Effect of an ATM Kinase Inhibitor on Thermo- and/or Radio-sensitization in Non-proliferating Normal Human Fibroblasts and Osteosarcoma Cells

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Abstract: This aim of the work described here was to determine the effect of an ataxia-telangiectasia mutated protein (ATM) kinase inhibitor on thermo- and/or radio-sensitivity in normal human fibroblasts cells and in osteosarcoma cells in the plateau or confluent phase of cell growth. Cell survival and chromosome aberrations were observed when cells were exposed to a heat shock at 45°C and/or γ-rays in the presence or absence of an ATM kinase inhibitor. Cell survival was measured with colony formation assays. Chromosome aberrations were observed using chemically induced premature chromosome condensation and fluorescence in situ hybridization. Cellular radiosensitivity was enhanced in both cell lines by a heat shock treatment at 45°C. Adding an ATM kinase inhibitor amplified this effect. Furthermore, thermosensitivity in both cell lines at 45°C was enhanced by treatment with an ATM kinase inhibitor. Heat shock treatments at 45°C enhanced the frequency of chromosome aberrations induced by γ-irradiation in both cell lines; in the presence of an ATM kinase inhibitor, the frequency of chromosome aberrations was enhanced. Thus, ATM kinase inhibitors increased the number of heat-induced aberrations. These results were observed in both, normal human fibroblasts cells and osteosarcoma cells. ATM kinase inhibitors can contribute to heat-induced cell killing and to heat-induced cellular chromosome aberration frequencies in the plateau or confluent phase of cell growth.

Key Words: hyperthermia, ATM kinase inhibitor, chromosomal aberrations, non-S phase cells

Introduction

Hyperthermia is an effective modality of cancer therapy. In particular, hyperthermia itself has several effects which increase the degree of tumor cell killing after exposure to ionizing radiation (IR)1-7. In combination with radiotherapy or chemotherapy, hyperthermia increases therapeutic efficiency markedly8,9, and many reports have described the clinical effectiveness of thermal radiation therapy10,11. Clinically, mild hyperthermia at temperatures of less than 42°C is used to enhance an organism’s immune...
activity\(^{(2)}\). However, hyperthermia itself can kill cells directly. While thermal radiation therapy is an effective treatment, many questions still exist concerning the exact cellular mechanisms which act during hyperthermia treatments. Recent studies have shown that DNA double-strand breaks (DSBs) lead to heat shock induced cell killing, because heat shock induces \(\gamma\)-H2AX foci formation\(^{(13)}\). However, it may be unclear whether \(\gamma\)-H2AX foci formation is a consequence of the formation of DNA DSBs.

DSBs are the most severe form of cellular damage induced by ionizing radiation, but this type of lesion can also arise during normal physiological processes such as DNA replication\(^{(14)}\). When DSBs occur, genetic information can be preserved or protected through the activation of cellular DNA damage response pathways, such as DNA repair, cell cycle check points, and apoptosis. The ataxia-telangiectasia mutated protein (ATM), is a member of the phosphoinositide 3-kinase family, and is mutated in the human disease ataxia-telangiectasia. This protein kinase plays a central role in the DNA damage response pathway. In unstressed cells, ATM exists as an inactive dimer. In response to DSBs, ATM rapidly autophosphorylates at Ser1981 and dissociates into active monomers. Active ATM phosphorylates many cellular targets, such as p53, Chk2, H2AX, and BRCA1\(^{(15–18)}\).

A recent study showed that, even in the presence of hyperthermic conditions, ATM kinase is activated, and ATM is phosphorylated\(^{(19)}\). However, it is assumed that the heat-induced signaling pathway is different from that of the IR-induced pathway; thus, the observation of heat-induced ATM does not necessarily lead to the conclusion that hyperthermia induces DNA DSBs. Although radiosensitivity caused by heat shock is observed throughout the cell cycle, the effect is maximal in S phase\(^{(20)}\). In addition, heat-induced DSBs are observed only in S phase\(^{(21)}\), but the number of breaks is very low, and it is not clear whether hyperthermia induces DNA DSBs.

One of the most reliable and sensitive methods of measuring IR-induced damage is the premature chromosome condensation (PCC) technique\(^{(22–24)}\). Recently, a chemically induced PCC technique was described using calyculin A by Gotoh et al.\(^{(25)}\) and Durante et al.\(^{(26)}\). This technique is easier to use than traditional cell fusion methods, and calyculin A can induce PCCs in many types of cells\(^{(25–28)}\). In a previous study, exponentially growing human fibroblasts were exposed to heavy ions, and the PCC technique was used to measure chromosome aberrations in G\(_2\)-phase cells\(^{(29,30)}\).

