Enhanced Expression of Transferrin Receptor Confers UV-resistance in Human and Monkey Cells

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**Transferrin receptor/UV-resistance/Apoptosis/Human cells.**

One of the most intriguing biological subjects is cell-surface molecules that regulate the susceptibility of human cells to cell-killing effects after irradiation with far-ultraviolet light (UV, principally 254 nm wavelength). Human RSa cells have unusual sensitivity to UV-induced cell-killing. We searched for molecules on the cell-surface of RSa cells that were present in different amounts as compared to a variant of these cells, UV-r-1 cells, which have increased resistance to UV cell-killing. Among the 21 molecules examined, the amount of transferrin receptor (TfR) protein was found to be 2-fold higher in UV-r-1 cells compared with in RSa cells. The amounts of this protein were also higher in the UV-resistant hematopoietic cell lines, CEM6 and Daudi, as compared to the UV-sensitive cell lines, Molt4 and 697. Culturing of UV-r-1 cells in a medium containing anti-transferrin antibodies resulted in sensitization of the cells to UV cell-killing as demonstrated by colony formation assay. Similar results were observed by treatment of the cells with TfR siRNA. In contrast, overexpression of TfR protein led to a resistance to UV cell-killing in RSa cells and monkey COS7 cells as demonstrated by both colony formation and apoptosis assay. In TfR-overexpressing cells, reduction of p53 and Bax protein was observed after UV-irradiation. Thus, TfR expression appears to be involved in the regulation of UV-resistance, possibly via modulation of the amount of p53 and Bax protein.

**INTRODUCTION**

Irradiation of human cells with UV induces various genetic and cellular events such as DNA damage, mutation and cell death. The events are suggested to be causally related to the activation of several cellular signaling pathways, such as p53 and mitogen-activated protein kinase (MAPK) pathways. The p53 and MAPK pathways were reported to modulate cellular signaling by transcriptional upregulation of signaling molecules such as Bax. On the other hand, some cell surface molecules are involved in UV-susceptibility by modulating several signaling pathways. Activation of the epidermal growth factor (EGF) receptor provides a survival signal against UV-induced apoptosis via activation of a MAPK member, extracellular signal-regulated kinase. Similarly, activation of the insulin-like growth factor-I (IGF-I) receptor mediates an anti-apoptotic signal against UV-induced apoptosis through p38 signaling pathways.

Previous findings suggested the involvement of cell surface molecules such as syndecan-1 in UV-susceptibility in human cells. To investigate such molecules, cell lines with different susceptibilities to UV cell-killing, such as human fibroblast cell lines, RSa and UV-r-1, are valuable. RSa cells were established from human embryo-derived fibroblastic cells that were doubly infected with simian virus 40 and Rous sarcoma virus. These cells were later characterized as being unusually sensitive to UV cell-killing. UV-r-1 cells were established as a variant cell line with increased resistance to UV cell-killing from RSa cells mutagenized with ethyl-methanesulphonate and then irradiated with UV. A comparative study of the characteristics of these UV-sensitive and -resistant cells may yield valuable information about molecular events of UV cell-killing effects.

In the present study, we searched for cell surface molecules that have different expression levels between RSa cells and UV-r-1 cells using immunofluorescence staining and flow cytometric analysis. Among the molecules examined, we found that the expression levels of TfR protein were more abundant in UV-r-1 cells than in RSa cells. Correlation between the TfR expression levels and UV-resistance was

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further examined in UV-sensitive and -resistant human hematopoietic cell lines. Consequently, to determine how the expression levels of TIR protein are involved in UV-resistance, we examined the effects of TIR overexpression in RSa cells and monkey COS cells.

