The HTLV-1 Tax Oncoprotein Attenuates DNA Damage Induced G1 Arrest and Enhances Apoptosis in p53 Null Cells

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Transformation of cells by the human T cell leukemia virus type 1 occurs via mechanisms unique among oncogenic retroviruses. A prevailing hypothesis for HTLV-1-mediated cellular transformation is that expression of the viral transactivator, Tax, induces genomic instability. Tax-mediated failure in the cellular repair response is one possible mechanism for loss in genomic integrity. Here we have examined the in vivo repair response of Tax-expressing cells to determine the underlying defects that contribute to loss of genomic integrity. In these studies we examined the effects of de novo Tax-expression in naive “pre-neoplastic” REF52 cells. DNA-damage-induced p53 stabilization and concomitant transient stabilization of p21 were clearly evident in Tax-expressing cells. Likewise, the damage-induced apoptotic response of Tax-expressing cells was normal. However, the damage-induced G1 checkpoint was abrogated in either p53+ or p53− cellular backgrounds. Although nucleotide excision repair (NER) of asynchronous Tax-expressing cells was impaired, cell-cycle-independent assessment of NER in the global excision repair assay demonstrated comparable NER activity in Tax-expressing cells, suggesting that the failure of G1 checkpoint contributes to NER deficiency. Interestingly, we observed a dramatic increase in apoptosis and UV sensitivity of Tax-expressing p53−/− cells when compared to Tax-expressing p53+/+ cells. These data demonstrate that Tax-mediated cellular genomic instability arises from attenuation of cell-cycle checkpoint and imply a clonal dependence on p53 status separate from genomic integrity.

Key Words: retrovirus; cell cycle; G1 checkpoint; apoptosis.

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) is associated with adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986; Poiesz et al., 1982). CD4+ T cells are the main target for infection by HTLV-1 and the cellular transformation process is believed to be in large part a consequence of expression of the viral transactivator Tax (Yoshida, 2001). Tax functions to transactivate viral transcription through interaction with the 5′ long terminal repeat (LTR) (Chen et al., 1985; Felber et al., 1985; Seiki et al., 1986). In addition, Tax can activate and/or repress a variety of cellular promoters with potential impact upon cell growth (Franklin and Nyborg, 1995; Neuveut and Jeang, 2000). Previously, Tax expression has been shown to reduce cellular genomic stability (Majone et al., 1993; Semmes et al., 1996b), prompting speculation that induction of genomic instability may facilitate HTLV-1-mediated cellular transformation.

Several studies have attempted to determine how Tax might influence overall cellular genomic integrity. One proposed explanation theorizes that genomic instability arises from cellular repair defects resulting from a direct effect of Tax on the transcription of repair gene products, such as β-polymerase (Jeang et al., 1990) and Proliferating Cell Nuclear Antigen (PCNA) (Ressler et al., 1997). In these cases Tax repressed or stimulated transcription of the cognate cellular promoter, respectively. Another possible mechanism involves direct interaction of Tax with cellular proteins that monitor/regulate genome integrity (Jin et al., 1998; Majone and Jeang, 2000; Suzuki et al., 2000). An example of this model is presented by the demonstration that Tax binds to HsMAD 1, thus disturbing spindle assembly/disassembly and progression through M, presumably via molecular sequestration. Tax also has both positive and negative effects on cell cycle, each of which may contribute to genomic instability. The positive effects on the cell cycle include activation of kinases and repression of cell-cycle inhibitors (Iwanaga et al., 2001; Lemoine and Marriott, 2001; Neuveut et al., 1998; Santiago et al., 1999; Schmitt et al., 1998; Suzuki et al., 1999). The negative effects on cell-cycle movement could result from a repression of c-myc function (Semmes et al., 1996a) or from activation of cell-cycle inhibitors (Yoshida, 2001). All of these activities have been ascribed to Tax with a common functional goal as yet undefined. In addition to these various direct effects that may have an impact on genome integrity, Tax may

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elicit more global effects via its reported activities on the regulatory protein p53.

