ABSTRACT

CTLs specific to p53 were previously shown to efficiently eradicate p53-overexpressing tumor cells in vitro as well as in vivo. In this report, we demonstrate that these CTLs can also eliminate tumors that display moderate or even low levels of p53. Neither high steady-state levels of p53 nor elevated p53 synthesis is a prerequisite for recognition of tumors by p53-specific CTLs. Instead, our data show that a high p53 turnover rate is an important factor in determining the sensitivity of tumor cells to p53-specific CTLs. Our data suggest that p53 turnover is related to the MHC class I-restricted presentation of p53-derived epitopes at the tumor cell surface and indicate that CTL-mediated immunotherapy that targets p53 can be applied to a wider range of tumors than has thus far been anticipated.

INTRODUCTION

Mutations in the gene encoding the tumor suppressor protein p53 are found in approximately 50% of all human malignancies (1). The aberrant expression of p53 in a wide variety of tumors and its direct involvement in malignant transformation make it an attractive target for immunotherapy of cancer. Mutations in p53 in many cases result in an increased half-life and higher steady-state expression levels for this protein. Altered expression of p53 can also be the consequence of interaction with viral oncoproteins (2, 3). For instance, the adenovirus type 5 E1B gene product (Ad5E1B) forms stable complexes with wtp53 (4) in the cytoplasm (4), which results in perinuclear inclusion bodies. The half-life of p53 is increased from ~20 min (5) to several hours (2). Furthermore, the complex formation prevents p53 from transcriptionally activating genes that induce cell cycle arrest (6, 7) or apoptosis (8, 9). In contrast, complex formation of p53 with the human papillomavirus type 16 E6 (HPV16 E6) oncoprotein leads to an increased ubiquitin-dependent and proteasome-mediated degradation of p53 (10). Over the past years, several laboratories have investigated the feasibility of p53-directed immunotherapy of cancer (11–16). A study by Gnjatic et al. (17) showed that overexpression of p53 in breast adenocarcinomas and melanoma was required for recognition by peptide-induced wtp53-specific CTLs. It was postulated that the overexpression of p53 in tumor cells, in contrast to the low and ubiquitous expression in normal tissue (18), creates a therapeutic window allowing wtp53-specific CTLs to eradicate a tumor without damage to normal tissue. This notion was supported by our study in which wtp53-specific CTLs, raised in p53−/− mice, eradicated p53-overexpressing tumors in p53+/+ mice without detectable damage to normal tissue (16). Therefore, experimental data reported thus far indicated that the efficacy of such immunotherapy would require the tumor to express greatly elevated levels of p53 as determined by IHC. This latter parameter is routinely used as a diagnostic marker in cancer treatment (19).

In the present study, we investigated whether T-cell-mediated immunotherapy of cancer exploiting p53 as a target antigen would indeed require the tumor to express greatly elevated levels of p53, or whether more modest expression levels would suffice to sensitize such tumors for immune attack by CTLs. Analysis of a panel of murine tumors, expressing different p53 steady-state levels, for sensitivity to p53-specific CTLs revealed that tumors expressing low levels of p53 could also be efficiently lysed by these CTLs. Accordingly, overexpression of p53 by the tumor is not a prerequisite for therapeutic efficacy of adoptively transferred p53-specific CTLs in tumor-bearing mice. Comparison of Ad5- and HPV16-transformed cells revealed that, in inverse correlation with p53 steady-state levels, HPV16-transformed cells are efficiently lysed by p53-specific CTLs, whereas their Ad5-transformed counterparts are not killed. Complex formation with the Ad5E1B protein sequesters and stabilizes p53 in Ad5-transformed cells, whereas in HPV16-transformed cells, p53 is rapidly degraded by the proteasome. Our data suggest that the rate of p53 processing rather than the p53 steady-state levels determine whether a given tumor is a good target for CTL-mediated immunotherapy.

