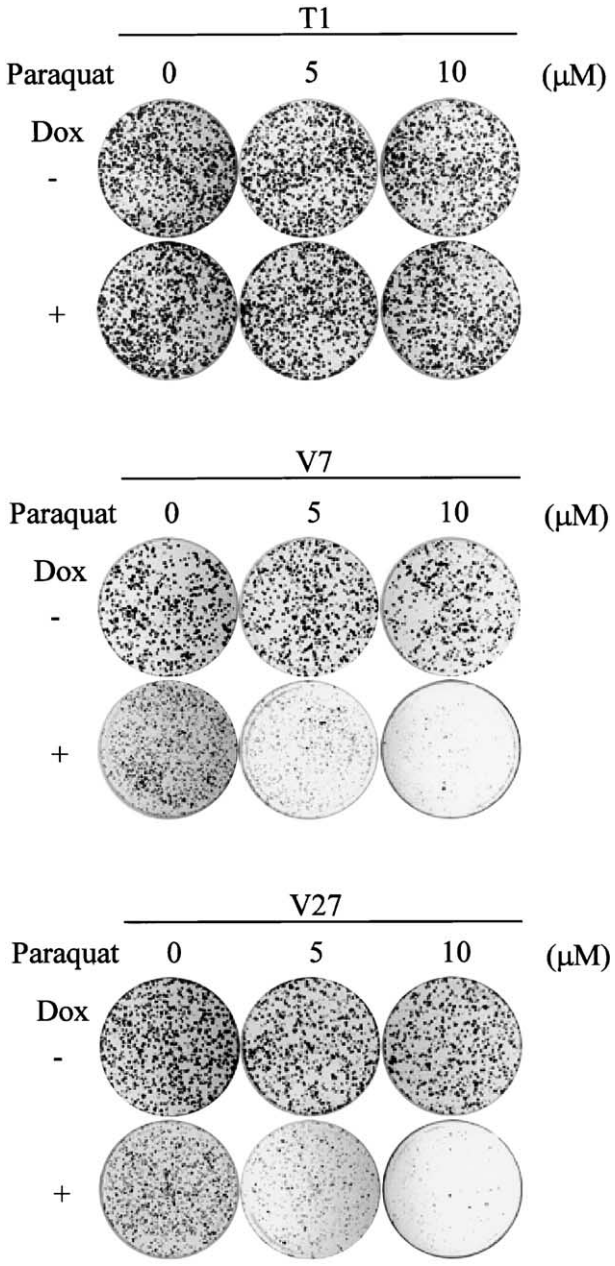

Fig. 4. Sensitivity to paraquat in liquid medium. T1, V7, and V27 cells were pretreated with 1 μg/ml Dox for 2 days and replated in medium containing 1 μg/ml Dox and various concentrations of paraquat. After culture for 10 days, colonies were stained with Coomassie Brilliant Blue R-250.

upregulated by vitamin D_3 in HL-60 cells [18] and identified by us as one of the senescence-associated genes [17]. After starting this study, VDUP1 was shown to bind to TRX [19]. Thus, VDUP1 seems to have a role in regulating redox state in a cell because TRX reduces disulfide bonds of proteins. Since oxidative stress accelerates cellular senescence and aging of various organisms, we tried to find a function of VDUP1 as regards cellular senescence.

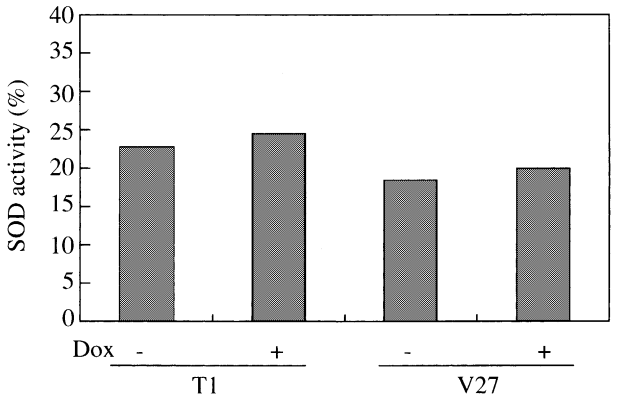

Fig. 5. Measurement of SOD activity. T1 and V27 cells were cultured for 2 days in the presence and absence of 1 μg/ml Dox. Cell extracts were prepared and assayed for SOD activity as described in Materials and methods. Values are means of two experiments.

We established two clones V7 and V27 that overexpress VDUP1 mRNA in response to Dox. Both clones immediately and sufficiently expressed the mRNA by addition of Dox but did not give rise to marked growth retardation, a change in morphology, or induction of senescence-associated β-galactosidase. In another experiment, we co-transfected plasmid encoding VDUP1 driven by cytomegalovirus promotor with pSV2neo to normal human fibroblasts. Transfectant cells selected by resistance to G418 did not show senescent cell morphology or induce senescence-associated β-galactosidase. Thus, VDUP1 did not seem to have a direct role in senescence in normal human fibroblasts.

We found that V7 and V27 did not form colonies on semi-solid medium in the presence of Dox. We hypothesized that overexpression of VDUP1 sensitizes cells to aerobic conditions. As expected, both V7 and V27 were sensitive to paraquat, but not to plumbagin, hydrogen peroxide, or MMC upon addition of Dox. As paraquat and plumbagin are known to generate superoxide in the cell, the reason for the above difference is not known. Paraquat is thought to generate superoxide by nonenzymatic reduction by NADPH. On the other hand, plumbagin, a quinone derivative, is known to be reduced enzymatically [33]. A quinone compound MMC is shown to generate superoxide anion [34], but its DNA cross-linking effect was much more prominent.

We measured SOD activity in the cell extracts because it affects paraquat sensitivity. However, the activity did not change by addition of Dox. SOD converts superoxide anion to dioxide and hydrogen peroxide, but is not always related with resistance to paraquat [35,36]. Recently it has been reported that VDUP1 is a mediator of oxidative stress by inhibiting TRX activity [37]. Preliminary experiments, however, showed that ectopic expression of TRX does not seem to rescue the sensitivity to paraquat conferred by overexpression of VDUP1 mRNA.

In this study, we showed that overexpression of VDUP1 mRNA inhibit colony formation under aerobic conditions and sensitizes to paraquat in HeLa cells. Unfortunately, however, the reason for these phenomena is not known at present. Further studies will be needed to elucidate a molecular basis for the phenomena. Nonetheless, the novel observation as regards VDUP1 may facilitate understanding of defense mechanisms against oxidative stress in mammalian cells.

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