p53 is an important regulator of cellular genome stability (Lane, 1992). Induction of p53 following DNA damage can result in activation of repair, cell-cycle arrest, and apoptosis (Levin, 1997). In light of the central role of p53 in preserving cellular genomic integrity, it is not unexpected that loss or inactivation of p53 has been causally associated with oncogenic transformation (Donehower, 1996; Donehower and Bradley, 1993). Several recent reports have demonstrated that Tax expression results in inactivation of p53 through several suggested mechanisms. For example, p53 inactivation by Tax is associated with hyperphosphorylation of serine 15, a residue located in the transcriptional activation domain (TAD) of the protein (Pise Masison et al., 2000). In addition, other laboratories have reported that inhibition of p53 function can result from squelching of CREB-binding protein (CBP) by HTLV-1 Tax protein (Suzuki et al., 1999b; Van Orden et al., 1999). In either case p53 inactivation, which may be expected to impact both DNA repair and clonal survival, could be one of the primary events leading to the clonal expansion of HTLV-1-infected cells. The ability of Tax to repress p53 transcriptional activity coupled with the observation that mutations in tumor suppressor genes is frequent in ATL (Hatta et al., 1995, 1997; Nishimura et al., 1995; Sakashita et al., 1992) has supported the contention that p53 has a role in Tax-mediated cellular transformation.

The above studies have concentrated on a mechanistic understanding of Tax repression of p53 transcription. Several additional studies have suggested an impact of Tax expression on downstream p53 function. By summarizing these data, the addition of exogenous p53 can

**FIG. 1.** Efficiency of viral transduction. (A) A depiction of the pHRTaxiGFP transducing vector construct and the control pHRGFP vector. (B) REF52 cells were transduced with pHRTaxiGFP providing bicistronic Tax and green fluorescent protein (GFP) expression, shown on the left. A corresponding phase contrast microscope view from the same field is shown on the right. (C) REF52 cells were transduced by either pHRGFP alone, or bicistronic pHRTaxiGFP, a mutated form of Tax (deletion of 30 amino acids from the carboxyl terminus) pHRTaxΔ334–354. The cell extracts were loaded as follows: pHRGFP (lane 1), pHRTaxiGFP (lane 2), pHRTaxD334–354 (lane 3), pHRTax (lane 4), and detected using anti-Tax. The wild-type form of Tax has an apparent molecular weight of 40 kDa and the deleted form runs quicker as indicated.
compensate for Tax-induced repair defects (Kao et al., 2000) and Tax-expressing p53 null cells fail to arrest in G1 following addition of exogenous p53 (Mulloy et al., 1998). Implied in the above studies is that Tax induces genomic instability via impairment of p53. Clearly it is necessary to determine whether the increased cellular genomic instability of Tax-expressing cells arises from impairment of p53 or via p53-independent mechanisms. Although a useful model for some p53-dependent activities, the addition of exogenous p53 does not address the functional intactness of an endogenous p53-mediated response. Furthermore, stable lines expressing Tax likely represent selected/adapted cellular changes to compensate for Tax expression and may not represent direct Tax effects. Here we attempt to establish the effect of de novo Tax expression upon the UV-induced DNA damage response in naive cells containing normal functional wild-type p53 signaling response.

RESULTS

Viral transduction results in efficient de novo expression of Tax

To test the efficiency of the viral transduction for de novo expression of Tax, we transduced REF52 cells to express either Tax and green fluorescent protein (GFP) or GFP alone. The pHRTaxiGFP vector used for delivery of the bicistronic message expressing Tax and GFP and the corresponding control vector expressing GFP alone are shown in Fig. 1A. We determined the transduction efficiency of this system as a measure of the resulting expression of the delivered gene. Tax-expressing REF52 cells were identified via their bicistronic expression of both Tax and GFP (Fig. 1B). Titration of the viral supernatant to a level resulting in 50% of cells expressing GFP resulted in endpoint measurement of transduction titer. By this definition, typical viral titers were $1 \times 10^6$ infectious units per milliliter without supernatant concentration. We also verified the expression of Tax in the target cells by Western blotting. We show in Fig. 1C the expression of Tax following transduction of the pHRTaxiGFP cDNA using anti-Tax antibody. Thus efficient production of Tax and GFP were achieved and can be quantitated using the described viral-mediated transduction method.

HTLV-1 Tax-expressing cells display an impaired nucleotide excision repair

In examining the biological response of Tax-expressing cells to UV-induced DNA damage, we first tested for efficient nucleotide excision repair (NER) ability. We used the host cell reactivation assay (HCRA) as a measure of NER capacity in Tax-expressing REF52 cells. As a control, we evaluated the differential repair competency of the Xeroderma Pigmentosum complementation group-A (XP-A) cell line GM04429 and the non-XP cell GM00010B of the same lineage. When examined for NER capacity in the HCRA, the XP-A cell line showed dramatically reduced repair capacity when compared to the non-XPA cell GM00010B (Fig. 2A). When Tax-expressing REF52 cells are compared to control GFP-expressing REF52, the Tax-expressing cells were significantly reduced in NER capacity (Fig. 2B). Thus, Tax expression results in reduced NER capacity as measured by HCRA and these results are in accordance with earlier findings (Kao et al., 2000).