**MATERIALS AND METHODS**

**Cell culture**

RSa cells and UV-r cells were cultured in Eagle’s minimum essential medium (Invitrogen, Grand Island, NY) containing 10% calf serum (Intergen, NY). T lineage lymphoid cell lines, Molt4 and CEM6, and B lineage cell lines, 697 and Daudi, were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) containing 10% fetal bovine serum (Intergen). COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

**Antibodies and immunofluorescence analysis**

Phycocyanin (PE)-conjugated antibodies (Abs); anti-CD4, anti-CD8, anti-CD11b, anti-CD14, anti-CD22, anti-CD25 and anti-CD34, and fluorescein isothiocyanate (FITC)-conjugated Abs; anti-CD2, anti-CD3, anti-CD5, anti-CD18, anti-CD19 and anti-CD138, were purchased from Becton Dickinson (San Jose, CA). PE-conjugated anti-CD69 Abs and FITC-conjugated anti-CD23 Abs and anti-CD95 Abs were obtained from Beckman Coulter (Tokyo, Japan). PE-conjugated anti-CD45 Abs and anti-CD81 Abs were obtained from Immunotech (Tokyo, Japan). The PE-conjugated Abs; anti-CD38, anti-CD44 and anti-TfR, were obtained from PharMingen (San Diego, CA). Isotype control FITC- and PE-conjugated Abs were obtained from Becton Dickinson. Polyclonal anti-human transferrin Abs were obtained from INTER-CELL TECHNOLOGIES INC. (Hopewell, NJ). Mouse anti-human TIR antibody (clone H68.4) was obtained from Zymed laboratory Inc (South San Francisco, CA). The anti-actin (C-2) monoclonal Abs and anti-Bax (B-9) monoclonal Abs used for Western-blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-p53 monoclonal Abs used for Western-blot analysis were obtained from Transduction Laboratories (Lexington, KY).

For immunofluorescence analysis, cells suspended in phosphate-buffered saline (PBS, 10 mM sodium phosphate and 150 mM NaCl, pH 7.2) were incubated with PE- or FITC-labeled Abs on the ice for 30 min. Labeled cells were then analyzed using a FACScan flow cytometer (Becton-Dickinson).

**UV-sensitivity tests**

The ratio of apoptotic cells was estimated by measuring the ratio of sub-G1 fraction using a FACScan analyzer or by analyzing the ratio of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells using an apoptosis in situ detection kit (Wako, Tokyo, Japan) according to an instruction manual. For TUNEL analysis, cells were seeded on glass chamber slides (Nalgen Nunc International, Tokyo, Japan), then irradiated as described. The percentage of cells in sub-G1 fraction after UV-irradiation was represented relative to those of mock (0 J/m²)-irradiated controls.

UV irradiation of cells and colony formation assays were performed as described previously. After 14 days of UV-irradiation, colonies with a diameter greater than 3 mm (containing more than 50 cells) were scored. The survival fraction was determined as the ratio of colony numbers after UV-irradiation per the number after mock-irradiation.

For the measurement of caspase-3 activity, the CaspACE™ Assay System Fluorometric Kit (Promega Co, WI) was used as described previously.

**siRNA transfection**

siRNA was designed to target the 1,062–1,082 bp of the TIR mRNA sequences with a corresponding AUG translation initiation codon of 264 bp. The sense (top) and antisense (bottom) sequences of the TIR siRNA duplex were as follows:

- **5'** GGUUGCAAUGCUAGAAAGCTT
- **3'** TTCAACGUUUAACGCUCUUGC

As a nonspecific control, a NC siRNA duplex with random sequences was designed as follows:

- **5'** AGUUCAAGUGUAACGTGTT
- **3'** TTUACAGCUUCACUAGCUUGC

The 2-nucleotide 3' overhang in the two siRNA duplexes was composed of 2'-deoxy thymidine according to a previous report. These siRNA duplexes were purchased from FASMAC Co., Ltd. (Kanagawa, Japan).