A specific inhibitor of ATM kinase, 2-morpholin-4-yl-6-thiantherin-1-yl-phyran-4-one (KU55933), inhibits ATM kinase activity, decreases cellular ATM autophosphorylation, and inhibits repair of DNA DSBs\(^{(31)}\). In this study, the effect of KU55933 on cellular sensitivity and chromosome aberrations was examined after treatment with heat shock, IR, and both in human normal fibroblasts and osteosarcoma cells in the plateau or confluent phase of growth.

Materials and methods

Cell lines

The AG1522 cell line, a normal human fibroblast culture derived from the foreskin of 3-day-old males, was obtained from the NIA Aging Cell Repository, and was used at passages 10-13. MG63, a human osteosarcoma cell line\(^{(32–34)}\), was obtained from the American Type Culture Collection (ATCC). Both cell lines were grown in minimum essential medium with 15% fetal bovine serum at 37\(^\circ\)C in a humidified (95%) 5% CO\(_2\) atmosphere.
Irradiation and/or heat shock

Cells were used in when confluent. One hour before irradiation and/or heat shock, KU55933 (1 µl/ml) was added. Cells in T-25 flasks were exposed to γ-ray doses from 0 Gy to 6 Gy, or to a heat shock by being immersed in a water bath. In treatments with heating alone, cells were heated at 45°C for up to 60 min. In combined treatments with heating and IR, cells were irradiated immediately after heat exposure at 45°C for 20 min.

In previous experiments, it was determined that there was no significant effect on survival rates after growth at 37°C and exposure to heat at 45°C for 20 min in either cell line. In addition, there were no significant differences in survival rates in the presence or absence of KU55933.

Cell survival

Cell survival was measured with colony formation assays. After treatments with IR and/or heating, cells were incubated at 37°C for 24 h, and then trypsinized and plated (at different densities depending on the irradiation or heat dose used) in 100 mm dishes. The concentration of cells in suspensions was measured using a Coulter counter. Cells were incubated for 14 days at 37°C, colonies were fixed with 100% methanol, and stained with crystal violet. Survival was assessed by counting colonies containing at least 50 cells.

Premature chromosome condensation (PCC)

A technique using chemically induced PCCs was used to evaluate G0/G1 phase chromosomes. Briefly, 24 h after irradiation and/or hyperthermia, cells were transferred from a T-25 flask to a T-75 flask. Colcemid (0.05 µg/ml) was added to the medium 18 h after subculturing to increase the frequency of mitotic cells. After a further incubation at 37°C, calyculin-A was added (Wako Chemicals, Osaka, Japan; stock concentration 0.1 mM in ethanol; storage was at -20°C until use; final concentration was 50 nM). G2 chromosomes were condensed by incubating the cells for 30 min at 37°C. The parameter used to estimate the number of G2/M chromosomes expected to be present was determined using the equation below after different incubation times.

(Number of G2- and M-phase cells) / (total cells observed) x 100%

The optimal collection time for the post-irradiation G2- and M cells was determined from the G2 and M phase index curve generated from cells collected after various incubation times.

FISH analysis

After treatment with calyculin-A, cells were transferred to a tube and centrifuged for 5 min at 2,000 rpm. The pellet was carefully resuspended in 8 ml of 75 mM KCL then incubated at 37°C. After 20 min, 2 ml of a freshly prepared fixative solution (methanol : glacial acetic acid, 3 : 1 vol./vol.) was slowly added to the solution, and the tubes were again centrifuged. A final wash and fixation in fresh fixative was performed before dropping cells onto a glass slide. Cells were incubated overnight at 37°C on a slide warmer. AG1522 was hybridized in situ with fluorescent DNA whole chromosome probes 1 and 3, and MG63 was hybridized with chromosome probe 18 (Cambio, Cambridge, British). The cells were counterstained with DAPI, and chromosome aberrations were viewed with a Zeiss Axioskop fluorescence
microscope.

**Statistical analysis**

Data were expressed as the mean±SD. Comparisons of each of the surviving fractions were performed with Dunnett’s test. Comparisons of the number of chromosome aberrations were done with the Student’s t-test. A p-value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using Excel 2007 (Microsoft, Washington, WA, USA).

