Cancer Research

Hyperthermia Selectively Targets Human Papillomavirus in Cervical Tumors via p53-Dependent Apoptosis

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Abstract

Human papillomavirus (HPV) is associated with cervical cancer, the third most common cancer in women. The high-risk HPV types 16 and 18 are found in over 70% of cervical cancers and produce the oncoprotein, early protein 6 (E6), which binds to p53 and mediates its ubiquitination and degradation. Targeting E6 has been shown to be a promising treatment option to eliminate HPV-positive tumor cells. In addition, combined hyperthermia with radiation is a very effective treatment strategy for cervical cancer. In this study, we examined the effect of hyperthermia on HPV-positive cells using cervical cancer cell lines infected with HPV 16 and 18, *in vivo* tumor models, and *ex vivo*–treated patient biopsies. Strikingly, we demonstrate that a clinically relevant hyperthermia temperature of 42°C for 1 hour resulted in E6 degradation, thereby preventing the formation of the E6–p53 complex and enabling p53-dependent apoptosis and G_2-phase arrest. Moreover, hyperthermia combined with p53 depletion restored both the cell-cycle distribution and apoptosis to control levels. Collectively, our findings provide new insights into the treatment of HPV-positive cervical cancer and suggest that hyperthermia therapy could improve patient outcomes. *Cancer Res; 75(23); 5120–9. ©2015 AACR.*

Introduction

Human papillomavirus (HPV)–associated cancers are responsible for approximately 5% of all tumors worldwide (1). Over 100 different HPV types have been identified, although only a few are oncogenic. These high-risk HPVs, such as types 16 and 18, can develop precancerous lesions. If these lesions remain untreated, they can process into cancer. HPV is associated with several types of cancers, such as carcinoma of the head and neck, anus, vagina, vulva, and penis (2–4). HPV is particularly notorious for causing cervical cancer, which is the third most common cancer in women (www.cancer.gov) and the second most frequent cause of cancer-related death in women (5). The high-risk HPV types 16 and 18 are found in over 70% of cervical cancers (6–8).

The standard treatment for cervical carcinoma is radical surgery and/or concurrent cisplatin chemoradiotherapy. Hyperthermia is clinically applied as concomitant therapy with either radiotherapy or chemotherapy, particularly as an alternative treatment if cisplatin-based chemotherapy is contraindicated or for recurrent cancers in a previously irradiated area (9). Hyperthermia is given simultaneously with cisplatin-based chemotherapy and shortly before or after radiotherapy. Several phase III studies on cervical cancer have shown a significantly better overall survival after radiotherapy combined with hyperthermia compared with radiotherapy alone (10–13). Because a high percentage of cervical tumors are HPV infected, we examined the response on HPV-positive cells to hyperthermia.

The high-risk HPV types 16 and 18 both produce early protein 6 (E6), which binds to the tumor suppressor protein p53 (14–18). E6 mediates ubiquitination of p53 and as a result targets p53 as well as itself for proteasomal degradation protein complex (14, 19). As a consequence, both p53-induced cell-cycle arrest and apoptosis are abrogated (20). Therefore, E6 might be an interesting target in cancer therapies (21, 22). In this study, we describe that hyperthermia interferes in this degradation process and allows p53 to escape proteasomal degradation, reactivating its tumor-suppressive capacities.

Materials and Methods

Cell culture

The cervical carcinoma cell lines: SiHa, HeLa, C33A, Caski, C4I, and HT3; the human prostate carcinoma cells lines: Du145, LNCaP, and PC3 were all obtained from the American Type Culture Collection. Colon carcinoma cell lines RKO, RC10.1, and RC10.2 were kindly provided by Dr. Kathleen Cho, University of Michigan (Ann Arbor, MI), and HCT116 p53wt and p53null were obtained from Horizon Discovery, Cambridge, UK. All cell lines were passed for fewer than 6 months after receipt, except for RKO, RC10.1, and RC10.2. Their p53 status has been tested regularly using Western blots. Human cervical carcinoma cells SiHa (HPV 16-infected), HeLa (HPV 18-infected), and C33A cells

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(HPV-negative) were grown in Media Modified Eagle's Medium (BioWhittaker/Lonza). HPV 16–infected cervical carcinoma Caski cells were grown in RPMI-1640 (Gibco-Brl Life Technologies). HPV-negative human cervical carcinoma cell line C4I was grown in Waymouth's medium (Gibco-Brl Life Technologies). HPV-negative human cervical carcinoma cells line HT3, colon carcinoma cell lines HCT116 p53wt and p53null, RKO, RC10.1, and RC10.2 were grown in McCoy's 5a (Gibco-Brl Life Technologies). All media contained 25 mmol/L Hepes (Gibco-Brl Life Technologies) supplemented with 10% heat-inactivated FBS and 2 mmol/L glutamine. Cells were maintained at 37°C in an incubator with humidified air supplemented with 5% CO_2. The doubling time of these cells during exponential growth is approximately 24 to 60 hours.

Xenograft tumors

Human SiHa cells were injected into the hind leg of athymic mice. When tumors had grown to a volume of about 100 mm^3, mice were treated with or without hyperthermia. The animals were cooled, which prevented an increase of the body core temperature. During all treatments, the animals were anesthetized with a mixture of 2.5% isoflurane in oxygen. Directly after treatment mice were sacrificed and the tumors were taken out, both untreated and treated tumors were prepared for either Western blotting or immunofluorescence.

Animal experiments were approved by the animal welfare committee of the Academic Medical Center as required by Dutch law LEX102767.

Patient biopsies

Cervical carcinoma (AMC/MEC 03/137) biopsies of patients were divided into two parts, one half was left untreated, whereas the other half was treated *ex vivo* with hyperthermia. Four biopsies were sliced and sonificated into cell suspensions to perform Western blotting. Three biopsies were submerged in paraformaldehyde, to be used for paraffin coupes.