Cell-cycle arrest and induction of apoptosis in HTLV-1 Tax-expressing cells

In addition to repair, at least two key events occur in the UV-induced cellular DNA damage response. These are initiation of cell-cycle arrest and induction of apoptosis. We assessed the ability of Tax-expressing REF52 cells to undergo cell-cycle arrest and apoptosis. Tax-expressing and control cells were subjected to UV irradiation and examined for apoptotic events and cell-cycle profile. Prior to exposure to UV, the percentage of non-apoptotic, apoptotic, and necrotic cells was examined. The three populations were identified using the Vybrant assay. In the absence of UV, Tax-expressing cells, similar to control REF52 and XP-A cells, showed a low percentage of apoptotic cells (Fig. 3; −UV). Following UV exposure, both REF52 and Tax-expressing REF52 displayed a normal apoptotic response to DNA damage as indicated by the moderate increase of apoptosis. The same results were seen using several Tax-expressing cells, including GM00010B (data not shown). However, the NER repair-deficient XP-A cells showed a significantly increased apoptotic response as was expected (Fig. 3; +UV). Thus, Tax-expressing cells show a normal apoptotic response, which is vastly more conservative than would be expected from NER-deficient cells.

We next measured the cell-cycle response of Tax-expressing cells. In this assay, asynchronous cells were exposed to UV and incubated with cytochalasin B 1 h later. The cell population was examined 36 h later and assessed for division events. Those cells that fail to arrest will appear as binucleated cells, whereas those that arrest prior to M will appear as mononuclear cells. As expected, cells which were not exposed to UV light continued to divide and resulted in greater than 98% binucleated cells (Fig. 4A). In contrast REF52 cells exposed to UV light initiated cell-cycle arrest and presented a predominately (>90%) mononuclear cell population (Fig. 4B). However, 75% of Tax-expressing REF52 cells, exposed to UV, were binucleated cells, indicating a failure to arrest in cell cycle in response to UV-induced DNA damage (Fig. 4C). In total, these results suggest that Tax-expressing cells have an intact early apoptotic response but fail to arrest in cell cycle.
Global nucleotide excision repair of Tax-expressing cells is normal in G1-synchronized cells

A failure in damage-induced cell-cycle arrest would result in reduced repair efficiency and could explain the Tax-associated NER deficiency. This would imply that the actual NER pathway in Tax-expressing cells is intact and would predict that restoration of a competent cell-cycle arrest would restore NER capacity. To test this hypothesis, we examined global NER in G1 synchronized cells. Tax-expressing rat embryonic fibroblasts (REF52) cells intermixed with REF52 were accumulated in G0 by serum starvation for 72 h. The cells were then released from G0 and allowed to advance for 4 h into G1. The cells were then exposed to UV and incubated with BrdU. Incorporation of BrdU is restricted to cells undergoing DNA synthesis, which is unscheduled during G1. The unscheduled DNA synthesis directly relates to NER-mediated repair synthesis in UV exposed cells. Under these conditions, the UV-induced unscheduled DNA synthesis in Tax-expressing cells was indistinguishable from control cells (Figs. 5A and 5B). Those cells unexposed to UV did not incorporate BrdU and demonstrate the successful synchronization out of S phase (Figs. 5C and 5D). We conclude that NER is intact in G1 synchronous Tax-expressing cells.

Stabilization of p53 and induction of p21 in response to UV exposure in Tax-expressing cells

A key player in initiation of the UV-repair response and activation of cell-cycle arrest is p53. Activation of p53 is signaled by an accumulation of p53 in the nucleus of cells exposed to UV light. To examine whether Tax-expressing cells display this early p53 response, we transduced REF52 cells with suboptimal viral titers to produce an estimated 50% transduction efficiency. The cells were exposed to UV and examined for nuclear expression of p53. The Tax-expressing REF52 cells displayed a strong nuclear accumulation of P53 in response to UV damage when compared to the same UV-treated REF52 cells not expressing Tax (Fig. 6A). REF52 cells not exposed to UV did not show nuclear accumulation of P53 (data not shown). These results demonstrate that one early step in p53 activation, nuclear accumulation, is intact in Tax-expressing cells.