The transfection of siRNA was carried out as follows: 24 h before transfection UV-r cells were trypsinized, suspended in fresh medium (1 × 10⁶ cells/ml), and transferred to 24-well plates (400 µl per well). The next day, the medium was replaced with OPTI-MEM (Invitrogen, Carlsbad, CA). Then, 0.8 µg of siRNA duplex was complexed with 2 µl of Lipofectamine 2000 (Invitrogen) according to instruction manual, applied to the cells and incubated for 3 h. The medium with complexes was replaced with fresh culture medium and cells were further incubated for 33 h. Cells were collected by trypsinization, re-seeded and cultured further for 24 h. Then, Western-blot analysis and irradiation for colony survival assays were performed.

**Western-blot analysis**

Cells were lysed in a buffer [0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfoxide, 100 µM leupeptin, 100 µM antipain, 100 µM pepstatin A and 100 µM N-acetyl-Leu-Leu-Val (ALLN)] for 10 min at 4°C and centrifuged at 13,000 × g.
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g for 10 min at 4°C, yielding protein samples. The samples were separated by SDS-polyacrylamide gel electrophoresis, and then immunoblot analysis was performed using an ECL system (Amersham, Buckinghamshire, England) as described previously. The intensity of each band was semi-quantified by scanning the X-ray films with a densitometer (Hoefer Scientific Instruments GS300).

Transfection of TfR

The full-length TfR cDNA was provided by Dr. Lukas C. Kuhn (Swiss Institute for Experimental Cancer Research, Switzerland). The cDNA was ligated into the pcDNA3.1 (+) expression vector (Invitrogen) with Eco RV and Xba I restriction sites, yielding pcDNA/TfR. The null plasmid vector was used as a control. These plasmids were transfected into RSa cells and COS7 cells with Effectene™ Transfection Reagent (Qiagen, Hilden, Germany). The expression of TfR of each cell was measured by flow cytometric analysis as described above. UV-irradiation of the transfectants was performed after 48 h of transfection.

RESULTS

Abundant expression of TfR in UV-resistant human cell lines

Consistent with UV-susceptibility, the ratio of apoptotic cells induced by UV irradiation was larger in the RSa cells compared to the UVr-1 cells (Fig. 1A). For the UV-sensitive

Fig. 1. Increased expression of TfR in UV-resistant cell lines. (A), (C) and (E), After 48 h of UV-irradiation, the ratios of apoptotic cells were determined by flow cytometric analysis, as described in Materials and Methods. Open bar: no irradiation, closed bar: UV (20 J/m²)-irradiation, hatched bar: UV (40 J/m²)-irradiation. Values are means ± S.D. of three independent results. (B), (D) and (F), Determination of cell surface TfR expression levels with flow cytometry. Cells were stained with FITC-labeled anti-TfR antibody and fluorescence intensities were analyzed using flow cytometry, as described in Materials and Methods.
RSa cell line and its variant, the UV-resistant UV^r-1 cell line, the expression levels of 21 cell surface molecules were comparatively examined by flow cytometric analysis with their specific Abs (data not shown). Among the molecules tested, only TfR appeared to be expressed more abundantly in UV^r-1 cells than in RSa cells; the mean fluorescence intensity of UV^r-1 cells was about 2-fold higher than that of RSa cells (Fig. 1B). To determine the relationship between the ratio of apoptotic cells induced by UV irradiation and TfR expression levels, four human lymphocytic cell lines were employed; T lineage Molt4 and CEM6 cells, and B lineage 697 and Daudi cells, respectively. Both CEM6 and Daudi cells showed greater decreases in ratios than either Molt4 or 697 cells (Figs. 1C and 1E). As expected, the former UV-resistant CEM6 and Daudi cells showed about 3-fold higher

![Graph showing survival fraction (%) of mock](image)

**Fig. 2.** Reduced UV-resistance in UV^r-1 cells by depletion of transferrin. After UV-irradiation, cells were cultured with or without anti-transferrin Abs in the medium for 14 days. Colony survival assay was performed, as described in Materials and Methods. ○, □ and ■ represent untreated, 9 μg/ml of Ab-treated and 27 μg/ml of Abs-treated cells, respectively. Values are means ± S.D. of three independent experiments.

