Heat Induces Histone γ H2AX Formation under Hypoxia

YUKARI YOSHIDA^{1†}, SHINTARO TOMINAGA^{1†}, LIQIU MA¹,
AKIHISA TAKAHASHI^{1*}

¹Gunma University Heavy Ion Medical Center, 3-39-22, Showa-machi, Maebashi, Gunma 371-8511, Japan

Abstract: Tumor hypoxia is a negative prognostic and predictive factor for radiotherapy, and hyperthermia therapy is clinically useful for overcoming radioresistance in hypoxic tumors. However, the mechanism for the hyperthermia-induced cell death observed in hypoxic tumors remains unknown. We aimed to clarify the relationship between heat sensitivity and heat-induced DNA double-strand breaks (DSBs), reflecting DNA damage, in tumor cells under hypoxia. HeLa human cervical epithelial adenocarcinoma cells were subjected to heat treatment or X-ray irradiation under hypoxia or normoxia. Control cells were left untreated. The formation of DSBs was evaluated by immunocytochemistry for histone γ H2AX foci, given that one histone γ H2AX focus reflects one DSB. Cell survival was evaluated by colony-formation assays. The colony-formation assays revealed that hypoxic cells showed greater radioresistance, as expected, but only slightly higher heat resistance than normoxic cells. Under normoxia, heat-treated or X-ray-irradiated cells showed larger amounts of γ H2AX foci formation than control cells, reflecting increased DSB formation and more DNA damage. Under hypoxia, heat-treated cells showed a less remarkable decrease in γ H2AX foci formation than X-ray-irradiated cells, reflecting sustained levels of DSB formation and DNA damage. The present findings indicate that heat treatment can induce DNA damage *via* DSB formation reflected by γ H2AX foci formation under hypoxia. The findings provide further support for an important role of heat-induced DSB damage in cell killing in hypoxic tumors that show radioresistance. Hyperthermia therapy can be beneficial for the prognosis of cancer patients through increased DNA damage leading to tumor cell death.

Key Words: hyperthermia, hypoxia, DNA double-strand break (DSB), γ H2AX, heat-sensitivity

Introduction

During the last 20 years, responses to radiation-induced DNA damage have attracted much interest, based on the detection of DNA double-strand breaks (DSBs) by immunocytochemistry for histone γ H2AX^{1,2)}. Specifically, radiation-induced one γ H2AX focus can be correlated with one DSB. The frequency of radiation-induced γ H2AX foci formation depends on the oxygen concentration, which decreases with distance from blood vessels in a solid tumor³⁾. Furthermore, tumor hypoxia is considered a negative prognostic and predictive factor for radiotherapy.

Received 6 August, 2018, Accepted 29 August, 2018:

[†]Yukari Yoshida and Shintaro Tominaga contributed equally to this work.

*Corresponding author: Tel, +81-27-220-7917; Fax, +81-27-220-7919;

e-mail, a-takahashi@gunma-u.ac.jp

doi: 10.3191/thermalmed.34.45

©2018 Japanese Society for Thermal Medicine

Hyperthermia therapy is well known to be clinically useful for overcoming the radioresistance of hypoxic tumors^{4,5)}. Hyperthermia therapy also allows clinicians to reduce the doses of radiotherapy and anticancer drugs delivered to patients⁶⁾, thus helping to minimize the adverse side effects of anticancer therapy. However, the exact mechanism for cell death under hypoxia remains unknown. We previously reported that heat can induce γ H2AX foci formation⁷⁾. Although the intensities of γ H2AX staining differed, heat-induced γ H2AX foci were observed in many different mammalian cell lines⁸⁾. While some researchers believe that heat does not lead to DNA damage and that γ H2AX is a byproduct of the cellular response to stress⁹⁻¹¹⁾, others have proven that heat can provoke DSB formation using comet assays^{7,12)}.

Based on these observations, we aimed to clarify the relationship between heat sensitivity and heat-induced DSBs under hypoxia.

Materials and methods

Cell culture

HeLa human cervical epithelial adenocarcinoma cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium containing high glucose and L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), and HEPES (10 mM) at 37°C in a humidified atmosphere of 5% CO₂.