To further analyze the p53 response in Tax-expressing cells, we examined both Tax-expressing and control cells for stabilization of steady-state p53 and transient induction of p21. Since induction of p21 is a key event in p53-mediated cell-cycle arrest, analysis of the p21 levels in Tax-expressing cells could determine the integrity of this signal. We exposed Tax-expressing and control cells to UV and harvested for Western analysis preexposure and at 8 and 24 h postexposure. In response to UV-induced DNA damage, both cell groups demonstrated stabilization of steady-state protein levels of p53 (Fig. 6B). Examination of the same cells for p21 expression revealed similar p53-induced transient p21 induction between Tax-expressing and control cells (Fig. 6C). Thus, one p53-dependent signal for cell-cycle arrest, transient induction of p21, was intact.

Attenuation of G1 checkpoint in p53−/− Tax-expressing cells

The results from the above experiments suggested that the deficiency in the repair capacity of Tax-expressing cells is due to a failure in cell-cycle checkpoint and that this defect is independent of p53 signaling. To test this hypothesis, we examined the cell-cycle arrest response in Tax-expressing and control p53−/− cells. HeLa and p53−/− cells were transduced to express Tax. Each of the experiments was conducted in two ways: complete transduction of Tax to 100% or target cells and transduction of Tax to approximately 25% of target cells.
Transduction of 100% of the target population allows for documentation that the Tax "effect" was universal. Transduction of approximately 25% of the target population allows for presentation of the Tax effect adjacent to control cells. Tax-expressing and control cell groups were subjected to the cytokinesis block cell-cycle progression assay as described under Materials & Methods. The cells were examined after 36 h for the presence of binucleated dividing cells. The results clearly showed that Tax-expressing cells in a p53/H11002 background failed to generate a DNA-damage cell-cycle arrest response (Fig. 7). This was true for either p53 "functionally repressed" or p53 "deleted" cell lines expressing Tax (Figs. 7B and 7C). Both p53 repressed and p53 deleted cell lines not expressing Tax showed efficient cell-cycle arrest, as has been reported elsewhere (Fig. 7B). Thus, the failed cell-cycle arrest is not dependent upon the presence of p53 and is evidence of failure in a p53-independent DNA damage response.

To determine whether the failure in cell-cycle arrest was due to a failed G1 checkpoint, we synchronized cells at G0 by serum starvation. The cells were then cultured in 20% serum to stimulate return to cell cycle following UV treatment. Cell cycle was determined by total DNA content as described under Materials & Methods. Tax-expressing cells showed a consistent and significant increase in the percentage of cells leaving G1 (Fig. 8). This was true for either a wild-type or a deleted p53 background. Therefore, the failure in cell-cycle arrest is due at least in part to a failed G1 arrest.

Tax-expressing p53−/− cells are hypersensitive to UV treatment

We reasoned that if Tax-expressing cells display impaired damage response in a p53-independent manner then cells both deleted for p53 and expressing Tax would be more prone to apoptotic cell death in response to UV. To test this hypothesis, we examined the apoptotic response of both Tax-expressing and control p53−/− cells. Tax-expressing and control cells were exposed to sublethal doses of UV and examined for percentage surviving cells 24 h after treatment. We observed that Tax-expression in a p53+/+ or p53-mutant background showed no decreased percentage of surviving cells over the appropriate control cells. However, Tax-expression in the p53−/− background resulted in significant cell death in response to UV exposure (Fig. 9). We also examined the early apoptotic response of Tax-expressing p53−/− cells. In this series of experiments the cell groups were exposed to 0, 20, and 50 J/m² and apoptosis measured at 12 and 24 h (Fig. 10). Significant apoptosis is seen in Tax-expressing cells at each time point in response to doses of UV, which result in only modest induction of apoptosis in control p53−/− cells. This level of UV-induced apoptosis is equivalent to that seen in cells defective in NER (Fig. 3).

**DISCUSSION**

One of the most compelling hypotheses to explain HTLV-1-mediated cellular transformation is that Tax expression induces genomic instability. In such a model,
the viral-induced genomic instability gives rise to increased potential for acquiring discrete genetic changes needed to support leukemogenesis. The initial reports of Tax-induced genomic instability suggested that loss in cellular genomic integrity was the result of the accumulation of genetic damage arising from nonmitotic DNA breaks (clastogenic) or failure in mitotic segregation (aneuploidogenic) nature (Majone et al., 1993; Semmes et al., 1996b). There is convincing evidence that Tax alone induces a state of genomic instability in the target cell; however, the exact mechanism for this process is unknown.