MATERIALS AND METHODS

Mice, Tumor Cell Lines, and Culture Conditions. C57BL/6 (B6, H-2b) mice were obtained either from the Netherlands Cancer Institute (Amsterdam, the Netherlands) or from Charles River (L’Arbresle, France). B6MEC were transformed by transfection with the oncogenes: mut.p53 + N-ras (tumor cell line 5D) and mut.p53 + H-ras (tumor cell line 42; Ref. 20). The tumor cell lines 5D and 42 express high levels of mutant p53 as tested by cytospin. The cell line C3 was made by transfecting B6MEC with HPV16 E+L and pEJ-E6/E7 and ras (21). The cell line XC3 was obtained by transfection of B6MEC cells with the Ad5 E1 region (22). The cell line Ad/TYSPLNLK (aa 122–130, 130) together with Ad5E1B peptide TYSPPLNKL (aa 122–130), together with Ad5E1B. The p53koMEC (ko, knockout) cell line is a MEC line obtained from p53−/− mice. Cell line 5D) and murine colon adenocarcinoma (26). Mouse cell lines were cultured in Iscove’s modified Dulbecco’s medium (BioWhitaker, Glasgow, United Kingdom) supplemented with 8% FCS, penicillin (100 IU/ml), and 2-β-mercaptoethanol (2 × 10−5 m) at 37°C in humidified air containing 5% CO2. Targets used for anti-p53 release assay were treated with 10 units/ml murine γ-IFN (Pepro Tech; Portwood, England) for 48 h before use. Isolation and culture of the CTL clones used in this study have been described elsewhere [wtp53-specific CTL clone 1H11 (16), HPV16 E7-specific CTL clone 9.5 (27), and Ad5E1B-specific CTL clone 0.1 C2 (28)]. Adoptive Transfer of wtp53-specific CTLs. wtp53-specific CTL clone 1H11 (2 × 104) was i.v. injected in either tumor-bearing or nonchallenged p53−/+ or C57BL/6 nude mice and C57BL/6 immunocompetent mice. CTL administration was accompanied by 6 × 105 IU of rIL-2 emulsified in 50%
IFA, administered s.c. (on the day of adoptive transfer and 1 week later). Control mice received either IL-2 alone or nothing.

**Eu** Release Cytotoxicity Assay, Cytokine ELISA. Experimental procedures to measure cell-mediated cytotoxicity in a Eu release assay have been described elsewhere (29). For cytokine ELISA, supernatant of specifically restimulated T-cell cultures was harvested after 3 days. Production of γ-IFN by the T cells was measured by a sandwich ELISA performed in maxisorp plates (Nunc, Roskilde, Denmark) using antimouse-γ-IFN-specific Abs [clones R4–6A2 (capture) and biotinylated XMG1.2 (detection); Pharmingen, San Diego, CA], streptavidin-conjugated poly-horseradish peroxidase (Central Laboratory for Blood Transfusion, Amsterdam, the Netherlands), and 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (Sigma, St. Louis, MO) as a substrate. Absorbance at 415 nm was measured using kineticalc 2.12 software in an EL312e Biokinetics ELISA plate reader (Biotek Instruments, Winooski, VT).

**IHC.** Tumor cells were spun down on a slide, air-dried, permeabilized, fixed with acetone, and washed in PBS. The cells were incubated at room temperature with 15 μg of Ab pAb 122 (30) reactive against p53. The cells were then labeled with a biotinylated secondary Ab directed against one of the p53 Abs. They were subsequently incubated with a freshly prepared biotinylated horseradish peroxidase/streptavidin complex (DAKO, Glostrup, Denmark) and developed with 3,3'-diaminobenzidine, which forms a very stable brown end product. This was followed by a Mayer Heamatoxylin nuclear staining.

**Immunoprecipitations.** Cell labeling and immunoprecipitations were performed as described previously (4) with minor modifications. In brief, for each immunoprecipitation, 2 × 10^6 cells were labeled for 1 h with 500 μCi EXPRE35 S protein labeling mix (New England Nuclear) in 2 ml of methionine-free medium containing 5% of dialyzed FCS (Life Technologies, Inc.), after which cell lysates were prepared (t = 0 h), or cells were washed once with standard culture medium and chased for indicated periods (t = 1 h, t = 4 h). Before lysis, cells were washed once with PBS, after which they were lysed in 1 ml of immunoprecipitation buffer (30 mM HEPES/NaOH (pH 7.8), 140 mM NaCl, 1% Triton X-100, phenylmethylsulfonyl fluoride, and trypsin inhibitor). Immunoprecipitations were performed with protein G Sepharose (Pierce) precoated with the Abs of choice. pAb 240 and pAb 246 were less efficient in immunoprecipitating p53 from lysates than was pAb 122. This difference is most likely related to the weaker affinity of protein G for murine IgG1 subclass Ab (pAb 240, pAb 246) than for murine IgG2b subclass Ab (pAb 122; see Pierce manual). Separation of immunoprecipitates on SDS polyacrylamide gels was performed as described previously (4). The gels were analyzed using a Molecular Dynamics PhosphoImager 445 SI with Imagequant 4.2 software.