**Results**

**Cell survival after hyperthermia plus irradiation**

Survival curves after exposures to a combination of heat shock, KU55933, and IR are shown in Fig. 1. Heat exposure conditions were at 45°C for 20 min. Although the survival rate of the AG1522 and MG63 cell lines was lower after an exposure to heat shock plus IR, and to IR plus KU55933 than to an exposure to IR alone, the decrease was more pronounced when these cell lines were exposed to IR plus KU55933. These results indicate that the extent of IR-induced cell killing was larger after suppressing ATM kinase activity than after a combined treatment with heat shock. In addition, KU55933 also enhanced the level of cell killing observed after an exposure to IR plus a heat shock.

![Fig. 1. Surviving fractions in confluent normal fibroblasts (AG1522) and osteosarcoma cells (MG63) after exposure to γ-rays. A heat shock (HS) was delivered by an exposure to 45°C for 20 min. KU55933 was added at a concentration of 1 μl/ml. Each point represents the mean and standard error of three independent experiments. Asterisks (*, ** and *** ) indicate statistically significant differences (P < 0.05, 0.01 and 0.001, respectively) using the Dunnett’s test.](image)

**FISH analysis after hyperthermia plus irradiation**

Cellular chromosome aberrations observed after an exposure to 6 Gy of γ-rays are shown in Fig. 2. Results in normal cells and osteosarcoma cells were similar to those seen with the survival curves. The number of cellular chromosome aberrations induced by heat shock plus IR was more than 4 times the number seen after exposure to IR alone. In addition, in the presence of KU55933, the number of aberrations was about two times greater than the number of aberrations seen in the absence of KU55933.
**Fig. 2.** Number of chromosome aberrations in AG1522 and MG63 cells irradiated with 6 Gy. A heat shock (HS) was produced by exposure to 45°C for 20 min. KU55933 was added at a concentration of 1 μl/ml. Bars indicate the standard error of the mean value. Asterisks indicate statistically significant differences ($P<0.05$) using the Student's t-test.

**Fig. 3.** Damage induced in MG63 cells by a 6 Gy irradiation and heat shock in the presence of KU55933. A heat shock was produced by exposure to 45°C for 20 min in plateau phase cells. KU55933 was added at a concentration of 1 μl/ml. Green indicates chromosome No.18. Chromosomal aberrations are indicated by the arrows.

Fig. 3 shows a photograph of chromosome aberrations observed after MG63 cells were exposed to heat shock plus IR in the presence of KU55933; numerous aberrations can be seen.
Cell survival after hyperthermia

Survival curves after a heat shock are shown in Fig. 4. The hyperthermic temperature was 45°C, the survival rate was measured after each heating period, and the survival rate decreased with increasing heating times. The survival rate showed larger decreases in the presence of KU55933 than with hyperthermia alone. Similar results were obtained with both cell lines. These results demonstrate that ATM kinase contributes to heat-induced cell killing.

![Graph of AG 1522 and MG 63 cell survival](Image)

**Fig. 4.** Surviving fractions in confluent normal fibroblasts (AG1522) and osteosarcoma cells (MG63) as a function of heating time. A heat shock was produced by exposure to 45°C. Each point represents the mean and standard error from three independent experiments. Asterisks (*, ** and ***) indicate statistically significant differences ($P<0.05$, $0.01$ and $0.001$, respectively) using Dunnett’s test.

![Graph of AG 1522 and MG 63 chromosome aberrations](Image)

**Fig. 5.** Number of heat-induced chromosome aberrations produced by exposure to 45°C for 60 min. KU55933 was added at a concentration of 1 μl/ml. The bars indicate the standard error of the mean. Asterisks (*) and **) indicate statistically significant differences ($P<0.01$ and $0.001$, respectively) using the Student’s t-test.
**FISH analysis after hyperthermia**

Cellular chromosome aberrations observed after a heat shock at 45°C for 60 min are shown in Fig. 5. These aberrations occurred after a heat shock alone. In the presence of KU55933, the number of aberrations increased by about a factor of two. These results indicate that ATM contributes to the repair of heat-induced chromosome aberrations. However, the number of chromosome aberrations induced by hyperthermia alone was very small, and was less than half of the number induced by 6 Gy of γ-rays.

![Heat shock alone](image)

Fig. 6. Damage induced by hyperthermia alone in AG1522 cells. A heat shock was produced by exposure to 45°C for 60 min. Red indicates chromosome No.1 and green indicates chromosome No.3. The yellow circle shows a chromosome aberration. The lower left photograph shows the same image before dyeing with a probe.

