Hyperthermia and superoxide generation from cancer cells · S. Kokura et al.

Original Contribution

Hyperthermia Enhances Free Radical-dependent Cytotoxicity of Gamma-linolenic Acid on AH109a Rat Hepatocellular Carcinoma Cells

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Abstract: Previous studies have indicated that polyunsaturated fatty acids can induce the suppression of cancer growth and that this action is free radical dependent. Recently, we demonstrated the antitumor efficacy of hyperthermia combined with gamma-linolenic acid in vivo. In the present study, we examined the effect of hyperthermia (43°C) on the generation of superoxide from rat hepatocellular carcinoma cells stimulated by polyunsaturated fatty acids. The cytotoxicity of gamma-linolenic acid for the cancer cells was markedly stronger than that of other polyunsaturated fatty acids (oleic acid, linoleic acid, or alpha-linolenic acid) at 37°C, and greatly enhanced at 43°C. In addition, both superoxide dismutase and α-tocopherol were able to inhibit the gamma-linolenic acid-induced cytotoxicity at 43°C, indicating a role for superoxide in this process. Compared with oleic acid, linoleic acid, and alpha-linolenic acid, gamma-linolenic acid significantly increased the generation of superoxide from hepatocellular carcinoma cells at 43°C. Moreover, the generation of superoxide from AH109a carcinoma cells stimulated by gamma-linolenic acid or alpha-linolenic acid was significantly higher at 43°C than 37°C. The extent of polyunsaturated fatty acids-induced cytotoxicity significantly correlated with that of polyunsaturated fatty acids-induced superoxide generation. Therefore, this study suggests that hyperthermic enhancement of gamma-linolenic acid-induced generation of superoxide contributes to the hyperthermic potentiation of gamma-linolenic acid-induced cytotoxicity.

Key words: hyperthermia, superoxide, gamma-linolenic acid, hepatocellular carcinoma, polyunsaturated fatty acid

Introduction

Recent studies have suggested that cis-unsaturated fatty acids such as gamma-linolenic acid (GLA) and eicosapentaenoic acid (EPA) can kill cancer cells selectively120) and that this cis-unsaturated fatty acids-induced tumoricidal action is free radical reaction dependent and lipid peroxidation dependent35).

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Our previous studies indicated that free radical reactions and lipid peroxidation contribute to the antitumor effect of hyperthermia, and that oral administration of EPA specifically increases the susceptibility of liver tumor tissue to attack by lipid peroxidation, and hence enhances the antitumor effect of hyperthermia. We also reported that the combination of hyperthermia with gamma-linolenic acid enhanced the antitumor effect of gamma-linolenic acid in vivo. Thus, understanding the mechanisms by which hyperthermia enhances the cytotoxicity of gamma-linolenic acid is important.

Ramesh G. & Das UN reported that GLA and EPA augmented the generation of superoxide anion in methylcholanthrene-induced sarcoma cells. Carcinoma cells and neutrophils are known to generate superoxide in response to several stimulations. Our previous study further suggested that superoxide generation from neutrophils was temperature-dependent, and that neutrophils most strongly generate superoxide at 41°C. These findings led us to hypothesize that hyperthermia enhances the gamma-linolenic acid-induced generation of superoxide, which contributes to the hyperthermic potentiation of gamma-linolenic acid-induced cytotoxicity.

Thus, the major objectives of the present study were to investigate the mechanisms by which hyperthermia enhances gamma-linolenic acid-induced cytotoxicity.

Materials and Methods

Cell lines

The rat hepatocellular carcinoma cell line, AH109a, was supplied by the Cancer Cell Repository of the Research Institute for Tuberculosis and Cancer, Tohoku University (Sendai, Japan). Rat AH109a carcinoma cells were injected intraperitoneally into rats. For experiments, cells were collected from the ascites of these rats, washed in Hanks’ balanced salt solution (HBSS), and seeded at 1.2 x 10^6 cells/sample in plastic tubes and incubated at 37.0°C or 43.0°C for 30 min by immersing the tubes containing the 3 ml cell suspension into a precision controlled water bath. Culture media containing various concentrations of polyunsaturated fatty acids [oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (ALA), or gamma-linolenic acid (GLA), Sigma Chemical Co., St. Louis, MO] was added to the cell suspensions in the plastic tubes just before heating.

Cell viability assay

After hyperthermia with various concentrations of polyunsaturated fatty acids, the cells were assayed for their viability using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) test as described previously.