Hyperthermia

Hyperthermia of the cells was performed by submerging the culture dishes in a thermostatically controlled water bath (Lauda aqualine AL12, Beun de Ronde) for 1 hour at 40, 41, or 42°C and 30 minutes at 42°C. Temperature was checked in parallel dishes, and the desired temperature (±0.1°C) was reached in approximately 5 minutes. The atmosphere was adjustable by a connection with air and CO_2 supplies. All cell cultures were heated in a 5% CO_2/95% air atmosphere and air inflow of 2 L/min.

Hyperthermia of mouse tumors was performed by submerging the tumor-bearing hind leg in a thermostatically controlled water bath for 1 hours at 42.7°C, resulting in a tumor temperature of 42°C, as confirmed by thermocouple measurements at the tumor surface. Hyperthermia (1 hour at 42°C) of patient biopsies was performed in culture dishes in the same thermostatically controlled water bath as described for the *in vitro* cultures.

Radiation treatment

Cells were irradiated with or without prior hyperthermia treatment. In order to allow observation of an additional effect of hyperthermia, all radiation treatments were performed with single dose (4 Gy) of gamma rays from a ^{137}Cs source at a dose rate of about 0.5 Gy/min. For survival curves, cells were irradiated with single doses up to 8 Gy.

Inhibitors

Proteasomal degradation was tested using an inhibitor (MG132, 10 μmol/L for 1 hour; Sigma). For lysosomal degradation, a lysosomal inhibitor, chloroquine (100 μmol/L for 16 hours; Sigma), was used and a second compound inhibiting fusion between autophagosomes and lysosomes, so called bafilomycin A1 (200 nmol/L for 4 hours; Sigma). To check the effect of hyperthermia on the p53–E6 interaction, an E6-siRNA (Santa Cruz Biotechnology) transfection was performed. Furthermore, cells were transfected with p53-siRNA and scrambled p53-siRNA (Cell Signaling Technology) prior to hyperthermia in order to study the p53-dependent apoptosis. Cells were also incubated with MG132 in order to investigate the localization of E6 after radiation. To test whether the accumulated p53 was produced *de novo*, cycloheximide (1 μmol/L for 1 hour; Sigma-Aldrich) was used, which prevents translation from taking place.

Survival assays

Clonogenic assays were conducted to investigate the radiosensitization of hyperthermia. Experiments were performed in HPV 16 and 18–positive cervical cancer cell lines. Cells were plated before treatment into six-well culture plates (Costar). Dishes were placed in an incubator with 5% CO_2 at 37°C until sufficiently large colonies were formed. Afterwards, the medium was removed and cells were washed with PBS. A mixture of 6.0% glutaraldehyde and 0.5% crystal violet was added for at least 30 minutes at room temperature (20°C). After removing the mixture of glutaraldehyde and crystal violet, plates were washed with water and eventually dishes were dried in normal air at 20°C. Colonies were counted under a light microscope. Survival fractions were calculated by dividing the plating efficiency of treated cells by that of control cells ± SEM (23, 24).

Surviving fractions after dose D, [S(D)/S(0)], were corrected for the cytotoxicity of hyperthermia alone, and survival curves were analyzed to calculate values of the linear and quadratic parameters α and β, using SPSS 14.0 statistical software by means of a fit of the data by weighted linear regression, according to the linear-quadratic formula: $\mathrm{Ln}(S(D)/S(0)) = -(\alpha D + \beta D^2)$ (25–28). Data on clonogenic assays and apoptosis were analyzed using a *t* test.

Western blotting

To understand the additional effect of hyperthermia to radiation on HPV-positive tumors, Western blots were carried out to study p53 levels. Controls and treated cells were harvested 4 hours after treatment. Pellets were lysed in ice-cold RIPA buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin) for 30 minutes on ice with protein inhibitors (29). Laemmli buffer with 2-mercaptoethanol (355 mmol/L) was added to the supernatant (1:1) and heated in boiling water for 2 to 5 minutes. Finally, samples were sonificated (Sonics & Materials Inc.). One microgram of protein was resolved by 10% SDS-PAGE precast gels (BioRad) and transferred to PVDF membranes. Equal protein loading was checked by Ponceau S staining (29). Immunodetection was performed for p53 mAb (Dako) in combination with a horseradish peroxidase–conjugated secondary anti-mouse IgG (Southern Biotech). Housekeeping protein ERK2 was detected using mAb (Bethyl Laboratories) and a secondary anti-rabbit mAb (Invitrogen Life Technologies). All samples were enhanced using chemoluminescence (Amersham Pharmacia

Biotech). Finally, blots were analyzed using LAS4000 (GE, Healthcare Life Sciences).

Cell cycle and apoptosis analyses

The Nicoletti assay (30) is another way to study p53 functionality. Apoptosis was studied in all cell lines using this assay. At 48 hours after treatment (p53-siRNA with or without hyperthermia), cells were collected and pellets were resuspended in Nicoletti buffer (0.1% w/v Sodium citrate, 0.1% v/v Triton X-100 in demi water, pH 7.4). We have also performed additional apoptotic assays (cleaved caspase-3 and Annexin V) on SiHa and HeLa cells. Analyses were made using flow cytometry (FACS Canto; BD Biosciences). Statistical analysis was performed using a *t* test.

Immunocytochemistry and immunohistochemistry

SiHa and HeLa cells were grown on coverslips. Inhibitors chloroquine, bafilomycin A1, and MG132 were added to study the breakdown of p53 and E6, in normal conditions and after hyperthermia. Also, a transfection with HPV 16/18-E6-siRNA was performed to investigate whether the same results were found after hyperthermia and a specific E6-siRNA transfection. Afterwards, cells were fixed with 4% paraformaldehyde for 20 minutes on ice. Before blocking cells (PBS containing 0.1% triton x-100 and 5% normal goat serum), cells were washed with PBS. Cells were incubated overnight at 4°C in HPV 16/18-E6 protein (Santa Cruz Biotechnology) or p53 mAb (Transduction Labs). The next day, samples were washed and incubated for 1 hour at room temperature with a secondary antibody Alexa Fluor 488 (Invitrogen Life Technologies), washed and incubated for 10 min in PBS containing 1 μg/mL DAPI (Sigma-Aldrich). After washing, coverslips were stuck to slides using ProLong Gold anti-fade reagent (Invitrogen Life Technologies).