Hypoxia treatment

Cells were subjected to hypoxic conditions (0.1% O₂) using a workstation (INVIVO2 500; Baker Ruskinn, Sanford, ME, USA) for 6 h. The workstation was able to regulate not only O₂ but also CO₂ at 37°C.

Heat treatment

Cells were heat-treated by sandwiching between two dry baths (H₂O₃; AS ONE Co. Ltd., Osaka, Japan) in a normoxic CO₂ incubator or hypoxic workstation (Fig. 1A). The temperature was maintained at 45°C (Fig.

Fig. 1. Heat treatment. A, Heating devices. B, Dish temperatures.

1B). Under these experimental conditions, no marked change in pH was detected in the medium during the treatment.

Irradiation

Cells were irradiated with X-rays using a 200-kVp X-ray generator (MultiRad225; Faxitron Bioptics LLC, Tucson, AZ, USA) with total filtration of 0.5 mm in aluminum and 0.5 mm in copper. Cells were sealed in a bag with a clip to maintain the oxygen concentration during exposure to X-rays, and kept at 37°C using a dry bath.

Colony-formation assay

Cell survival was measured by colony-formation assays. Briefly, 6-cm-diameter dishes were used, and four experiments were repeated for each survival point. Three hundred cells were seeded into dishes at 6–8 h before hypoxia treatment. After treatment, cells were cultured under normoxia. Colonies obtained after 12 days were fixed with ethanol and stained with 2% Giemsa solution. Microscopic colonies composed of more than approximately 50 cells were counted as surviving cells.

H2AX phosphorylation assay

Cells (2.0×10^4 cells/well) were seeded on 13-mm-diameter cover glasses in a 15-mm Nunc™ multidish (Thermo Fisher Scientific, Waltham, MA, USA) at 24 h before treatment. Thirty minutes after treatment, cells were fixed with methanol and sequentially incubated with an anti-phospho-H2AX monoclonal antibody (JBW301; EMD Millipore, Billerica, MA, USA; 1: 300) and Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Thermo Fisher Scientific Inc.; 1: 400). The fluorescence intensity of histone H2AX phosphorylated at Ser139 per cell was analyzed using a MetaMorph system (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data were presented as mean $\pm$ standard deviation. Student's *t*-test was performed to calculate the statistical significance of differences in data. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Cell survival under hypoxia

Hypoxic cells were more radioresistant, but only slightly more heat-resistant than normoxic cells (Fig. 2). At the 10% survival point, the oxygen enhancement ratio values for X-ray (Fig. 2A) and heat (Fig. 2B) treatment were 1.6 and 1.1, respectively.

Fig. 2. Comparison of the effects of radiation (A) and hyperthermia (B) on cell survival under normoxic and hypoxic conditions. OER: oxygen enhancement ratio.

Heat induces γ H2AX formation under hypoxia

Under normoxia, γ H2AX foci were formed more frequently when cells were heated or irradiated with X-rays compared with control cells. X-ray-induced γ H2AX foci was less in hypoxic cells than in normoxic cells (Fig. 3). In contrast, γ H2AX intensity differences between normoxic and hypoxic cells under hyperthermia treatment are not statistically significant, although that of hypoxic cells slightly decrease in comparison with normoxic cells (Fig. 3B).

Fig. 3. Comparison of the effects of radiation and hyperthermia on γ H2AX foci formation under normoxic and hypoxic conditions. **A**, Immunocytochemistry. **B**, Relative intensities of γ H2AX fluorescence. Scale bar, 10 μ m. * $p < 0.05$.

Discussion

A previous study showed that hypoxic cells were slightly more sensitive to heat than their normoxic counterparts⁴). It was also reported that trypsinization after heating can enhance the effect of hyperthermia in cells¹³). To clarify the specific effect of heating under hypoxia, heat sensitivity was analyzed without trypsinization in this study.

We previously reported that high temperatures increase γ H2AX foci formation⁷). Furthermore, γ H2AX foci were formed more frequently when cells were heated in S phase compared with G₁ and G₂ phases⁷). The rate constant curves appeared very similar between γ H2AX foci formation and cell killing⁷). These observations led us to conclude that heat sensitivity was dependent on heat-induced γ H2AX foci, which probably reflects the formation of DSBs. We previously considered that heat-induced DSBs produced through radicals attack DNA bases^{7,14}). Early studies reported that increased levels of base damage and other types of damage were detected after heat treatment through the production of reactive oxygen species^{15,16}). Base modification may induce nick formation by incision enzymes during base excision repair. DNA synthesis polymerase β enzymes are known to be heat-sensitive^{17,18}). Nicks would accumulate during heat treatment. If

nicks are formed on the opposite strand, DSBs may occur. Therefore, high temperature and long heating time may lead to the formation of many DSBs^{7,14}. In S phase, a single nick can lead to a DSB during the replication step. Therefore, heat induces DSBs more frequently in S-phase cells^{7,14}.