In our present study, we have confirmed previous observations (Kao and Marriott, 1999) and demonstrated that de novo Tax expression resulted in a reduction in cellular NER capacity as measured by the HCRA. When we examined the UV-induced repair-response of Tax-expressing cells, several key early damage-induced cellular events such as p53 nuclear accumulation and induction of apoptosis were intact. Interestingly, we clearly observed increased steady-state levels of both p53 and p21 in Tax-expressing cells without UV treatment, a result consistent with Tax-induced stabilization of this pathway. However, a notable UV-mediated damage-induced p53 accumulation and transient stabilization of p21 was still evident. Thus, although NER capacity was reduced, the endogenous p53-mediated damage-response events were normal in Tax-expressing cells. The only functional defect we could observe in the cellular damage-response was an inability to accommodate cell-cycle checkpoint. It has been reported that expression of Tax abrogates G1 arrest induced by exogenous p53 (Mulloy et al., 1998) and subsequently concluded that this result is evidence for alteration of p53 function by Tax. However, induction of G1 arrest by exogenous p53 is dependent on status of endogenous p53 perhaps representing cellular adaptation to loss of p53. Specifically, only p53 null lines demonstrate efficient G1 arrest in response to exogenous p53 (Cascallo et al., 1999; Kagawa et al., 1997; Morretti et al., 2000; St John et al., 2000), implying that there is a biological difference between damage-induced endogenous G1 checkpoint and exogenous p53-induced G1 arrest. It should be noted that damage-induced G1 arrest can occur through p53-independent pathways and indeed we report here attenuation of damage-induced G1 checkpoint in p53 null cells by Tax. Our results, therefore, support a mechanistic model in which Tax directly alters cell-cycle checkpoint in a manner independent from p53. Synchronizing Tax-expressing cells to G1 resulted in decreased activity of exogenous p53. This result allows for a different interpretation of earlier studies in which exogenous addition of p53 partially restores NER activity of Tax-expressing cells. Since

FIG. 4. Cell-cycle response to UV irradiation in REF+Tax cells. REF52 and REF+Tax cells were subjected to the cytokinesis block cell-cycle arrest assay to determine the intactness of the damage-induced checkpoint. (A) REF52 cells unexposed to UV show progression through the cell cycle and presentation as binucleated cells. (B) REF52 cells exposed to UV light initiated cell-cycle arrest and presented over 95% mononuclear cell population. (C) REF+Tax cells, exposed to UV, showed nearly 75% of the population as binucleated cells, indicating a failure to arrest in cell cycle. For each data point 1000 cells were analyzed. Tax-expressing cells unexposed to UV exhibit the same profile as in the absence of Tax, showing binucleated cells (not shown).

FIG. 5. Excision repair of Tax-expressing cells is normal in G1-arrested cells. REF52 and REF+Tax cells were synchronized in G0 by serum starvation. The cells were UV irradiated and labeled with BrdU-dUTP. The cells were then fixed and immunostained with anti-Tax rabbit polyclonal and anti-BrdU mouse monoclonal antibodies. Secondary antibodies were anti-rabbit Texas red and anti-mouse FITC-conjugated, respectively. Both REF52 (unlabeled in A) and REF+Tax (red in A, arrow) were actively undergoing unscheduled DNA synthesis (green in B) following UV irradiation. A and B are paired images of the same field of view. No unscheduled DNA synthesis was observed in the absence of UV for either REF52 (unlabeled in C) or REF+Tax (red in C). The transduction efficiency was titered to 25% so that REF52 and REF+Tax cells could be viewed adjacent. A total of 25 adjacent events were examined.
exogenously added p53 can induce G1 arrest, rescue of NER by exogenous p53 may result from reinforced G1 arrest and not from restoration of p53 function. Interestingly, we observed a failure in cell-cycle arrest in the presence of a competent p21 induction and in cells regardless of p53 status. The loss of cell-cycle checkpoint in p53−/− cells implies that the effect of Tax is p53 independent and suggests that Tax might abrogate G1 arrest signals downstream to or independent of p53. This could occur via a direct interaction with and sequestration of cell-cycle progression/checkpoint components or transcriptional repression of the corresponding gene product. We are currently pursuing this possibility.