**RESULTS**

**Lysis of Tumor Cells by wtp53-specific CTLs Does Not Require High Steady-State Levels of p53.** The relation between p53 expression levels and sensitivity to lysis by wtp53-specific CTLs was

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4 Pierce Technical Manual for Immunopure Immobilized Protein G9, Rockford, IL.
investigated with a panel of murine tumor cells of C57BL/6 origin expressing different levels of p53. Expression of p53 was measured by IHC using the p53-specific Ab pAb 122 (30). Cytotoxicity assays were performed with the previously described wtp53-specific CTL clone 1H11. A–B, CTL clone 01 recognizing the AdE1B-derived epitope VNRNCY1 (15 and lightfaced lines) was a control for the lysability of the Ad5E1-transformed tumor cells. C–D, CTL clone 9.5 recognizing the HPV16E7-derived epitope RAHYNVTF (15 and lightfaced lines) was a control for the lysability of the HPV16 E6/E7-transformed tumor cells. Two Ad5E1-transformed tumor cell lines were tested: A, XC, (n = 4) and B, Ad/TYS (n = 4); and two HPV16 E6/E7-transformed tumor cell lines were tested: C, TC-1 (n = 8) and D, C3 (n = 8). n, number of independent tests performed (each test was performed in triplicate); abscissa, the E:T ratio; ordinate, percentage of specific lysis. In E, the γ-IFN secretion of wtp53-specific CTLs was measured to establish T-cell receptor-mediated recognition of tumor cells; abscissa, the different tumor cells; ordinate, the amount of γ-IFN produced.

**Fig. 2. Inverse correlation of steady-state p53 expression and recognition by wtp53-specific CTLs.**

For graphs A–D, peptide-pulsed (● and lightfaced lines) and nonpulsed (○ and lightfaced lines) p53koMEC are taken along as a reference for CTL activity. ▲ and boldfaced lines, the specific lysis of the tumor cell line by wtp53-specific CTL clone 1H11. A–B, CTL clone 01 recognizing the AdE1B-derived epitope VNRNCY1 (● and lightfaced lines) was a control for the lysability of the Ad5E1-transformed tumor cells. C–D, CTL clone 9.5 recognizing the HPV16E7-derived epitope RAHYNVTF (○ and lightfaced lines) was a control for the lysability of the HPV16 E6/E7-transformed tumor cells. Two Ad5E1-transformed tumor cell lines were tested: A, XC, (n = 4) and B, Ad/TYS (n = 4); and two HPV16 E6/E7-transformed tumor cell lines were tested: C, TC-1 (n = 8) and D, C3 (n = 8). n, number of independent tests performed (each test was performed in triplicate); abscissa, the E:T ratio; ordinate, percentage of specific lysis. In E, the γ-IFN secretion of wtp53-specific CTLs was measured to establish T-cell receptor-mediated recognition of tumor cells; abscissa, the different tumor cells; ordinate, the amount of γ-IFN produced.

Inversed Relationship between p53 Steady-State Levels and Recognition by wtp53-Specific CTLs. The relationship between p53 overexpression and sensitivity for wtp53-specific CTLs was further addressed by analyzing the recognition of Ad5E1 and HPV16 E6/E7-transformed tumor cells. We selected these cells because wtp53 levels and turnover are known to be strongly modulated by the oncoproteins expressed. The Ad5E1B protein sequesters and stabilizes p53 (4), whereas the HPV16E6 protein drives the proteasome-dependent degradation of p53 (10). In accordance with previous reports, we found that p53 steady-state levels were very high in Ad5-transformed cells (Fig. 2, A and B) and very low in HPV16-transformed cells (Fig. 2, C and D). Intriguingly, the sensitivity to lysis by p53-specific CTLs did not correlate with p53 expression levels, in that the HPV16-transformed cells were lysed more efficiently (Fig. 2, C and D) than their Ad5-transformed counterparts (Fig. 2, A and B). The poor sensitivity of the Ad5-transformed cells was not attributable to overall resistance to CTL lysis, inasmuch as these cells were efficiently lysed by Ad5E1B-specific CTLs (Fig. 2, A and B) as well as by p53-specific CTLs when exogenously loaded with the relevant peptide epitope (not shown). To confirm that T-cell receptor triggering, rather than overall sensitivity of targets to lysis, dominated the outcome of our experiments, we tested the secretion of γ-IFN (31) by the p53-specific CTLs on incubation with HPV16- or Ad5-transformed cells. Also in this assay, the p53-specific CTLs are better triggered by the HPV16-transformed cells despite the fact that steady-state p53 levels in these cells are very low (Fig. 2E). Because in Ad5-transformed cells the p53 protein is reported to be very stable, whereas in HPV16-transformed cells p53 was shown to be rapidly degraded, our data suggest that degradation rate rather than steady-state levels determines whether these cells constitute good targets for p53-specific CTLs.

