DNA-PKcs subunits in radiosensitization by hyperthermia on hepatocellular carcinoma hepG2 cell line

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Abstract

AIM: To investigate the role of DNA-PKcs subunits in radiosensitization by hyperthermia on hepatocellular carcinoma HepG2 cell lines.

METHODS: Hep G2 cells were exposed to hyperthermia and irradiation. Hyperthermia was given at 45.5 °C. Cell survival was determined by an in vitro clonogenic assay for the cells treated with or without hyperthermia at various time points. DNA DSB rejoining was measured using asymmetric field inversion gel electrophoresis (AFIGE). The DNA-PKcs activities were measured using DNA-PKcs enzyme assay system.

RESULTS: Hyperthermia can significantly enhance irradiation-killing cells. Thermal enhancement ratio as calculated at 10 % survival was 2.02. The difference in radiosensitivity between two treatment modes manifested as a difference in the α components and the almost same β components, which α value was considerably higher in the cells of combined radiation and hyperthermia as compared with irradiating cells (1.07 Gy-1 versus 0.44 Gy-1). Survival fraction showed 1 logarithm increase after an 8-hour interval between heat and irradiation, whereas DNA-PKcs activity did not show any recovery. The cells were exposed to heat 5 minutes only, DNA-PKcs activity was inhibited at the nadir, even though the exposure time was lengthened. Whereas the ability of DNA DSB rejoining was inhibited with the increase of the length of hyperthermic time. The repair kinetics of DNA DSB rejoining after treatment with Wortmannin is different from the hyperthermic group due to the striking high slow rejoining component.

CONCLUSION: Determination with the cell extracts and the peptide phosphorylation assay, DNA-PKcs activity was inactivated by heat treatment at 45.5 °C, and could not restore. Cell survival is not associated with the DNA-PKcs inactivity after heat. DNA-PKcs is not a unique factor affecting the DNA DSB repair. This suggests that DNA-PKcs do not play a crucial role in the enhancement of cellular radiosensitivity by hyperthermia.

INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the most difficult tumors to treat[1-15]. About 90 % of patients are unresectable at presentation because of tumor size, location, or underlying parechymal disease[16-20]. Those patients are sometimes recommended to receive non-surgical therapies, including radiotherapy[21-28], radiofrequency hyperthermia[29,30], or the hyperthermia as an adjuvant to radiation in the treatment of local and regional disease[31]. Thermoradiotherapy currently offers the most significant advantages in the treatment of certain types of cancer[32]. Numerous uncontrolled studies have been performed in which comparable lesions were treated with either radiation alone or combined with hyperthermia[33]. Although many of these studies are difficult to evaluate, they give strong evidence that adjuvant heat treatment increases the probability of complete response and, consequently, tumor control. The cause of this radiosensitization has not been firmly established, however, in part this sensitization is thought to be through inhibition of repair of radiation induced DNA damage[34-36]. The mode of this repair inhibition is still unclear. Protein denaturation and aggregation appear to be the most relevant process underlying the biological effects of hyperthermia.

Several studies have shown that hyperthermia could inhibit both recovery of radiation induced potentially lethal radiation damage (PLD) and sublethal damage (SLD)[37]. Such inhibition was dependent on the time, temperature, and sequence of hyperthermia treatment. It was shown that polymerase β may be one of the mechanisms involved in thermoradiosensitization[38]. In addition, DNA-dependent protein kinase (DNA-PKcs) plays a central role in the repair of DSB[39]. DNA-PKcs is a complex consisting of three proteins: Ku70 and Ku80 and the catalytic subunit, DNA-PKcs[40]. The Ku70 and Ku80 proteins are involved in binding to the DNA ends at DSB and this binding activates the DNA-PKcs[41]. A possible mechanism for hyperthermic radiosensitization is mediated through the heat lability of Ku subunits of DNA-PKcs[42]. To support this mechanism, we have used Hep G2 cells to study the relationship of DNA-PKcs activity in thermal radiosensitization and the kinetics of DNA DSB rejoining with the time after irradiation, addressing the main question that the role of DNA-PKcs subunits in thermal radiosensitization.
MATERIALS AND METHODS