![Western blot analysis](image)

**Fig. 3.** Reduction of UV-sensitivity by transfection with TfR siRNA in UV^r-1 cells. (A), Western blot analysis. Sixty h after transfection with TfR siRNA or NC siRNA, cells were lysed and Western-blot analysis was performed using TfR and β-actin Abs in sequence (upper panel). Protein levels of TfR were expressed relative to the level of untransfected cells after normalization by the signal of actin (lower panel). Values are means ± S.D. of three independent results. (B), Colony survival assay. Sixty h after transfection with TfR siRNA or NC siRNA, the cells were irradiated with UV and colony survival assay was performed. ○, □ and ■ represent untransfected, NC siRNA-transfected and TfR siRNA-transfected cells, respectively. Values represent the percentage of colony numbers relative to those of the mock-irradiated cells. Values are means ± S.D. of three independent experiments.
**Fig. 4.** Acquisition of UV-resistance in TfR-overexpressing cells. (A) and (B), Expression of cell surface TfR was determined using flow cytometry with FITC-labeled anti-TfR Abs, as described in Materials and Methods. (C) and (D), Colony survival assay after UV-irradiation was performed as described in Materials and Methods. In C, ○, △, ■ and □ represent untransfected RSa cells, RSa-pcD cells, RSa-TfR cells and UV⁻¹ cells, respectively. In D, ○, △ and ■ represent untransfected COS7 cells, COS7-pcD cells and COS7-TfR cells, respectively. Untransfected RSa, UV⁻¹-1 and COS7 cells were also mock-treated in the same manner as transfected cells. Values represent the average percentage of colony numbers relative to those of mock-irradiated cells. Values are means ± S.D. of three independent experiments.

**Fig. 5.** Decrease in apoptotic cells by UV-irradiation in TfR-overexpressing cells. (A), Ratio of apoptotic cells after 48 h of UV-irradiation in null vector-transfected control cells (open bars) and in TfR-transfected cells (black bars) was measured by TUNEL assay as described in Materials and Methods. Values represent the average percentage of TUNEL positive cells. (B), Caspase-3 activity in null vector-transfected cells (open bars) and TfR-transfected cells (black bars). Values represent the average percentage of caspase-3 activity relative to those of mock-irradiated null vector-transfected cells. Values are means ± S.D. of three independent experiments.

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expression levels of TfR compared with those of the latter UV-sensitive Molt4 and 697 cells (Figs. 1D and 1F). Thus, abundant TfR expression in UV-resistant cells was not a phenomenon restricted to UV$^r$-1 cells derived from RSa cells, but rather a general phenomenon.

**Sensitization of UV$^r$-1 cells to UV by anti-transferrin Abs**

To determine whether UV-resistance is associated with the function of TfR, specific Abs against transferrin were used for a depletion assay. When cultured in the presence of anti-transferrin Abs after irradiation, UV-resistance of UV$^r$-1 cells abated in a dose-dependent manner (Fig. 2). There was no significant difference of cloning efficiency under mock-irradiation between the Abs-treated cells and non-treated cells. Thus, the results suggested that binding of TfR to transferrin is involved with UV-resistance.

**Sensitization of UV$^r$-1 cells by TfR siRNA**

To investigate the role of TfR in UV-sensitivity, we employed RNA interference with UV$^r$-1 cells. To this end, we designed siRNA duplex that was homologous to a region within the TfR coding sequence to the start site of translation. TfR siRNA treatment exhibited a strong inhibition of TfR accumulation, whereas NC siRNA treatment had no effect on TfR expression (Fig. 3A). The endogenous protein levels of actin were also not significantly affected by any of the siRNA duplexes (Fig. 3A).