However, it is well known that hypoxia can suppress indirect actions of free radicals. Nevertheless, we found that heat can induce γ H2AX foci formation regardless of hypoxia (Fig. 3). Therefore, the mechanism involving free radicals may be not dominant. Thus, we need to consider another mechanism (Fig. 4). Recently, Velichko *et al.*¹²) reported that hyperthermia in S phase leads to topoisomerase 1-dependent formation of single-strand breaks, some of which can be converted into DSBs after several hours. However, heat stress is known to immediately induce DSB formation in G₁, S, and G₂ phases⁷. It is also well known that topoisomerase 2 generates temporary DSBs¹⁹. Thus, the most probable mechanism for the immediate heat stress-induced formation of DSBs was suggested to involve inhibition of DNA topoisomerase II ligation activity²⁰. We anticipate that future studies will shed light on this issue.

Taken together, we conclude that heat induces γ H2AX foci formation, which probably indicates DSB formation regardless of hypoxia. These findings provide support for the concept that DSBs play an important role in heat-induced cell killing under hypoxia.

Fig. 4. Model illustrating how radiation and hyperthermia induce γ H2AX foci formation under hypoxia.

Acknowledgments

This work was supported by grants from JSPS KAKENHI (JP16K10341) and Research Projects with Heavy Ions at Gunma University Heavy Ion Medical Center (GHMC). The authors thank Alison Sherwin, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript and helping to draft the abstract.