We speculated that if the Tax-mediated effect were upon a p53-independent pathway then Tax-expressing p53−/− cells would lack both p53-mediated and p53-independent repair responses. Such a cell, not given the opportunity to adapt to these defects, would display a hypersensitivity to DNA damage. In fact, we saw a dramatic increase in cell death in response to UV light in Tax-expressing p53−/− cells. The sensitivity to UV in the Tax-expressing p53−/− cells was comparable to that seen in NER-deficient XP-A cells and is in stark contrast to Tax-expressing p53+/+ cells. This result suggested that survival of, and in turn mutational pressure on, Tax-expressing cells is dependent on p53 status and may provide a framework for Tax and p53 interaction. Thus, although the attenuation of UV-mediated cell-cycle arrest by Tax does not appear to arise from a direct effect on p53, the status of p53 has profound impact on Tax-expressing cells. Furthermore, since our results were observed following de novo expression of Tax, we would propose that loss of p53 is selected against in Tax-expressing cells.

FIG. 6. Nuclear accumulation, stabilization of p53, and induction of p21 in response to UV-irradiation in Tax-expressing cells. (A) REF52 cells were transduced with pHRTaxiGFP to 25% efficiency and mounted on coverslips. Forty-eight hours later, the cells were UV-irradiated (20 J/m²), fixed, and immunostained with a mouse monoclonal anti-P53 and rabbit polyclonal anti-Tax antibody. Secondary antibodies were anti-mouse FITC-conjugated and anti-rabbit TRITC-conjugated, respectively. Shown are separate images of the same field of view which encompasses two cells. The arrows indicate the nucleus of each cell. Both REF52 and REF+Tax cells showed equivalent nuclear accumulation of P53 (left). (B) REF52 cells were transduced with either pHRGFP or pHRTaxiGFP, then exposed to 20 J/m² UV (+) and harvested, and subjected to Western blot analysis. When probed with anti-P53 antibody, both cell groups demonstrated stabilization of p53, resulting in increased steady-state protein levels. The same cells from (B) were harvested after 0, 8, and 24 h. The immunoblots were prepared as described above. The blots were probed with anti-p21 antibody. Shown are extracts from both pHRGFP (−) and pHRTAXiGFP (+) transduced cells.

FIG. 7. Failed cell-cycle arrest in Tax-expressing cells with deleted p53. HeLa and p53 deleted (p53d) cells were subjected to the cytokinesis block cell-cycle arrest assay. Each cell group was exposed to UV and incubated with cytochalasin B 1 h later. The cells were examined after 36 h for the presence of binucleated dividing cells. (A) HeLa cells were transduced to express Tax at 25% efficiency. Tax-expressing HeLa cells were identified by immunofluorescence using anti-Tax mouse monoclonal antibody and FITC-conjugated anti-mouse secondary antibody. The nuclei were stained with propidium iodide. HeLa (red) and Tax-expressing HeLa (yellow) formed binuclei, indicating normal division. (B) When exposed to UV, HeLa cells (red) arrested as mononucleated, whereas Tax-expressing cells (yellow) continued to divide and formed binucleated cells. (C) Similar results were obtained with the p53-deleted cell line. Shown is a population of cells transduced to express Tax at 100% efficiency. Tax-expressing p53d cells fail to arrest in response to UV and form binucleated cells. In the experiments using partial transductions of Tax (A+B), 100 pairs of adjacent Tax-expressing and nonexpressing cells were examined.
expressing naive cells. The hypersensitivity due to the loss of p53 in de novo Tax-expressing cells may help explain the long period between HTLV-1 infection and cellular transformation. In fact Kao et al. (2001) have shown that stable Tax-expressing p53 null cells display a moderate apoptotic response, suggesting that Tax-expressing cells can adapt to survive p53 loss. Selection against loss of the tumor suppressor p53, which is frequently associated with the phenotype of HTLV-1 transformed cells, would prolong the pre-transformed state of HTLV-1-infected cells. This might explain why Tax has evolved a mechanism for interacting with cellular p53 pathways and why close to 50% of ATL patients present p53 mutations.

