Heat Induces Histone $\gamma$H2AX Formation under Hypoxia

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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 $\gamma$H2AX foci, given that one histone $\gamma$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 $\gamma$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 $\gamma$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 $\gamma$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), $\gamma$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 $\gamma$H2AX1,2). Specifically, radiation-induced one $\gamma$H2AX focus can be correlated with one DSB. The frequency of radiation-induced $\gamma$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.
Hyperthermia therapy is well known to be clinically useful for overcoming the radioresistance of hypoxic tumors. Hyperthermia therapy also allows clinicians to reduce the doses of radiotherapy and anticancer drugs delivered to patients, 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 γH2AX foci formation. Although the intensities of γH2AX staining differed, heat-induced γH2AX foci were observed in many different mammalian cell lines. While some researchers believe that heat does not lead to DNA damage and that γH2AX is a byproduct of the cellular response to stress, others have proven that heat can provoke DSB formation using comet assays.

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 µg/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. 1).

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 ± 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 γH2AX formation under hypoxia

Under normoxia, γH2AX foci were formed more frequently when cells were heated or irradiated with X-rays compared with control cells. X-ray-induced γH2AX foci was less in hypoxic cells than in normoxic cells (Fig. 3). In contrast, γH2AX 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).

A previous study showed that hypoxic cells were slightly more sensitive to heat than their normoxic counterparts. It was also reported that trypsinization after heating can enhance the effect of hyperthermia in cells. 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 γH2AX foci formation. Furthermore, γH2AX foci were formed more frequently when cells were heated in S phase compared with G1 and G2 phases. The rate constant curves appeared very similar between γH2AX foci formation and cell killing. These observations led us to conclude that heat sensitivity was dependent on heat-induced γH2AX foci, which probably reflects the formation of DSBs. We previously considered that heat-induced DSBs produced through radicals attack DNA bases. 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. Base modification may induce nick formation by incision enzymes during base excision repair. DNA synthesis polymerase β enzymes are known to be heat-sensitive. Nicks would accumulate during heat treatment.

![Fig. 3. Comparison of the effects of radiation and hyperthermia on γH2AX foci formation under normoxic and hypoxic conditions. A, Immunocytochemistry. B, Relative intensities of γH2AX fluorescence. Scale bar, 10 μm. *p < 0.05.](image)
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\textsuperscript{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\textsuperscript{7,14).}

However, it is well known that hypoxia can suppress indirect actions of free radicals. Nevertheless, we found that heat can induce γH2AX 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 \textit{et al.}\textsuperscript{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 G\textsubscript{1}, S, and G\textsubscript{2} phases\textsuperscript{7).} It is also well known that topoisomerase 2 generates temporary DSBs\textsuperscript{19).} Thus, the most probable mechanism for the immediate heat stress-induced formation of DSBs was suggested to involve inhibition of DNA topoisomerase II ligation activity\textsuperscript{20).} We anticipate that future studies will shed light on this issue.

Taken together, we conclude that heat induces γH2AX 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 γH2AX foci formation under hypoxia.](image)

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温熱は低酸素下でもヒストンγH2AX生成を誘導する

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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γH2AXフォーカスは、一つのDSBsに相当することから、DSBsの生成は、γH2AX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、γH2AX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。

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