Paraffin-embedded coupes were made from xenograft tumors and patient biopsies. They were deparaffinized and rehydrated. Afterwards, a heat-induced antigen retrieval at pH 9.0 for 20 minutes was performed, followed by a 30-minute cooling period. Next, a 15-minute PO block including H_2O_2 was performed. Then coupes were incubated overnight at 4°C with HPV 16/18-E6 protein or p53 mAb (Transduction Labs). Next, tissue was embedded in Alexa Fluor 488 (Invitrogen Life Technologies), after washing in PBS. DAPI was used to stain the nuclei blue before covering tissue with ProLong Gold antifade reagent (Invitrogen Life Technologies) and a coverslip.

In order to study the cleaved caspase-3 in immunohistochemistry stainings, the heat-induced antigen retrieval was performed at pH 6.0. Primary antibody cleaved caspase-3 antibody (Cell Signaling Technology) was diluted in Primary antibody dilution (KliniPath) and incubated overnight at 4°C. Afterwards, tissue was embedded in Powervision Poly-HRP-GAM/R/R IgG (Immunologic, Immunovision Technologies). For counterstaining Eosin-hematoxyline (Fluka) was used, before covering tissue in mounting solution (Pertex) and a coverslip.

Results

Restoration of p53 after hyperthermia

To investigate changes in p53 levels after radiation and after hyperthermia, Western blots and immunocytochemistry stainings were performed. Highly elevated levels of p53 were found in SiHa and HeLa cells after 1 hour of hyperthermia at 42°C and after a combinational treatment of hyperthermia and radiation, but not after a treatment of radiation alone. A temperature of 42°C is required to achieve a significant increase of p53 and either 40°C or 41°C was not sufficient to cause induction of p53 (Fig. 1A). The effectiveness of radiation, hyperthermia, and their combination on p53 levels is tested on HPV-positive (Supplementary Fig. S1A) and HPV-negative cells (Supplementary Fig. S1B). In HPV-positive cells, a treatment including hyperthermia was required to induce p53, whereas radiation alone was sufficient in upregulation p53 levels in HPV-negative p53wt cells. The HPV-negative p53mut or p53null cells did not show any upregulation of p53. Accumulation of p53 was observed immediately after hyperthermia (at 42°C for 1 hour) and lasted until 4 hours after treatment (Fig. 1B).

In immunocytochemistry staining, p53 was only seen after hyperthermia or after the combinational treatment of radiation and hyperthermia (Fig. 1C), p53 was neither observed in controls nor after radiation treatment only. Moreover, *in vivo*–treated tumors of SiHa cells grown in athymic mice and *ex vivo*–treated patient biopsies show a p53 induction after a 60-minute *in vivo* treatment with 42°C (Fig. 1D–G). Results of more patients are shown in Supplementary Fig. S1C and S1D. Quantification of immunofluorescence staining is presented in Supplementary Fig. S1E.

Hyperthermia causes E6 downregulation

Protein E6, produced by human papillomavirus 16 and 18, is present in the untreated SiHa and HeLa, as shown with immunocytochemistry (Fig. 2A and B), whereas E6 has disappeared immediately after hyperthermia (42°C for 1 hour). In order to test the role of E6 in HPV-infected cells after hyperthermia, an E6-siRNA transfection was used. After transfection with E6-siRNA, neither E6 nor p53 is observed in immunocytochemistry staining. Next, cells were transfected with E6-siRNA and irradiated (4 Gy). Again, there was no E6 measurable, but now an accumulation of p53 was observed, which suggests that hyperthermia also causes stress to the cells that induce p53. Similar results were obtained with Western blots examination as shown in Supplementary Fig. S2A.

In SiHa cells grown in athymic mice, E6 was present in the cells of untreated tumors, but no E6 was detected after treatment with hyperthermia (Fig. 2C). Furthermore, after *ex vivo* treatment of patient biopsies, E6 disappeared after hyperthermia treatment, which was similar to the outcomes of the cell and xenograft experiments (Fig. 2D and Supplementary Fig. S2B and S2C).

Cells were also treated for 30 minutes at 42°C to understand the time needed before hyperthermia is fully effective on both E6 and p53 (Supplementary Fig. S2D). In the untreated situation, all cells were E6 positive, whereas none of the cells express p53. After a 30-minute treatment, only a few cells were completely positive for p53, and E6 expression is lower compared with the control. This shows that a 30-minute treatment is not sufficient and 60-minute treatment at 42°C is needed to induce p53 in all cells and to degrade E6 completely. Quantification of immunofluorescence staining is presented in Supplementary Fig. S2E.

Besides functional p53, degradation of p53 still occurs mainly via the proteasomal pathway

To examine whether p53 accumulation is newly produced or due to interference with the degradation, cells were treated with 1 μmol/L cycloheximide for 1 hour. This did not result in a p53