Central to this discussion is the reconciliation of our results with several recent reports implicating impairment of p53 transcriptional activation function in the Tax-mediated transformation process (Pise Masison et al., 2000; Van Orden et al., 1999). In one study, overexpression of exogenous p53 restored the impaired NER capacity of Tax-expressing cells (Kao et al., 2000). Implied in these studies is that loss of p53 function would lead to inefficient repair process. However, examination of p53-knockouts have revealed no increase in mutation rate (Griffiths et al., 1997), as would be expected if repair capacity were reduced. In general, early loss of p53 has been associated with improved clonal survival following mutation events. Interestingly, when p53−/− ATLL were examined, there was no evidence of higher mutagenic rates (Takamoto et al., 2000). Furthermore, studies of Tax transgenic mice have demonstrated that there is minimal increase in tumor formation events contrasting with significant increase in disease progression (Portis et al., 2001). However, de novo Tax expression results in increased mutation rates as we have measured by can resistance in yeast (Semmes et al., 1999) and as demonstrated in hprt resistance in mammalian cells (Miyake et al., 1999). Therefore, “early” Tax-induced genomic instability, associated with increased mutation frequency, may precede a “late” stage signified by loss in p53 function, which contributes to clonal survival of the newly generated clones. Indeed the two stages are likely interdependent.

Our system of retroviral transduction allows for examination of de novo expression of Tax and would therefore reflect early cellular responses to Tax expression. Events occurring in long-term or stable Tax-expressing systems could represent adaptations of the cell to Tax expression and may reflect long-term Tax effects. In this light we do not address the potential role of impaired p53 transcription activity as reported by others (Pise Masison et al., 2000; Van Orden et al., 1999). We propose that an impairment of the cellular damage-repair response through bypass of appropriate cell-cycle checkpoint is a mechanistic route to Tax-induced cellular genomic instability. We also demonstrated that loss of p53 function severely impacts on the survival of Tax-expressing cells, a result that provides an explanation for the interdependence between Tax activity and p53 function.

MATERIALS AND METHODS

Plasmids, cells, and antibodies

The plasmids used for retroviral transduction are as previously described (Naldini et al., 1996). pMD.G is used for the production of the envelope protein G of vesicular stomatitis virus. pCMVD8.2 is the packaging construct and is used for the production of human immunodeficiency virus gag, pol, and regions of env. The delivery construct pHRTax was made by inserting the tax ORF into the XhoI and BglII site of pHRCMV and produces

![FIG. 9. Increased cell killing in response to UV irradiation in p53-deleted cells. Tax-expressing and control cells were exposed to sublethal doses of UV and examined for percentage surviving cells 24 h after treatment. Tax expression in a p53+/− (REF+Tax) or p53 mutant background (HeLa+Tax) showed no decreased percentage of surviving cells over the appropriate control cells. However, Tax expression in the p53−/− background (p53d+Tax) resulted in significant cell death in response to UV exposure.](image-url)
“packageable” viral RNA. pHRTaxiGFP and pHRGFP produce either Tax-GFP and GFP packageable RNA. pRSV-CAT contains the chloramphenicol acetyltransferase (cat) reporter gene under the control of the Rous sarcoma virus (RSV) promoter, and pMSV-Luc containing the luciferase gene under the control of Moloney sarcoma virus (MSV) promoter. The REF52 cell line was a gift from Thomas Parson (University of Virginia), the p53−/− cell line was a gift from Bert Vogelstein (Johns Hopkins University), and the XP-A (Xeroderma pigmentosum complementation group A) cell line GM04429 was obtained from Coriell Cell Repositories (NIGMS). The cells were maintained at 37°C in Iscove’s modified Dulbecco’s medium with 10% fetal calf serum and 1% penicillin–streptomycin (Gibco-BRL). The anti-Tax rabbit polyclonal antibody was raised against amino acids 104 to 120 and was affinity purified with the same peptide. Antibodies against p53 (DO-1) and p21 (F-5) were purchased from Santa Cruz Biotechnology. Anti-BrdU (BU-3) was purchased from Sigma.

Immunoblot analysis

Approximately 2 × 10⁶ cells were harvested and proteins extracted with 200 μl M-Per mammalian protein extraction reagent (Pierce). An amount of 50 μl of 4X Laemmli buffer was added to the lysate and 30 μl of the lysate was electrophoresed through a 10% SDS–polyacrylamide gel. The proteins were electroblotted onto an Immobilon-P membrane (Millipore) and probed with the indicated primary antibody and the appropriate secondary alkaline phosphatase conjugated antibody. Immunoreactivity was detected via Western Star chemiluminescence protein detection (Tropix).