References

- 1) Sedelnikova O.A., Rogakou E.P., Panyutin I.G., Bonner W.M.: Quantitative detection of ¹²⁵IdU-induced DNA double-strand breaks with gamma-H2AX antibody. *Radiat Res*, 158: 486–492, 2002.
- 2) Takahashi A., Ohnishi T.: Does γH2AX foci formation depend on the presence of DNA double strand breaks? *Cancer Lett*, 229: 171–179, 2005.
- 3) Zhu Y., Zhao T., Itasaka S., Zeng L., Yeom C.J., Hirota K., Suzuki K., Morinibu A., Shinomiya K., Ou G., Yoshimura M., Hiraoka M., Harada H.: Involvement of decreased hypoxia-inducible factor 1 activity and resultant G1-S cell cycle transition in radioresistance of perinecrotic tumor cells. *Oncogene*, 32: 2058–2068, 2013.
- 4) Gerweck L.E., Gillette E.L., Dewey W.C.: Killing of Chinese hamster cells *in vitro* by heating under hypoxic or aerobic conditions. *Eur J Cancer*, 10: 691–693, 1974.
- 5) Overgaard J., Grau C., Lindegaard J.C., Horsman M.R.: The potential of using hyperthermia to eliminate radioresistant hypoxic cells. *Radiother Oncol*, 20: 113–116, 1991.
- 6) Falk M.H., Issels R.D.: Hyperthermia in oncology. *Int J Hyperthermia*, 17: 1–18, 2001.
- 7) Takahashi A., Matsumoto H., Nagayama K., Kitano M., Hirose S., Tanaka H., Mori E., Yamakawa N., Yasumoto J., Yuki K., Ohnishi K., Ohnishi T.: Evidence for the involvement of double-strand breaks in heat-induced cell killing. *Cancer Res*, 64: 8839–8845, 2004.
- 8) Takahashi A., Mori E., Somakos G.I., Ohnishi K., Ohnishi T.: Heat induces γH2AX foci formation in mammalian cells. *Mutat Res*, 656: 88–92, 2008.
- 9) Hunt C.R., Pandita R.K., Laszlo A., Higashikubo R., Agarwal M., Kitamura T., Gupta A., Rief N., Horikoshi N., Baskaran R., Lee J.H., Löbrich M., Paull T.T., Roti Roti J.L., Pandita T.K.: Hyperthermia activates a subset of ataxia-telangiectasia mutated effectors independent of DNA strand breaks and heat shock protein 70 status. *Cancer Res*, 67: 3010–3017, 2007.
- 10) Laszlo A., Fleischer I.: The heat-induced gamma-H2AX response does not play a role in hyperthermic cell killing. *Int J Hyperthermia*, 25: 199–209, 2009.
- 11) Laszlo A., Fleischer I.: Heat-induced perturbations of DNA damage signaling pathways are modulated by molecular chaperones. *Cancer Res*, 69: 2042–2049, 2009.
- 12) Velichko A.K., Petrova N.V., Razin S.V., Kantidze O.L.: Mechanism of heat stress-induced cellular senescence elucidates the exclusive vulnerability of early S-phase cells to mild genotoxic stress. *Nucleic Acids Res*, 43: 6309–6320, 2015.
- 13) Bass H., Moore J.L., Coakley W.T.: Lethality in mammalian cells due to hyperthermia under oxic and hypoxic conditions. *Int J Radiat Biol Relat Stud Phys Chem Med*, 33: 57–67, 1978.
- 14) Takahashi A., Ohnishi T.: What is the critical hyperthermia target in cancer cells? *Thermal Med*, 22: 229–237, 2006.
- 15) Bruskov V.I., Malakhova L.V., Masalimov Z.K., Chernikov A.V.: Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. *Nucleic Acids Res*, 30: 1354–1363, 2002.
- 16) Cui Z.G., Wada S., Kondo T., Fujiwara Y., Zhao Q.L., Ogawa R., Nagaki Y., Shoji M., Tabuchi Y., Yuki H., Feril L.B., Jr.: Enhancement of hyperthermia-induced apoptosis by modification of intracellular oxidative stress. *Jpn J Hyperthermic Oncol*, 21: 71–80, 2005.
- 17) Spiro I.J., Denman D.L., Dewey W.C.: Effect of hyperthermia on isolated DNA polymerase-β. *Radiat Res*, 95: 68–77, 1983.
- 18) Jorritsma J.B., Kampinga H.H., Scaf A.H., Konings A.W.: Strand break repair, DNA polymerase activity and heat radiosensitization in thermotolerant cells. *Int J Hyperthermia*, 1: 131–145, 1985.
- 19) Nitiss J.L.: Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*, 9: 338–350, 2009.
- 20) Kantidze O.L., Velichko A.K., Luzhin A.V., Razin S.V.: Heat stress-induced DNA damage. *Acta Naturae*, 8: 75–78, 2016.

Abstract in Japanese

温熱は低酸素下でもヒストン γ H2AX 生成を誘導する

吉田由香里¹・富永信太郎¹・馬 立 秋¹・高橋 昭 久¹

¹群馬大学・重粒子線医学研究センター

要 旨: 固形腫瘍内の低酸素領域は、放射線治療の負の予後および予測因子である。ハイパーサーミア治療は、低酸素腫瘍による放射線抵抗性を克服するうえで臨床的に有用である。しかしながら、低酸素腫瘍で観察されるハイパーサーミアによって誘発される細胞死のメカニズムはよくわかっていない。そこで、腫瘍細胞における低酸素下での温熱感受性と温熱によって誘発される DNA 二本鎖切断 (DSBs) の関係を明らかにすることとした。ヒト子宮頸がん HeLa 細胞を、常酸素及び低酸素の条件で、温熱処理または X 線照射した。対照は未処理の細胞とした。一つの γ H2AX フォーカスは、一つの DSB に相当することから、DSBs の生成は、 γ H2AX の蛍光免疫染色によって評価し、細胞死は、コロニー形成法で評価した。低酸素では、明らかに放射線抵抗性で、わずかに温熱抵抗性を示した。 γ H2AX 蛍光強度は、低酸素において X 線では顕著に減少したのに対して、温熱では減少が顕著に抑えられた。今回の結果は、 γ H2AX フォーカス形成によって示される DSB によって、低酸素下でも温熱処理が DNA の損傷を誘発することができることを示している。これらのことから、ハイパーサーミア治療は、放射線抵抗性の低酸素細胞に対しても有効であることを裏付けることができた。

Thermal Med, 34[3]: 45-51, 2018.