Cell culture
HepG, cell line was obtained from the American Type Culture Collection (ATCC) and was grown in MEM medium supplemented with 100×10^5 U·L^−1 penicillin, 100 mg·L^−1 streptomycin, and 100 ml·L^−1 fetal calf serum at 37 °C in a humidified incubator, at an atmosphere of 50 ml·L^−1 CO_2 and 950 ml·L^−1 air. Cells were maintained in a phase of nearly logarithmic growth by subculturing every 4 days at an initial concentration of 2×10^6 cells in T-25 tissue culture flasks for both clonogenic assay and DNA DSB rejoining studies, 2×10^6 cells in T-75 tissue culture flasks for determination of DNA-PKcs activity. The cells were passed several times through a 20-gauge needle in syringe to make the clamp cells single in each subculturing.

Hyperthermia treatment
Hyperthermia was carried out by sealing cell cultures grown in tissue culture flasks with paraffin and immersing the flasks into a temperature control waterbath (±0.05 °C). The continuous heating experiments ranged from 5 to 30 minutes at an interval of 5 minutes. After heating at 45.5 °C, flasks were put into ice for 10 minutes for the DNA DSB rejoining and DNA-PKcs activity studies, or at 37 °C waterbath for 5 minutes to equilibrate to 37 °C for clonogenic assay. At this point, if required, the flasks were irradiated on the ice.

Induction and repair of DNA DSB
Cells for DNA DSB repair experiments were labeled with 3.7MBq·L^−1 ^14C-thymidine plus 2.5 μmol·L^−1 cold thymidine for the entire period of growth. The cells were used 3 days later as the concentration reached 1×10^6 cells/T-25 flask. When indicated by the experimental protocol, cells were treated with 20 μmol·L^−1 Wortmannin for 1 hour or hyperthermia at 45.5 °C for various times before irradiation. Cells were cooled to 4 °C prior to irradiation and were irradiated on ice. After irradiation, the medium was replaced with fresh growth medium pre-warm at 42 °C to rapidly restore to 37 °C, and then cells were quickly returned to the incubator at 37 °C to allow for repair. Cells were prepared for DNA DSB analysis at various time intervals thereafter.

After completion of the repair time interval, cells were trypsinized for 90 minutes in ice for the first 4 hours, and 10 minutes at 37 °C at later points. The cells were collected with 5 ml cold medium, centrifuged at 4 °C, and washed with 5 ml cold serum-free medium. The cells were resuspended in 165 μl cold serum-free medium. This cell suspension was mixed with an equal volume of 10 g·L^−1 agarose (InCert agarose, FMC) to reach a concentration of 3×10^6 cells·L^−1. The cell-agarose suspension was then pipetted into a 3 mm diameter glass tubes and placed into ice to allow for solidification. The solidified cell-agarose suspension was extruded from the glass tubes and cut into 3×5 mm cylindrical blocks containing approximately 1.5×10^6 cells/block[41]. Blocks were then placed in lysis buffer containing 10 mmol·L^−1 Tris, pH 8.0, 50 mmol·L^−1 NaCl, 0.5 mol·L^−1 EDTA, 2 g·L^−1 N-Lauryl Sarcosyl (NLS), A0.1 g·L^−1 proteasine E & O, and incubated first at 4 °C for 45 minutes and then at 50 °C for 16-18 hours. Following lysis, agarose blocks were washed for 1 hour at 37 °C in a buffer containing 10 mmol·L^−1 Tris, pH 8.0 and 0.1 mol·L^−1 EDTA, and were then treated for 1 hour at 37 °C in the same buffer, at pH 7.5, with 0.1 g·L^−1 RNAse A. Cells from identically treated non-irradiated cultures were also processed at pre-defined times to determine the signal generated by non-irradiated cells as background. For dose response, a similar protocol was also employed to determine the induction of DNA DSB except that in this case cells were embedded in agarose prior to irradiation with various doses on the ice, and were lysed immediately thereafter.