**Turnover of p53 Is an Important Factor in Determining the Sensitivity of Tumor Cells to wtp53-Specific CTLs.** To investigate the relation between CTL recognition versus p53 synthesis and deg-
radiation rates in more detail, p53 was immunoprecipitated from pulse-chase labeled lysates of several cell lines. Immunoprecipitation of p53 using pAb 122 from pulse-labeled cells (Fig. 3; t = 0 h) revealed that all of the cells expressed p53, but that the synthesis rates of p53 differed greatly between these cells. Fig. 3 shows different exposures of the same gel (see Fig. 3 legend for details). The p53 + H-ras-transformed cells, which were generated through transfection of a mutant p53 gene, showed, by far, the highest p53 synthesis rates. MC38, FBL-3, and EL-4 synthesized intermediate amounts of p53, whereas synthesis rates were very low in Ad5- and HPV16-transformed cells. Therefore, the turnover rates of p53 in the different cells are in accordance with the wild-type and/or mutant forms of p53 detected in these cells. Furthermore, the immunoprecipitation data can account for the steady-state levels of p53 as detected by IHC (Figs. 1 and 2). The cell lines with low p53 turnover rates accumulate high levels of intracellular p53, whereas cell lines with high p53 turnover show very little p53 in IHC (Table 1). From this summary of the data, it can also be seen that recognition of the cells by p53-specific CTLs does not show strong correlation with p53 steady-state levels, as exemplified by the observation that Ad5-transformed cells are poorly recognized. Instead, Table 1 reveals that these cells express mutant p53. Estimated overall p53 half-life in these cells is 4 h. It is highly conceivable that the decrease in p53 in these cells is attributable to turnover of the p53 fraction that exhibits the wild-type conformation, as previously reported for other cell lines in which these two forms of p53 coexists (34). Taken together, the turnover rates of p53 in the different cells are in accordance with the wild-type and/or mutant forms of p53 detected in these cells. Furthermore, the immunoprecipitation data can account for the steady-state levels of p53 as detected by IHC (Figs. 1 and 2). The cell lines with low p53 turnover rates accumulate high levels of intracellular p53, whereas cell lines with high p53 turnover show very little p53 in IHC (Table 1). From this summary of the data, it can also be seen that recognition of the cells by p53-specific CTLs does not show strong correlation with p53 steady-state levels, as exemplified by the observation that Ad5-transformed cells are poorly recognized. Neither does CTL recognition require elevated p53 synthesis rates, because HPV16 MEC, FBL-3, and EL-4 are efficiently recognized despite modest p53 synthesis levels. Instead, Table 1 reveals that these CTLs especially recognize target cells that display relatively high p53 turnover rates (HPV16 MEC, FBL-3, and EL-4), or cells that combine increased p53 half-life with increased p53 synthesis levels. This is particularly true for p53 + H-ras MEC and, to a lesser extent, for MC38. In conclusion, elevated synthesis and/or steady-state levels of p53 are not a prerequisite for recognition of tumor cells by p53-specific CTLs, whereas high p53 turnover is an important factor determining sensitivity of tumor cells to p53-specific CTLs.
Eradication of Established Tumors with No or Moderate p53 Expression by wtp53-specific CTLs. We have previously demonstrated that wtp53-specific CTLs can control the outgrowth of p53-overexpressing tumors (cell lines 4J, 5D) in p53+/+ mice (18). The in vitro cytolytic assays (Figs. 1 and 2) suggest that the in vivo efficacy of these CTLs may not be limited to tumor cells that express greatly elevated levels of p53. We, therefore, challenged mice with tumor cells expressing moderately increased (MC38) or barely detectable steady-state levels (FBL3) of p53 (Fig. 1, D and E), and analyzed whether treatment of mice with adoptively transferred anti-wtp53 CTLs and rIL-2 could prevent the outgrowth of these tumors. In accordance with our observation that the CTLs can lyse both of these tumor cells in vitro (Fig. 1, D and E), administration of these CTLs resulted in tumor-free survival of the majority of the mice challenged with either of the tumor cells (Figs. 4 and 5). These results imply that CTL-mediated immunotherapy that targets p53-derived epitopes can be applied to tumors displaying a wide range of p53 expression levels.