A decreased ratio of colony survival after UV-irradiation was observed in TfR siRNA-transfected cells but not in NC siRNA-transfected cells (Fig. 3B). There was no significant difference of UV-sensitivity between NC-transfected cells and untransfected cells (Fig. 3B).

**Increased UV-resistance of cells by overexpression of TfR protein**

We next examined the effect of overexpression of TfR on cell viability after UV-irradiation in RSa cells and COS7 cells. After 24 h of transfection, higher levels of cell surface TfR expression were obtained in both pcDNA/TfR-transfected RSa cells and COS7 cells, RSa-TfR and COS7-TfR, respectively, than in null vector-transfected cells, RSa-pcD and COS7-pcD, respectively (Figs. 4A and 4B). An incre-
ased ratio of colony survival after UV-irradiation was observed in TfR-overexpressing cells but not in null vector-transfected cells (Figs. 4C and 4D). There was no significant difference of cloning efficiency between pcDNA/TfR-transfected cells and null vector-transfected cells; 35%–40% in RSa cells and 50%–60% in COS cells, respectively.

Furthermore, the fraction of TUNEL-positive cells decreased significantly in TfR-overexpressing cells than in null vector-transfected cells (Fig. 5A). Concurrent with the decreased apoptosis ratio, caspase 3 was not activated in RSa-TfR and COS7-TfR cells by UV-irradiation (Fig. 5B), suggesting that overexpression of TfR interrupts apoptotic events.

**Decrease of p53 and Bax expression after UV-irradiation in TfR-overexpressing cells**

We analyzed the expression levels of apoptosis-related proteins, p53, Bax and Bel-2, up to 8 h after UV-irradiation. No dual decrease in the expression levels was observed in RSa-pcD, whereas the amount of p53 and Bax protein in RSa-TfR gradually decreased during the duration examined (Fig. 6A). The gradual decrease of p53 and Bax protein was also observed in COS7-TfR cells, but only a slight decrease was transiently detected in COS7-pcD cells (Fig. 6B). In contrast, the expression levels of Bel-2 did not change significantly after UV-irradiation in both TfR-transfected cells and null vector-transfected cells (data not shown). Taken together, these results suggest that overexpression of TfR attenuates the apoptotic events through decreases in p53 and Bax proteins after UV-irradiation in the cells tested.

**DISCUSSION**

Higher amounts of TfR were found in UV-resistant cell lines compared with UV-sensitive ones, as categorized by their apoptotic response (Fig. 1). The transcriptional activity of TfR was reported to increase after UV-irradiation.21 TfR knock down by siRNA in UV*-1 cells resulted in sensitization of the cells to UV cell-killing (Fig. 3). Further, TfR overexpression by cDNA transfection in human RSa cells and monkey COS cells resulted in increased resistance of these cells to UV cell-killing and/or apoptosis, estimated by colony formation and the apoptosis assay; TUNEL and caspase-3 activity assay (Figs. 4 and 5). These findings suggest that expression levels of TfR may be related to UV-susceptibility of the cells tested.

TfR plays a major role in cellular iron uptake through binding and internalizing the carrier protein transferrin.22 TfR is also known to be a growth factor receptor. The anti-apoptotic function of growth factor receptors was reported recently. Cell surface molecules such as EGF receptor,10 IGF-I receptor,11 syndecan-1,12 and G protein receptor,23 mediate UV responses in human and other mammalian cells. Kitagawa et al.10 reported that the phosphorylation of EGF receptor mediates extracellular signal-regulated kinase activation, providing the cell survival against UV-induced apoptosis. The endothelial receptor tyrosine kinase Tie1, an orphan receptor tyrosine kinase, is stimulated and autophosphorylated by its ligand, induces activation of phosphatidylinositol 3-kinase and Akt, then inhibits caspase 3 activation, resulting in inhibition of UV-induced apoptosis.24 No studies have reported that TfR itself contains kinase domains,25 although a recent study showed that stimulation of TfR by its specific Abs led to the tyrosine phosphorylation of ZAP70 and then to T cell proliferation.25 Therefore, TfR might be a signal transducer that attenuates UV-induced apoptosis through inhibition of caspase 3-activation by modulating the activity of the downstream molecules, because the ratio of UV-induced caspase 3 activation was decreased by TfR-overexpression in both RSa and COS7 cells (Fig. 5B).