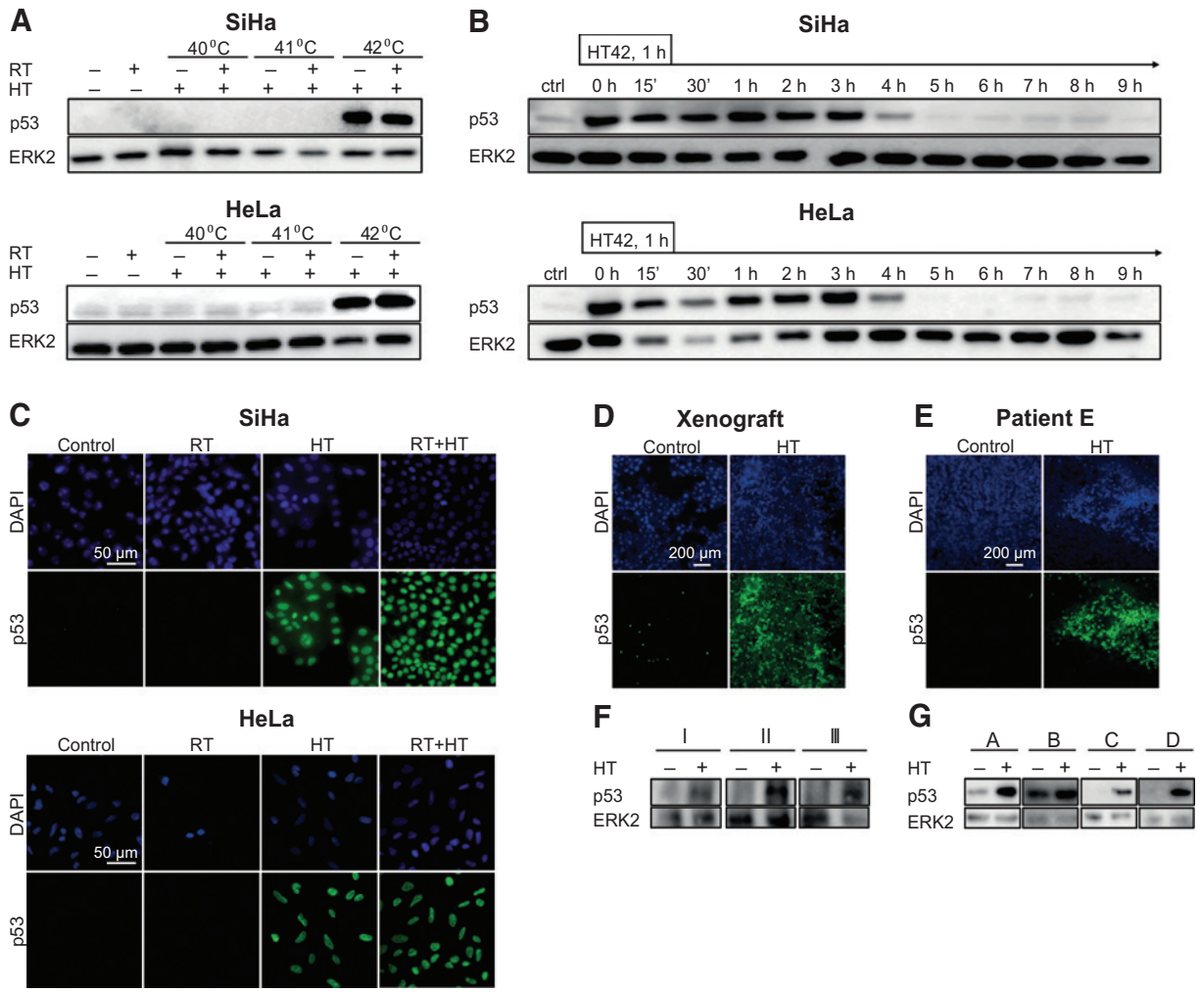

Figure 1.

HPV-positive cells are sensitive to hyperthermia (42°C for 60 minutes). A, Western blot analyses of p53 in cervical cancer cells are shown. SiHa and HeLa cells were treated with or without hyperthermia prior to radiation (4 Gy). Accumulation of p53 was only observed after a 60-minute treatment with a hyperthermia temperature of 42°C. B, the induction of p53 started immediately after hyperthermia and remained up to 4 hours after treatment. C, immunocytochemistry staining of DAPI (blue) and p53 (green) is presented for different conditions. Accumulation of p53 was observed after hyperthermia (HT) and radiation (RT) combined with hyperthermia, not after radiation alone. D and E, in SiHa cells grown in athymic mice (D) and in patient biopsies (E); p53 is accumulated after hyperthermia. F and G, the upregulation of p53 after hyperthermia is also observed in Western blots for three different xenografts (I, II, and III) and four *ex vivo*–treated patient biopsies (indicated with A, B, C, and D). HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia.

accumulation, whereas treatment with 10 μmol/L MG132 for 1 hour did show p53 accumulation (Fig. 3A). It was concluded that p53 is produced *de novo* after a hyperthermia treatment at 42°C for 60 minutes.

To gain insight into degradation of E6 and p53 after hyperthermia, cells were treated after incubation with a proteasomal inhibitor (MG132) or a lysosomal inhibitor (chloroquine). In untreated conditions, no p53 is detected in HPV-positive cells. After blocking the lysosome using chloroquine, no difference was observed using Western blot, but after blocking the proteasome using MG132, a substantial increase of p53 was observed. Compared with blocking of the proteasome alone, combined blocking of both lysosome and proteasome showed a similar increase in p53. After a 60-minute treatment at 42°C, the lysosomal inhibition with chloroquine resulted in a slight increase in the levels of p53. However, this effect was much more pronounced when the activity of the proteasomal pathway was blocked, indicating that the degradation of p53 is to a large extent dependent on the proteasome (Fig. 3B).

In order to study the localization of p53 within the cells, the proteasome was blocked using an inhibitor (MG132) to prevent degradation. After hyperthermia p53 is only present in the nucleus, whereas after radiotherapy p53 is also present in the cytoplasm (Fig. 3C and D, top).

After studying localization of p53, the localization of E6 has been investigated as well. As we cannot detect E6 after hyperthermia, a proteasomal inhibitor (MG132) was used before treating the cells with hyperthermia or radiation. After both radiation and hyperthermia treatment, in the presence of MG132, E6 is localized in the cytoplasm and nucleus (Fig. 3C and D, bottom).