Host cell reactivation assay

pSV2-CAT reporter plasmid was damaged ex vivo by exposure to 1000 j/m² of UV-C light using a UV chamber-GS Gene Linker (Bio-Rad). REF52 and XP-A cells were transfected with 4 μg of UV-irradiated or nonirradiated pSV2-CAT plasmid together with an undamaged reporter plasmid (pMSV-Luc), and with or without Tax plasmid. Forty-eight hours after calcium phosphate transfection, cells were pelleted and resuspended in 250 μl of 250 mM Tris pH 8.0. For the luciferase assay, 25 μl of the total cellular extract was added to 50 μl of luciferase substrate. Luciferase activity was quantitated in a luminometer. Cat assays were performed in parallel with the same cells as described (Semmes and Jeang, 1992). Cat activity was normalized to luciferase activity of the same extract. Repair activity was calculated by setting normalized cat activity from cells cotransfected with nonirradiated pSV2-CAT to 100%. The repair activity of duplicate cells cotransfected with irradiated pSV2-CAT was reported as a percentage of that activity.

Global nucleotide excision repair assay

REF52 cells were seeded onto glass coverslips and transduced to express Tax at 25% efficiency. Subconfluent Tax-expressing REF52 cells were cultured in 0.5% serum for 48 h, to synchronize at G0, prior to irradiation. The cells were released from G0 with addition of com-
complete medium. At 4 h postrelease, the cells were irradiated with UV light (20 J/m²) using a UV chamber-GS Gene Linker (Bio-Rad) and incubated for 30 min with 10 mM BrdU-containing medium. The cells were then washed four times with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized with 2 min incubation in 100% methanol and washed four times with PBS. The prepared cells were then reacted with both anti-Tax and anti-BrdU in PBS (containing 2% BSA). Primary antibodies were removed by washing four times with 1% Tween 20 in PBS (PBS–Tween). Secondary conjugated antibodies were reacted for 1 h at room temperature (RT). The cells were then washed with PBS-Tween and the slips were inverted onto slides with Vecta Shield (Vector Laboratories, CA). Incorporation of BrdU into the nucleus of cells at G1 corresponds to unscheduled repair synthesis.

Viral transduction

We used the lentiviral transduction system as described by Naldini et al. (1996). Three plasmids (pHRGFP or pHRGFP produces packagable RNA; pCMVD8.2 produces gag, pol, and accessory gene products; and pMD.G produces VSV G protein) were cotransfected into 293/T17, by the calcium phosphate method, to produce replication-defective viral particles. Viral titer was determined as relative to control GFP producing virus stock. The expression of GFP was assessed as a percentage of green fluorescent cells. The standard curve of p24 values associated with increasing expression efficiencies was used as an estimate of potential infectious units. p24 values were derived for each batch of virus supernatant.

Target cells were plated at 2–3 × 10⁵ cells per ml in serum-free medium. Supernatant containing lentiviral vector particles was added at a concentration corresponding to 1 × 10⁴ infectious units per milliliter. Cells were incubated for 24 h and then washed and cultured in vitro for 48 h to ensure maximal transgene expression. GFP expression was analyzed in target cells by fluorescence microscopy. When reduced transduction efficiencies were desired, the viral supernatants were titrated to achieve the desired infectious units per milliliter of culture media. The relative percentage of transduced cells was verified by observing the percentage of green cells.

Apoptosis studies

Tax-expressing cells were treated with different UV doses (0, 20, and 50 J/m²) and assessed at different time points postransduction (4, 8, 12, and 24 h). Cells were separated into living, necrotic, or apoptotic populations using the triple-source fluorescent labeling, Vybrant Apoptosis Assay kit (Molecular Probes), according to the manufacturer’s recommendations.

Cytokinesis block cell-cycle progression assay

Asynchronous cell cultures were seeded on coverslips and exposed to UV irradiation and allowed to recover for 1 h. Cytochalasin B was added and the cultures incubated for 36 h. The coverslips were fixed with paraformaldehyde/methanol and stained with propidium iodide. Slides were prepared and cell nuclei examined by microscopy. Binucleus cells were considered dividing.

Flow cytometry

For cell-cycle analysis, cells were collected by gentle scraping following a rapid EDTA rinse, concentrated by low speed centrifugation and washed in 1× PBS and fixed with cold 10% ethanol. Cells were stained with a PI solution [PBS 1×, RNase A (10 μg/ml), and propidium iodide (50 μg/ml)] followed by cell-sorting analysis. FACS data acquired were analyzed by CellQuest software.

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