Fig. 6 shows chromosome aberrations which resulted when AG1522 cells were kept at 45°C for 60 min in the presence of KU55933. Chromosome aberrations can be seen, even when cells are subjected to heat shock alone in the plateau phase of growth.

**Discussion**

Currently, thermal therapy is used widely in therapeutic efforts. Hyperthermia is known to increase an organism’s immune activity. This action is observed with so-called mild hyperthermia using temperatures of less than 42°C. Clinical thermal therapy involving mild hyperthermia is often used to increase an organism’s immune activity. However, hyperthermia can cause a direct cytotoxic reaction,
although the mechanism through which hyperthermia kills cells is not yet clear.

Many studies have shown that hyperthermia effectively increases radiosensitivity in cancer therapy\textsuperscript{33}. Although increases in the level of IR-induced cell killing by hyperthermia are observed throughout the cell cycle, it becomes maximal in S phase\textsuperscript{31}. According to several reports, the repair of IR-induced DNA damage is inhibited by hyperthermia at temperatures of 42°C or higher\textsuperscript{17}. In the present experiments, it was confirmed that the survival rate in normal and osteosarcoma cells treated with a combination of hyperthermia at 45°C plus IR was lower than after exposure to IR alone. In the presence of KU55933, the survival rate following heat shock plus IR was lower than with IR alone in both cell lines. When chromosomes were observed following a 6 Gy irradiation, the largest number of chromosome aberrations was observed after treatment with hyperthermia plus IR. These results suggest that heat shock inhibits IR-induced DNA damage repair, and that hyperthermia can be an effective means to increase radiosensitivity in the plateau growth phase in normal cells and osteosarcoma cells.

The experiments reported here showed that with a 45°C heat shock, the survival rate decreased linearly with exposure time. Furthermore, the survival rate showed a larger decrease in the presence of KU55933. These results were observed in both normal cells and osteosarcoma cells. In addition, cellular chromosome aberrations were seen after hyperthermia alone, but the number of aberrations increased in the presence of KU55933.

According to Hunt et al., hyperthermia appears to enhance ATM kinase activity and increase cellular ATM autophosphorylation\textsuperscript{39}. Their study showed that hyperthermia induced MDC1 foci formation; however, the formation of 53BP1 and SMC1 foci was not observed in heated cells but was seen in irradiated cells. Furthermore, their study showed that the induction of chromosomal DNA strand breaks was observed in IR-exposed but not in heated cells. In the present experiments, ATM kinase activity was inhibited by KU55933. From the fact that the number of cellular chromosome aberrations increased in the presence of KU55933, it can be hypothesized that ATM is associated with heat-induced cell killing, and that there is an induction of heat-induced DNA DSBs.

A recent study showed that heat-induced DSBs were observed only in S phase\textsuperscript{39}. According to Takahashi et al., hyperthermia induced γ-H2AX foci formation, and chromosomal DNA DSBs are induced by hyperthermia alone\textsuperscript{13,36,37}. In the present experiment, confluent cells were used, and most cells appeared to remain in the plateau or confluent phase. Thus, it appears that chromosomal DNA DSBs are induced by hyperthermia, and that these are observed in the plateau phase. The fact that cellular chromosomal aberrations caused by hyperthermia are observed in the plateau phase is an important observation. The central portion of solid tumors is hypoxic, and under these conditions, most cells are in the Go/G\textsubscript{1} phase. If cellular chromosome aberrations caused by hyperthermia occur in Go phase cells in vivo, this would be clinically significant.

At a temperature of 45°C, protein alterations occur\textsuperscript{39}. Both, the normal cells and the osteosarcoma cells which were used in the present experiments are adherent cells. It is thought that their survival rate decreases after a heat exposure due to cell membrane damage. Such damage could be caused by protein alterations and result in aberrant adhesion\textsuperscript{39}. However, even if this does occur, there is no explanation currently available to explain the decreased survival rate caused in the presence of KU55933. However, it can be seen that heat-induced ATM contributes to the repair of DNA damage caused by hyperthermia.
In examining the present results, the index indicating the number of chromosomes expected to be present in heat treated cultures, and thus the number of heat-induced cellular chromosome aberrations, is very low, and it is doubtful that all of the observed DNA damage occurred in plateau phase cells. Although confluent cells were used, it is likely that not all of the cancer cells remained in the $G_2/G_1$ phase, and aberrations were observed in at least a few cells which had progressed to S phase. Additional studies will be needed to examine this, and to determine if all of the observed aberrations were induced in confluent cells.

References