To understand the interaction of E6 and p53 after hyperthermia, immunocytochemistry stainings were performed using both

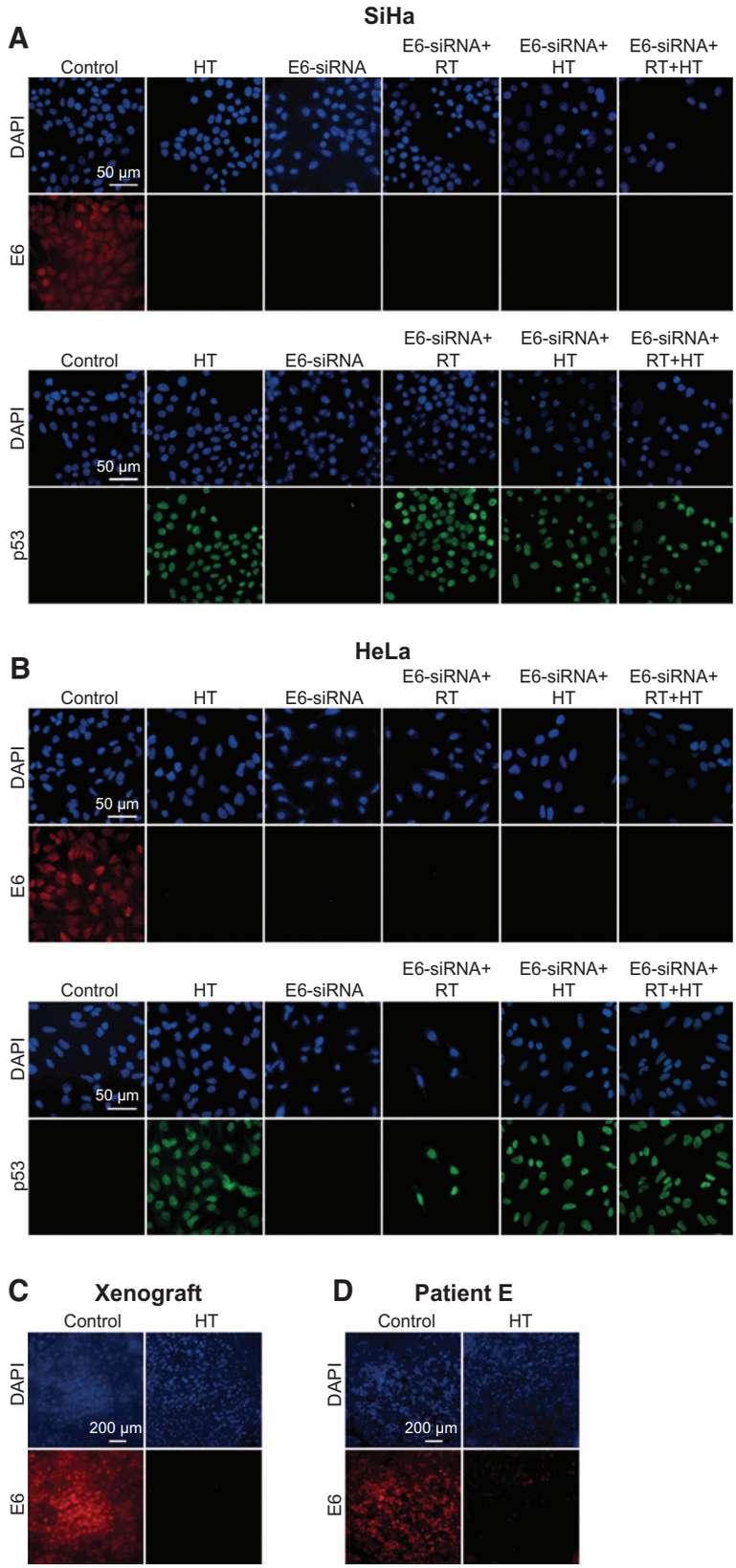

Figure 2.
E6 downregulation after hyperthermia (42°C for 60 minutes). A and B, immunocytochemistry staining of DAPI (blue), E6 (red), and p53 (green) is shown for different conditions on SiHa (A) and HeLa (B) cells. Untreated cells were positive for E6, but negative for p53. After hyperthermia accumulation of p53 was observed, there was no E6 anymore. After transfection with E6-siRNA, neither E6 nor p53 was observed. Any treatment after an E6-siRNA transfection causes induction of p53. C and D, after hyperthermia (HT), there was no E6 present in xenograft (C) and in a patient biopsy (D). HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia.