Pulsed-field gel electrophoresis
Asymmetric field inversion gel electrophoresis (AFIGE) was carried out in 5 g·L^−1 Seakem agarose (FMC), cast in the presence of 0.5 mg·L^−1 ethidium bromide, in 0.5×TBE (45 mmol·L^−1 Tris, pH 8.2, 45 mmol·L^−1 Boric Acid, 1 mmol·L^−1 EDTA) at 10 °C for 40 hours. During this time, cycles of 1.25 V·cm^−1 for 900 seconds in the direction of DNA migration alternated with cycles of 5.0 V·cm^−1 for 75 seconds in the reverse direction. The agarose gels were quantified to estimate DNA damage by means of a PhosphorImager (Molecular Dynamics). Gels were dried and exposed to radiation-sensitive screens for 48-96 hours. DNA DSB was quantitated by calculating the fraction of activity released (FAR) from the well into the lane in irradiated and non-irradiated samples. The FAR measured in non-irradiated cells (background) was subtracted from the results shown with irradiated cells. Gel images were obtained either by photographing ethidium bromide-stained gels under UV light, or from the PhosphorImager.

Repair kinetics were fitted assuming two exponential components of rejoining according to the equation FAR=Ae^{−bt}+Ce^{−bt}[42]. The first term in the equation was fitted to the slow and the second to the fast component of rejoining. Fitting was achieved using the non-linear regression analysis routines of a commercially available software package (SAS). Parameters A and C describe the amplitudes, and parameter b and d and the rate constants of the slow and the fast components of rejoining, respectively. From these parameters the half-time for the rejoining of the slow and the fast components were calculated as t_{0.5,slow}={ln2/b} and t_{0.5,fast}={ln2/d}, respectively. The fraction of DSB rejoined by fast kinetics was calculated as F_{fast}=A/A+C and F_{slow}=C/A+C.

Determination of DNA-PKcs activity
Cell extract preparation: Cells (2×10^6) were grown in the T-75 tissue culture flask for 5 days. After treatment, about 30×10^6 cells were collected in cold PBS after being trypsinized at 37 °C for 10 minutes, centrifuged at 4 °C, and resuspended with 1 ml cold PBS and transferred to Eppendorf tube. After spin 1 500 r·min^−1 for 5 minutes at 4 °C, PBS was replaced with 0.5 ml (about 4 volumes of cells) hypotonic buffer containing 10 mmol·L^−1 HEPES KOH pH 7.9 at 4 °C, 5 mmol·L^−1...
KCl, 1.5 mmol·L\(^{-1}\) MgCl\(_2\), 20 mmol·L\(^{-1}\) b-Glu, 0.2 mmol·L\(^{-1}\) phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol·L\(^{-1}\) diithiothreitol (DTT). The cells were put on ice for 10 minutes, frozen in liquid nitrogen and thawed for 3 cycles, adjusted salt being 50 mmol·L\(^{-1}\) KCl in hypotonic buffer (16 µl, 1.6 mol·L\(^{-1}\) KCl), and allowed to stay for 10 minutes on ice. After centrifugation (40 minutes at 14 000 r·min\(^{-1}\) at 4 °C), cytoplasm extract was obtained from the collection of supernatants. Nuclei were resuspended with 50 mmol·L\(^{-1}\) KCl hypotonic buffer (100 ml), the salt was adjusted to 400 mmol·L\(^{-1}\) KCl with 3 mol·L\(^{-1}\) KCl in the buffer (13 µl), mixed in the cold room (4 °C) for 30 minutes. After centrifuged at 14 000 r·min\(^{-1}\) for 15 minutes at 4 °C, nucleic extract was obtained from the collection of supernatants. Protein concentration of both cytoplasm and nucleic extracts were determined using Bio-Rad protein II assay.