UV*-1 cells showed slightly greater decreases in survival in Fig. 4C than in Fig. 2. The reason for this difference remains unclear. Most likely it is due to differences between the experimental conditions. In the experiment in Fig. 4C, untransfected cells were treated by the same method as transfected cells. One possible explanation for the decrease is that the EffectinTM reagent used in the transfection experiment may have inhibitory effects on cell survival. Despite sensitization, there was an apparent differences in UV-sensitivity between TfR- and mock-transfected RSa cells (Fig. 4C).

Although about 80% reduction in the TfR protein levels was observed in UV*-1 cells after siRNA transfection, the cells did not show the same ratio of colony survival after UV-irradiation as RSa cells (Fig. 3). These results may imply other factors that cause differential sensitivity of human cells to UV-irradiation, besides the TfR expression level itself. Interestingly, we found that increased expression levels of syndecan-1,12 nucleophosmin20 and HSP2727 correlate with increased resistance to UV cell-killing by comparative analysis between UV-sensitive RS cell lines and derivative UV-resistant cell lines. It will be necessary to clarify whether molecular functions of these molecules are interrelated with TfR expression on UV-susceptibility of human cells.

RSa and UV*-1 cells used in this study were established by SV40 and Rous sarcoma virus infection. The function of p53 in those cells may also be inactivated by this procedure because large T-antigen of SV40 is known as an inhibiting factor of p53 function.28 However, we reported sensitization of UV*-1 cells to UV by p53-overexpression,29 indicating that the amount of p53 is one of the factors that contribute to UV-susceptibility in those cells. Therefore, analysis of p53 function in both cell lines remains valuable.

In Fig. 6, the expression levels of p53 in whole cell lysates were not increased after UV-irradiation in RSa cells. In agreement with this result, the levels in cytoplasmic fractions were not significantly different between RSa and UV*-1 cells.
1 cells, and moreover the levels in both cells did not seem to be affected by UV irradiation. However, the levels of nuclear fractions in RSa cells were increased 1 h after UV irradiation, while those in UV-1 cells were not increased for up to 2 h. Therefore, further detailed studies of p53 metabolism, such as that in cellular components, will be necessary in order to clarify whether p53 metabolism is intimately related with TIR-involved UV susceptibility of human cells.

The expression levels of p53 and Bax protein were rapidly decreased 1 h after UV irradiation, then gradually decreased for up to 24 h in TIR-transfected RSa cells and COS7 cells (Fig. 6). The mechanism of modulation of p53 and Bax expression by TIR remains unclear. However, the decrease might play a role in UV-resistance by causing TIR overexpression, since UV induces the activation of p53 following upregulation of Bax expression, which executes the cell death pathway.

The ratio of apoptotic cell fraction of RSa cells by TUNEL analysis (Fig. 5A) showed a higher ratio than that by sub-G analysis (Fig. 1A). This difference may be caused by detection mechanisms of those analyses. TUNEL analysis detects not only the end of doubly broken DNA strands, but also nick of the strands. In contrast, sub-G analysis evaluates the amount of double strand nucleotides alone. Therefore, there may be some differences of the apoptosis ratio between the two analyses.

In summary, our study provides additional evidence of the modulation of UV-sensitivity by cell surface molecules. Human cells with discrepancy in the TIR expression levels will be useful for the elucidation of how TIR regulates cell survival against UV via modulation of p53 and Bax expression.

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