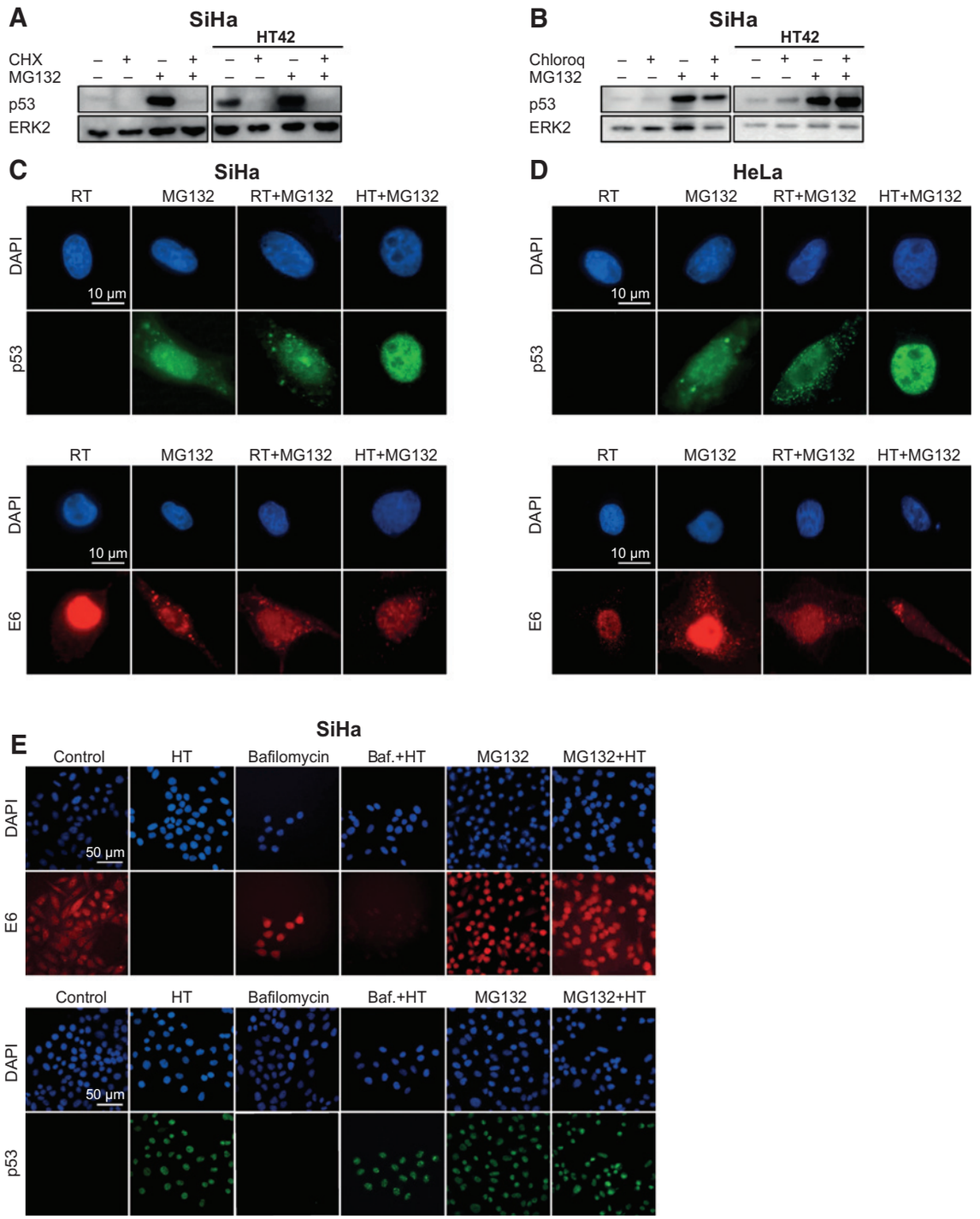

Figure 3.

De novo production of p53 and degradation of E6 after hyperthermia. A, Western blot analysis of p53 in SiHa cells. Treatment with 1 μmol/L cycloheximide (CHX) for 1 hour did not result in a p53 induction, whereas treatment with 10 μmol/L of a proteasomal inhibitor MG132 did; in combination with hyperthermia, similar results were found. B, without hyperthermia, p53 is degraded exclusively via the proteasomal pathway. After hyperthermia, accumulation of p53 was observed. After blocking the lysosome (chloroquine), no increase of p53 was observed, whereas an extremely higher increase of p53 was seen after blocking the proteasome (MG132). No significant differences were detected using ImageJ after blocking both the proteasome and lysosome prior to hyperthermia compared with blockage of the proteasome alone prior to hyperthermia, indicating E6 is degraded via the proteasomal pathway. C and D, immunofluorescence staining of DAPI (blue), E6 (red), and p53 (green) is shown for different conditions. To visualize p53 after radiation (RT), a proteasomal inhibitor (MG132) was used prior to radiation. Comparing p53 levels after hyperthermia and radiation, p53 was present in the nucleus after hyperthermia, while p53 was also present in the cytoplasm after radiation. E, after incubation with a proteasomal inhibitor (MG132), high levels of p53 were observed, whereas there was no p53 seen after incubation with the lysosomal inhibitor (bafilomycin) in SiHa cells. HT, hyperthermia (42°C for 60 minutes).

lysosomal and proteasomal inhibitors. In untreated SiHa cells, E6 was present in control, and also after blocking the lysosome (bafilomycin) and after blocking the proteasome (MG132). However, p53 was absent both in control and after blocking the lysosome, whereas p53 was detected after blocking the proteasome, showing p53 is only degraded via the proteasome after complex formation with E6. Both E6 and p53 were present after treating cells with hyperthermia before blocking either the lysosome or proteasome (Fig. 3E and Supplementary Fig. S3A), suggesting that after hyperthermia E6 is degraded via both the lysosomal and proteasomal pathways. Besides functional p53, it is observed that some p53 is still degraded via the proteasomal pathway.

Moreover, conditions containing components of the standard treatment (radiation with platin-based chemotherapy) were tested. Cisplatin (cDDP) or standard chemoradiation (cDDP+RT) did not result in an accumulation of p53 compared with untreated controls (Supplementary Fig. S3B). A treatment with hyperthermia was necessary to induce p53. Quantification of immunocytochemistry staining is presented in Supplementary Fig. S3C.

Accumulation of p53 results in p53-dependent apoptosis and G_2 arrest

To test whether the accumulated p53 is functional, apoptosis was tested by the Nicoletti assay. About 6% of the untreated SiHa and HeLa cells were apoptotic (Fig. 4A). No difference was observed after radiation or after transfection of p53-siRNA prior to hyperthermia. Both after hyperthermia and after hyperthermia combined with radiation, a significant increase in apoptosis was observed. The effects of more treatments and treatment combinations on apoptosis in SiHa and HeLa cells are summarized in Supplementary Fig. S4A. Additional HPV-positive cell lines (Supplementary Fig. S4B) show similar results as were found in SiHa and HeLa cells: induction of apoptosis occurs only if hyperthermia is part of the treatment. In the p53wt HPV-negative cell lines, radiation alone already caused a significant increase in apoptosis (Supplementary Fig. S4C), whereas there was no apoptosis observed in the p53mut or p53null cell lines. Cleaved caspase-3 immunohistochemistry staining has been performed on paraffin-embedded coupes to test the apoptotic induction in xenograft and patient biopsies. After hyperthermia, there is a clear upregulation of the cleaved caspase-3, indicating that apoptosis is induced (Fig. 4B).