Activity assay of DNA-PKcs: In 1.5 ml microfuge tube, the reaction buffer was set up on ice in a mixture containing 4 µl 5X kinase buffer (250 mmol·L\(^{-1}\) HEPES, pH 7.5, 50 mmol·L\(^{-1}\) MgCl\(_2\), 1 mmol·L\(^{-1}\) EGTA, 5 mmol·L\(^{-1}\) DTT), 2 µl 10X substrate peptide (2 mmol·L\(^{-1}\) ), 2 µl 10X sonicated calf thymus DNA (100 mg·L\(^{-1}\) ), 2 µl (7.4 MBq·L\(^{-1}\) ) of (γ-32P) ATP. In this reaction buffer mixture, an equal volume (5 µg in 10 µl) of nucleic extract was added and mixed quickly. The optimal incorporation time was found to be 15 minutes at 30 °C. After incubation, the reaction was terminated with 20 µl stop solution (300 g·L\(^{-1}\) acetic acid, 1 mmol·L\(^{-1}\) ATP). Out of 40 µl reaction mixture, 20 µl was spotted on Watermann P81 phosphocellulose paper, and washed 4 times with 150 g·L\(^{-1}\) acetic acid for 15 minutes each time. The filters were placed in scintillation vials, and the adsorbed radioactivity was quantitated. To calculate the specific activity of (γ-32P) ATP, we removed 5 µl from any two reaction tubes, and added to scintillation vial to count. Calculation of the specific activity of (γ-32P) ATP in cpm/pmol is shown as follows (40/5 × X/10 000 = X/250 [40 is the sum of the reaction volume (20 µL) + stop buffer (20 µL), 5 is the volume (µl) used for the specific activity of (γ-32P) ATP, X is the average counts, and 10 000 is the number of pmoles of ATP in the reaction]. Calculation of incorporated ATP (pmol) is (CPM\(_{reaction\ with\ DNA}\) - CPM\(_{reaction\ without\ DNA}\)) / The specific activity of [γ-32P] ATP in 10\(^{-3}\)cpm·mol\(^{-1}\).

RESULTS
The survival curves of HepG\(_2\) cell exposure to X-ray combined with or without hyperthermia are shown in Figure 1A and B. The mean dose of survival fraction at 2 Gy (SF\(_2\)) was 0.230±0.033 for the group of radiation alone, and 0.148±0.043 for the radiation combined with hyperthermia group. The linear-quadratic model S=\(e^{-\alpha D}\ - \beta D^2\) was applied to describe the survival data in Figure 1, where S is the fraction of cells surviving a dose D, and \(\alpha\) and \(\beta\) are constants. The \(\alpha\) and \(\beta\) values are 0.805±0.037 Gy\(^{-1}\) and 0.072 ± 0.012 Gy\(^{-2}\) for the group of radiation alone, and 0.950 ± 0.018 Gy\(^{-1}\) and 0.010 ± 0.001 Gy\(^{-2}\) for the group of radiation combined with hyperthermia. The \(\alpha/\beta\) ratios are 11.2 Gy and 97 Gy for the groups of radiation alone and combination modes, respectively. Thermal enhancement ratio as calculated at 10 % survival (TER\(_{10}\)) was 2.02. The difference in radiosensitivity between these two groups can be interpreted as being due to some phenomena, which manifest as a difference in the a components.

Figure 1B shows the survival curves for cells exposed for various periods of time to 45.5 °C. The combination of hyperthermia with 3 Gy X-ray significantly improved the killing effects in comparison of hyperthermia alone. Figure 1C shows a combination of hyperthermia at 45.5 °C for 15 minutes first and then 3 Gy of X-rays. When the time intervals between hyperthermia and radiation in existence, the cell survival increased with the time interval increase within 8 hours, but no significant change was observed 8 hours later.