Measurement of superoxide generation by the chemiluminescence assay

The chemiluminescence assay was performed using a Luminescence Reader (Aloka Co., Tokyo, Japan) and 2-methyl-6-(p-methoxyphenyl)-3,7-hydroimidazol[1,2-α]pyrazin-3-one (MCLA) as described previously. MCLA is a Cypridina luciferin analogue developed by Nakano et al, which is highly specific for superoxide. Diethylenetriaminepentaacetic acid (DETAPAC, final concentration 100μM, Sigma Chemical Co., St. Louis, MO) was added to rat AH109A carcinoma cells suspended in HBSS (1.2 x 10^6 cells / 3 ml) and incubated at 37.0°C or 43.0°C for 1 min. MCLA (final concentration 1μM)
was added, and the mixture then incubated for 2 min at the same temperature. OA, L.A, ALA, and GLA at a final concentration of 240μM were used as stimulators. The amount of luminescence was indicated as the maximum light intensity. That the observed luminescence is derived from the superoxide ions is confirmed by the fact that quenching of luminescence was observed in a reaction system pretreated with superoxide dismutase (final concentration 10 unit/ml, Nippon Kayaku Co., Tokyo, Japan).

Administration of free radical scavengers and α-tocopherol

CuZnSOD and/or catalase (Sigma Chemical Co., St. Louis, MO), and α-tocopherol (Sigma Chemical Co., St. Louis, MO) were added to the cell suspensions in plastic tubes just before the addition of GLA. The final concentrations of CuZnSOD, catalase, and α-tocopherol were 100 unit/ml, 100 unit/ml, and 100 μM, respectively.

Statistical analysis

Data represent mean ± SE of triplicate samples in a representative experiment. Data were compared using an analysis of variance (ANOVA) followed by Scheffe test. A level of p < 0.05 was accepted as statistically significant.

Results

Hyperthermic enhancement of polyunsaturated fatty acids-induced cytotoxicity

We examined the cytotoxicity of polyunsaturated fatty acids at 37.0°C or 43.0°C using the MTT assay. At 37°C, only GLA-treated AH109a cells showed a significant decrease in viability at 240μM GLA (Fig. 1a). Hyperthermia alone (43.0°C, 30 min) did not affect the viability of AH109a carcinoma cells (data not shown). On the other hand, both GLA- and ALA-treated AH109a cells showed a significant decrease in viability at 43.0°C (Fig. 1b).

![Graph 1a](image1.png)

(a)

![Graph 1b](image2.png)

(b)

Fig. 1. Enhancement of the cytotoxic effects of various poly-unsaturated fatty acids (PUFA in figure) with hyperthermia.

(a) Cytotoxicity of various concentrations of oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (ALA), and gamma-linolenic acid (GLA) at 37.0°C. (b) Cytotoxicity of various concentrations of poly-unsaturated fatty acids with hyperthermia (43°C). After 30 min treatment, cells were subjected to the MTT assay and the percentages of viable cells noted. Data represent mean ± SE of triplicate samples in a representative experiment. Similar results were obtained in three independent experiments. *p < 0.01, **p < 0.0001 compared with untreated control.
Hyperthermic enhancement of alpha- and gamma-linolenic acid-induced superoxide generation

Following stimulation by polyunsaturated fatty acids, chemiluminescent intensity derived from AH109a carcinoma cells gradually peaked but remained low thereafter. No luminescence was observed when SOD (10 units/ml) was added to the reaction system before stimulation. At 37°C, each polyunsaturated fatty acid resulted in a different chemiluminescent intensity, however all were under $5 \times 10^4$ counts/min. When AH109a carcinoma cells were incubated at 43.0°C for 5 min. before the chemiluminescence assay, the chemiluminescent intensity following ALA or GLA stimulation was significantly higher than that at 37°C. Furthermore, the chemiluminescent intensity following GLA stimulation was significantly higher than that following ALA stimulation at 43.0°C (Fig. 2).

The effects of CuZnSOD, catalase, and α-tocopherol on the cytotoxicity of gamma-linolenic acid combined with hyperthermia

We next examined the effects of CuZnSOD, catalase, and α-tocopherol on the cytotoxicity of GLA combined with hyperthermia. Hyperthermia alone, CuZnSOD plus catalase, and α-tocopherol alone did not affect viability compared to the control (37°C, 30 min incubation). In addition, GLA combined with hyperthermia treatment resulted in less than 25% viability. CuZnSOD and CuZnSOD plus catalase significantly blocked the GLA plus hyperthermia-induced cytotoxicity (Fig. 3a). α-tocopherol showed the same inhibitory effect (Fig. 3b).