Untreated SiHa cells showed a cell percentile distribution of 62.6 ± 3.1 in G_0–G_1, 15.8 ± 1.1 in S, and 26.0 ± 0.5 in G_2. After hyperthermia, a clear G_2-phase arrest is observed as the percentages change to 44.2 ± 1.2 in G_0–G_1, 11.0 ± 0.6 in S, and 49.1 ± 0.7 in G_2–M. Using p53-siRNA prior to hyperthermia, the G_2 arrest is abrogated again as the distribution of cell phases almost returned to control level, showing 65.6 ± 1.0 in G_0–G_1, 13.5 ± 0.6 in S, and 25.9 ± 1.2 in G_2–M. Similar results were found in HeLa cells: in untreated conditions, 65.2 ± 1.6 in G_0–G_1, 12.1 ± 1.2 in S, and 22.7 ± 0.4 in G_2–M; after hyperthermia, 44.0 ± 1.2 in G_0–G_1, 14.6 ± 0.2 in S, and 41.4 ± 1.3 in G_2–M. After transfection with p53-siRNA and hyperthermia, cell distribution almost returned to control levels 66.4 ± 0.5 in G_0–G_1, 11.5 ± 0.6 in S, and 22.2 ± 1.2 in G_2–M. These data in combination with more HPV-positive cell lines are shown in Supplementary Table SI. The effectiveness of hyperthermia or hyperthermia and radiation on HPV-negative cell lines is presented in Supplementary Table SII. Histograms of cell cycle show a clear G_2 arrest and induction of apoptosis after hyperthermia (Fig. 4C). Additional apoptotic assays performed on SiHa and HeLa (cleaved caspase-3 and Annexin V staining) confirm results found by the Nicoletti assay, only apoptosis induction after a treatment including hyperthermia (Supplementary Fig. S4D and S4E). In these figures, the functionality of p53-siRNA was demonstrated as well. Transfection with scrambled p53-siRNA prior to hyperthermia caused apoptosis, whereas the normal p53-siRNA prior to hyperthermia was able to prevent the induction of p53. This has been confirmed using Western blot (Supplementary Fig. S4F).

Cell survival of HPV-positive cells was tested using a clonogenic assay (24). The survival curves are shown in Fig. 4D. All cell lines show a consistently significant lower survival after radiotherapy combined with hyperthermia compared with radiation alone. This hyperthermic sensitization is shown for clinically relevant radiation doses. The corresponding α- and β values of the linear-quadratic analysis can be found in Supplementary Table SIII, showing an impressive enhancement of the linear parameter, α.

Discussion

In this study, we aimed to elucidate the cellular mechanisms that may explain why hyperthermia is particularly clinically effective in HPV-positive cancers. In HPV-positive carcinoma cells, E6 binds and ubiquitinates p53, after which this complex is degraded via the proteasomal pathway (14, 15, 17, 18). As a consequence, neither p53-dependent G_2 arrest nor apoptosis can be induced. We found that hyperthermia at 42°C for a duration of 60 minutes abrogates the interaction of p53 with E6, resulting in a major accumulation of p53. This was demonstrated in multiple HPV-positive cell lines, both *in vitro* and in xenografts and in patient cervical carcinoma biopsies. We found that hyperthermia restored p53 function inducing p53-dependent apoptosis and G_2 arrest in HPV-positive cells, which could be inhibited by p53-siRNA (Fig. 4A and C). Our data highlight the difference between HPV-negative and -positive cells, because the HPV-negative cells induce p53 and causes apoptosis after radiation alone, whereas HPV-positive cells require hyperthermia to accomplish these effects. G_2-phase arrest and apoptosis induction after hyperthermia at 42°C for 1 hour in HPV-E6–transfected cells were observed earlier (31). This important finding may explain why patients with HPV-positive cervical cancers respond particularly well to the combination of standard radiotherapy or chemotherapy with mild hyperthermia and may boost further clinical studies with hyperthermia in other HPV-associated cancers. Activation of the p53-dependent apoptotic pathway by hyperthermic degradation of E6 is specific for HPV-positive tumor cells, which may provide a therapeutic benefit to cancer patients by minimizing normal tissue damage compared with classical cisplatin-based treatments.

Hyperthermia induces *de novo* p53 (Fig. 3A). This induction of p53 is observed after 1-hour treatment at 42°C; treatment at lower temperatures did not show any accumulation of p53 (Fig. 1A). Furthermore, this p53 accumulation occurs immediately after hyperthermia and remains elevated up to 4 hours later (Fig. 1B). Also in immunofluorescence staining, p53 upregulation is observed directly after hyperthermia, in both HPV 16 and 18–positive tumor models (Fig. 1C). Previous studies support the hypothesis that blocking E6 might lead to reactivation of p53 (32). In these studies, blocking of E6 alone was not effective, and it was postulated that an additional stimulus would be necessary to