The DNA-PKcs activity was measured before and after irradiation with 40 Gy X-ray, or hyperthermia for 20 minutes at 45.5 °C, or both. All of DNA-PKcs values were expressed with both relative percentage at left side and pmol at right of figures in this paper. Figure 2A shows that DNA-PKcs activity was inhibited by about 70 % at heating. Radiation stimulated DNA-PKcs activity increased about 30 % in the cells treated with or without hyperthermia at the 2nd hour. In order to determine whether the level of DNA-PKcs activity recovers its activity after hyperthermia, the time point was extended to 24 hours to correspond to clonogenic survival in Figure 1C.

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As shown in Figure 2B, no restore was found in DNA-PKcs activity up to 24 hours. The DNA-PKcs activity was inhibited after the cells exposed to heat at 45.5 °C for 5 minutes (Figure 2C). The DNA-PKcs activity remained at almost the same level despite the hyperthermic time extending.

To understand the role of DNA-PKcs subunits in radiosensitization by hyperthermia, induction of X-ray induced DNA DSB was measured by AFIGE. Figure 3 shows the dose response curves for Hep G2 cells received radiation combined with or without hyperthermia. The upper panel shows a typical gel scanned with 14C-TdR, while the lower panel shows quantitative data as described in the Methods. The FAR increases almost linearly with dose up to 30 Gy but bends downward at higher doses. Similar increases in FAR as a function of dose were observed in radiation alone or combined with hyperthermia, suggesting similar yields of DNA DSB.

The rate of rejoining of radiation-induced DNA DSB was subsequently examined to determine whether differences in repair between radiation alone and combined with hyperthermia for different periods of time account for the results shown in Figure 2A that the DNA-PKcs activity was inhibited after heat. Figure 4 shows that DNA DSB repairs kinetics in Hep G2 cells. Cells were irradiated with 40 Gy and prepared for AFIGE after various periods of incubation at 37 °C to allow for repair. The upper panel in the Figure shows a typical AFIGE gel, while the lower panel shows its quantification as described in the Methods. The rejoining of DNA DSB decreased with increasing the lengths of hyperthermic time at 45.5 °C. Table 1 shows that the half-time for rejoining of the fast components was shorter in the control group or exposure to heat for 5 minutes than the exposure to longer time (15 and 20 minutes). However, the half-time of slow components in both control and hyperthermic groups were almost the same which ranged between 7 and 9 hours.

 Wortmannin inhibits the entire family of PI-3 kinases and probably also other cellular kinases. To evaluate the
contribution of DNA-PKcs to Wortmannin-induced inhibition of DNA DSB rejoining, we searched for Wortmannin treatment, which is able to compare heat-induced inhibition of DNA DSB rejoining. Figure 5 indicates the kinetics of DNA DSB rejoining after treatment with Wortmannin. We found that the slow rejoining component is strikingly high. The deficiency of DNA DSB rejoining was found in Wortmannin treatment cells after 2 hours. These results are different from those in hyperthermic groups.

Table 1 The half-time and fraction of DNA DSB rejoined by fast and slow kinetics

|                | $T_{50,\text{fast}}$ | $T_{50,\text{slow}}$ | $F_{\text{fast}}$ | $F_{\text{slow}}$ |
|----------------|----------------------|----------------------|-------------------|-------------------|
| Control        | 0.49                 | 7.07                 | 0.80              | 0.20              |
| 5 min          | 0.33                 | 4.15                 | 0.44              | 0.56              |
| 15 min         | 0.92                 | 9.37                 | 0.49              | 0.51              |
| 20 min         | 1.25                 | 9.24                 | 0.30              | 0.70              |
| 20 mmol·L$^{-1}$ Wort. | 0.87               | $7 \times 10^{8}$ | 0.44              | 0.56              |

DISCUSSION

Tumors of liver are among the most common malignancies in the world. Primary hepatocellular carcinoma was the second most common cancer and the leading cause of cancer deaths behind gastric cancer in China. Surgical resection has been accepted as the only curative therapy for primary liver cancer. Unfortunately, most patients were surgically unsectable[1-28]. Hyperthermia[29,30], radiation therapy[21-28], or combination of both[31,32] were introduced as an alternative therapeutic approach. The cause of this radiosensitization has not been firmly established.