### DISCUSSION

Although previous reports suggested that CTL-mediated immunotherapy targeting p53 would be primarily applicable to tumors that express greatly enhanced levels of p53, the data presented in this report indicate that p53-specific CTLs can eliminate tumors expressing a wide range of p53 levels. Our study, furthermore, shows that p53 turnover, rather than synthesis or steady-state levels, constitutes an important factor in determining the sensitivity of tumor cells for killing by wtp53-specific CTLs. Degradation of p53 has been examined in detail and is known to occur in a ubiquitin-dependent fashion via the proteasomes (35). A key mediator in p53 turnover is the protein mdm2, which associates with p53 and in this manner targets p53 for ubiquitin-dependent degradation. mdm2 expression is up-regulated at the transcriptional level by wtp53, thereby providing an efficient autoregulatory feedback loop on p53 expression. Degradation of p53 is strongly accelerated in HPV16-transformed cells because of the fact that the HPV16 E6/E6-AP complex functions as an additional ubiquitin-protein ligase in the ubiquitination of p53, thereby enhancing proteasome-mediated degradation of p53 (10). In contrast, p53 degradation is inhibited in Ad5-transformed cells because the Ad5E1B 55 kDa oncoprotein sequesters wtp53 into a highly compact complex (4), preventing it from inducing the expression of mdm2 (36) and, in addition, removing it from the pool of proteins available for ubiquitin/proteasome-mediated degradation. In accordance with these previously published observations, we found that Ad5-transformed cells were poorly recognized by p53-specific CTLs, despite their impressive steady-state levels of p53, whereas HPV16-transformed cells, which display barely detectable p53 levels, were efficiently recognized. This inverse correlation between p53 steady-state levels and sensitivity for p53-specific CTLs constitutes the ultimate illustration that: (a) high p53 expression neither is a prerequisite, nor holds a guarantee for recognition of tumors by p53-specific CTLs; whereas (b) a high p53 turnover rate does strongly correlate with CTL recognition. This can be explained by the aforementioned involvement of the proteasome complex in p53 degradation, which links this process to the processing of p53-derived epitopes into class I MHC.

In addition to HPV16-transformed cells, several other tumors (MC38, FBL-3, EL-4) expressing moderate-to-low levels of p53 were efficiently recognized by the wtp53-specific CTLs. Of these, FBL-3 and EL-4 also show high p53 turnover rates, which supports our notion that this parameter is of a high predictive value with respect to the recognition by p53-specific CTLs. The situation with MC38 is more complicated in that this tumor, like the p53-transfected tumors 41 and 5D, features a combination of increased p53 synthesis with increased p53 stability. Apparently, this setting also feeds sufficient p53 into the proteasome degradation pathway. Taken together, our data argue that overexpression of p53 can elevate, but does not necessarily result in an elevated presentation of, p53-derived peptides in surface MHC class I molecules. On the other hand, modest and even low steady-state p53 levels can suffice to sensitize tumors for immune attack by wtp53-specific CTLs, provided that the p53 turnover rates are high. Ultimately, techniques establishing the amount of...
p53-derived peptides presented by MHC class I molecules, like peptide/MHC-specific Abs (37), will determine whether tumors constitute good targets for p53-directed CTL-mediated immunotherapy. However, these techniques are currently not available. We conclude that determination of p53 steady-state expression levels in tumors, as analyzed with IHC, is a valuable diagnostic tool, but that caution should be taken to use p53 expression as an inclusion criterion for clinical immune intervention protocols that target p53 because patients who might benefit from such an intervention might otherwise be excluded.

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High Steady-State Levels of p53 Are Not a Prerequisite for Tumor Eradication by Wild-Type p53-specific Cytotoxic T Lymphocytes

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