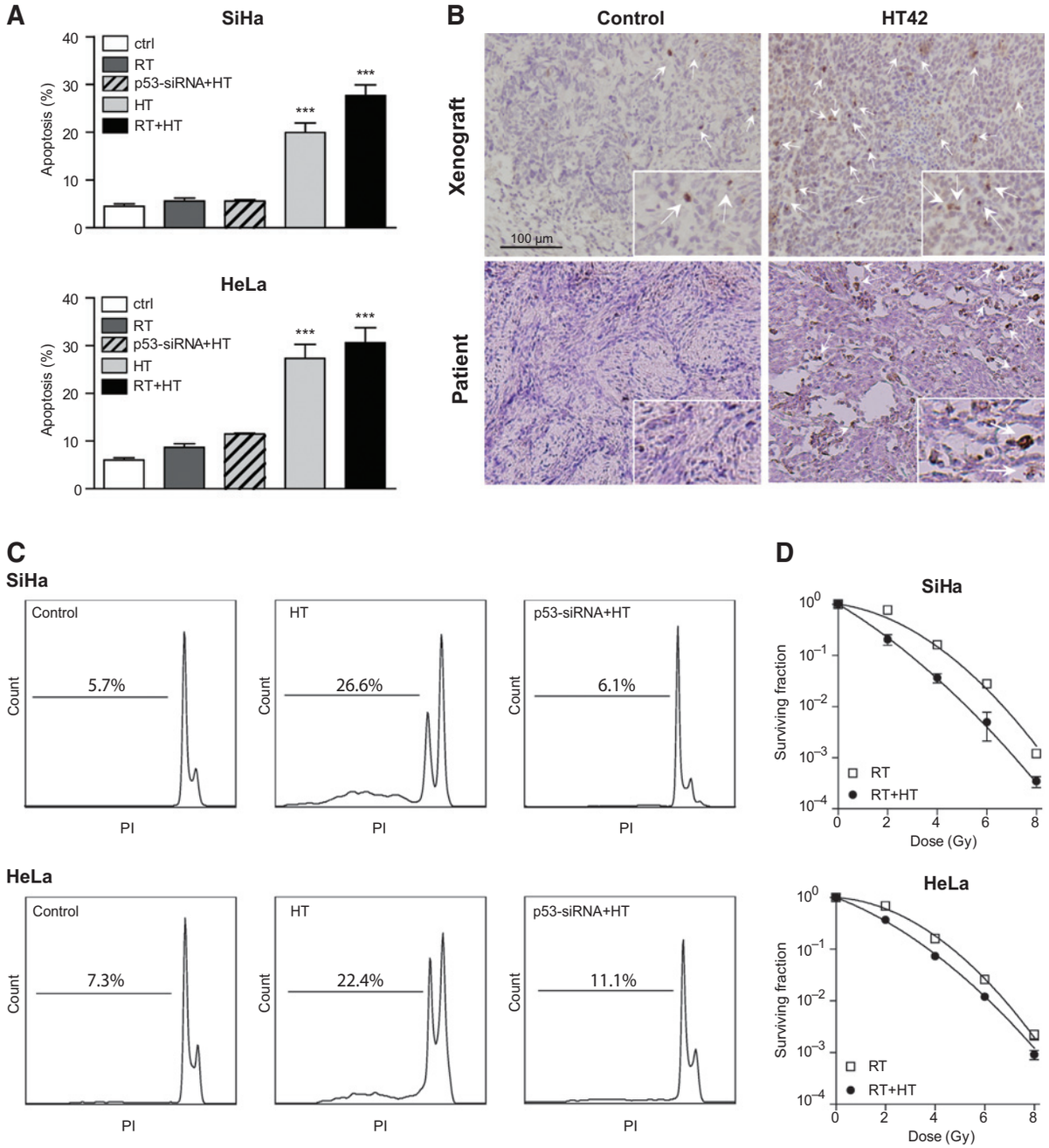

Figure 4.
Activity of p53 after hyperthermia results into G_2 arrest and apoptosis. A, Nicoletti assay on SiHa and HeLa cells. A low percentage of apoptosis was found in control samples. Hardly any difference is found after radiation or after p53-siRNA prior to hyperthermia. After hyperthermia or hyperthermia combined with radiation, a 3- to 5-fold increase in apoptosis was observed. B, cleaved caspase-3 was increased after hyperthermia in xenograft and *ex vivo*–treated patient biopsy in immunohistochemistry stainings. C, Nicoletti assay presenting cell-cycle distribution and apoptosis in SiHa and HeLa cells. In untreated samples, a high G_1 population and a very low G_2 population were observed. After hyperthermia, a substantial increase in G_2 population was noticed and a 3- to 4-fold increase of apoptosis was measured. Transfecting cells with p53-siRNA prior to hyperthermia resulted into more or less normal cell distributions. D, survival fractions of all HPV-positive cell lines were lower after radiation combined with hyperthermia compared with radiation alone. Values are shown ± SEM. HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia. ***, $P < 0.001$.

transport and activate p53. Our results confirm these earlier findings by showing that p53 is not elevated in E6-siRNA–transfected cells, but that p53 levels in these cells are highly increased after adding radiation. This p53 accumulation appears to be about similar in quantity to the p53 induction found after hyperthermia (Fig. 2).

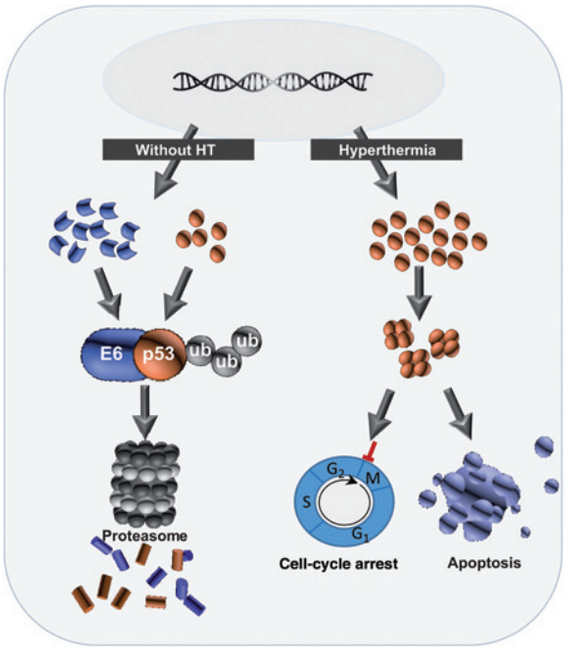

Figure 5.
Schematic overview. Hyperthermia affects HPV-infected cancer cell proteins E6 and p53. Left, in untreated conditions, HPV produces E6, which binds to p53. E6 ubiquitinates p53, resulting in proteasomal degradation of this complex. Right, after hyperthermia, p53 is accumulated and can get activated, causing p53-dependent G_2 arrest or p53-dependent apoptosis.

By blocking the proteasome or lysosome it can be observed that E6 is degraded via the proteasomal and lysosomal pathways, and p53 is degraded exclusively via the proteasomal pathway (Fig. 3). After hyperthermia, no significant differences in p53 levels were detected, suggesting that p53 degradation remains via the proteasomal pathway. Figure 5 presents a schematic overview summarizing these findings.

Reactivating or restoration of p53 function in tumor cells has long been studied as a rational cancer treatment (33, 34). Also, targeting E6 resulted into effective killing of HPV-positive cancer cells (35). Targeting of E6, resulting in reactivation of p53, has also been studied in HPV-positive head and neck tumors (36). The main difference between the present study and previous investigations is our use of hyperthermia; although this clinical treatment has been used for decades, the sensitization mechanism in tumor cells is only now starting to unfold (37).

Our study presents a possible biologic explanation for the tumor-specific radiosensitization by hyperthermia for HPV-positive tumor cells. These findings suggest that patients with HPV-positive tumors benefit from mild hyperthermia due to abrogation of the p53–E6 complex causing activation of the p53-dependent apoptotic pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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