The inhibition of DNA-PKcs by hyperthermia has been demonstrated in several studies[40, 43,44]. In our series, DNA-PKcs activity was inhibited by about 60 % after hyperthermia at 45.5 °C for 5 minutes, and no significant change was found after increasing hyperthermia time. This result is similar to other reports[40, 43]. Interestingly, the rejoining of DNA DSB was decreasing with the increase of the hyperthermic time at 45.5 °C. The decrease of DNA DSB repair did not correspond proportionally to the heat inhibited DNA-PKcs activity which was kept at a same low activity level between 5 and 30 minutes. This means DNA-PKcs is not critical for DNA DSB repair in heat.

The activity of DNA-PKcs depends upon the presence of double-stranded DNA ends, and is based on the similarity of their DNA-binding properties, and Ku was identified as the DNA-targeting component of this protein complex[45-51]. This can be used to explain the fact that DNA-PKcs activity increased by about 30 % at the 2nd hour after 40 Gy X-ray irradiation, which produced plenty of DNA DSB. After
hyperthermia at 45.5 °C for 15 minutes, DNA-PKcs activity was stable at a low level (30 %) up to 24 hours. We are eager to know whether the low level DNA-PKcs affects the clonogenic survival. The results showed that the effects of thermal radiosensitization were lowered, even though DNA-PKcs activity did not restore. This indicated that there was no correlation between thermal inactivation of DNA-PKcs and radiosensitization by heat.

Wortmannin is a fungal metabolite originally characterized as an irreversible inhibitor of PI3K-like family including DNA-PKcs[32]. The inhibition of DNA-PKcs by Wortmannin could increase the slow rejoining component, resulting in deficiency of DNA DSB repair. The kinetics of DNA DSB rejoining in hyperthermia groups different from that treated with wortmannin, inhibited the DNA-PKcs activity. This confirms that DNA DSB repair is not completely affected by DNA-PKcs, other factors might also involve in the repair.

DNA-PKcs is not critical for radiosensitization by heat. What might explain the synergistic interaction between heat and radiation? One possibility is that homologous recombination may impair after heat shock, but the contribution of this repair pathway in mammalian cells seems limited[39]. Another possibility is that heat-induced loss of activity of DNA repair enzymes other than DNA-PKcs has been observed and proposed previously as the mechanism that underlies DNA repair inhibition and radiosensitization. These enzymes include DNA polymerases[37], ATM system[38], ATR system[39], etc. Motsumoto found recently that heating up to 90 minutes at low doses and the less sensitive it is to dose fraction[39]. The contradictory results are due to the methods of measurement, which can not distinguish between inhibition of ATM and DNA-PKcs as the substrate, and peptide is good for both kinases.

In clonogenic assay, the difference in radiosensitivity between radiation and thermal radiation groups manifested as α difference in the α components. The dose range over which the linear component dominates in a linear-quadratic (LQ) survival relationship depends on the relative values of α and β: the higher the relative value of α, the more linear response at low doses and the less sensitive it is to dose fraction[39]. In hyperthermia group, the α/β ratio is high, with no detectable influence of the quadratic function over the first two decades of reduction in cell survival, implying that accumulation of sublethal injury plays a negligible role in cell killing by thermal radiosensitization clinically[39]. From these results, we can deduce that heat inhibits the repair of radiation-induced single-strand breaks and radiation-induced chromosome aberrations. This inability to repair molecular damage translates into the inability to repair both sublethal damage and potentially lethal damage produced by radiation.

In conclusion, when the DNA-PKcs activity was determined using the cell extracts and the peptide phosphorylation assay, DNA-PKcs activity was inactivated by heat treatment at 45.5 °C, and could not restore. Cell survival is not associated with the DNA-PKcs inactivity after heat. DNA-PKcs is not a unique factor affecting DNA DSB repair. This suggests DNA-PKcs does not play a crucial role in the enhancement of cellular radiosensitivity by hyperthermia.

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