![Fig. 2. Influence of hyperthermia on the luminescence generated from carcinoma cells stimulated by various polyunsaturated fatty acids. Final concentration of OA, LA, ALA, and GLA was 240µM. Data represent mean ± SE of triplicate samples in a representative experiment. Similar results were obtained in four independent experiments. *p < 0.01 compared with intensity of ALA or GLA at 37.0°C. # p < 0.01 between ALA and GLA at 43°C.](image)

![Fig. 3. Effects of (a) SOD and/or catalase, and (b) α-tocopherol on GLA combined with hyperthermia-induced cytotoxicity. Final concentrations of CuZnSOD, catalase, and α-tocopherol were 100 unit/ml, 100 unit/ml, and 100 µM, respectively. Data represent mean ± SE of triplicate samples in a representative experiment. Similar results were obtained in three independent experiments. *p < 0.0001 compared with control. # p < 0.001 compared with GLA-treated group.](image)
The relationship between superoxide generation and cytotoxicity of poly-unsaturated fatty acids in hepatocellular carcinoma

To define the relationship between the generation of superoxide and cytotoxicity of poly-unsaturated fatty acids, cytotoxicity was plotted as a function of the generation of superoxide in Fig. 4. This analysis revealed linear correlation ($r = -0.861$, $p < 0.001$) between superoxide generation by poly-unsaturated fatty acids and cytotoxicity after treatment.

Discussion

This study provided evidence to support the hypothesis that hyperthermia enhances GLA-induced superoxide generation from hepatocellular carcinoma cells. Many poly-unsaturated fatty acids including GLA induce superoxide generation by cancer cells, and the generation of superoxide is the main mechanism for the anti-cancer action of poly-unsaturated fatty acids. Neutrophils are known to generate superoxide in a temperature-dependent manner in response to several stimuli. Our previous study indicated that neutrophils most strongly generate superoxide at 41°C. On the other hand, in a cell-free system, DNA produces superoxide by stimulus of cisplatin, and hyperthermia enhances this production. The present study clearly shows that hyperthermia enhances the generation of superoxide from rat hepatocellular carcinoma upon stimulation by poly-unsaturated fatty acids, in particular GLA. Our previous studies have demonstrated that the combination of GLA with hyperthermia enhances the antitumor effect of hyperthermia on rat hepatocellular carcinoma in vivo. These previous studies also showed that the enhanced antitumor effect of the combination of hyperthermia and GLA is due to the increase in free radical reactions in tumor tissue. The present results showing that hyperthermia enhances the cytotoxicity of GLA and that SOD or a-tocopherol significantly attenuates the cytotoxicity of GLA suggest that hyperthermic enhancement of superoxide generation from carcinoma cells may contribute to their cytotoxicity. This scenario is supported by the present result that shows positive correlation between the ability of fatty acids to generate superoxide and cytotoxicity of fatty acids. Although the efficacy of combining polyunsaturated fatty acids and radiation, chemotherapy, or hyperthermia is relatively well known, the details of the mechanisms by which the cytotoxicity is enhanced remains incomplete. Our results indicate that the transferred GLA itself is responsible for the cell toxicity, although whether the GLA is taken up into the cell or remains on the membrane surface is unclear. The coexistence of a-tocopherol inhibits GLA-induced cytotoxicity in various cells such as Hela cells and leukemic cells. In the present study, we also confirmed that the coexistence of 100μM a-tocopherol inhibits the cytotoxicity induced by GLA.

![Fig. 4. Scatter plots showing the relationship between the cytotoxicity of poly-unsaturated fatty acids and superoxide generation by poly-unsaturated fatty acids in hepatocellular carcinoma cell line. There is inverse correlation between cytotoxicity after treatment and superoxide generation by poly-unsaturated fatty acids. $r = -0.861$, $p < 0.001$.](image)
combined with hyperthermia, suggesting that the mechanism of GLA-induced cytotoxicity is the same in various cell lines. Our results show that exogenous addition of SOD or SOD plus catalase inhibits GLA combined with hyperthermia-induced cytotoxicity. Thus, superoxide generated on the outside of the cells appears to be responsible for cytotoxicity. There have been reports that oxygen radicals contribute to apoptosis in various cells\(^{21,22}\). Activation of superoxide generation in neutrophils has been suggested to be associated with the influx of Ca\(^{2+}\) into the cytoplasm from extracellular space\(^{23,24}\). Oyamada\(^{13}\) also reported the importance of influx of Ca\(^{2+}\) into vascular endothelial cells in superoxide generation. Furthermore, poly-unsaturated fatty acids have been shown to increase the intracellular concentration of Ca\(^{2+}\)\(^{25,26}\). Recently, Kameda et al.\(^{27}\) reported that hyperthermia increases the intracellular concentration of Ca\(^{2+}\) in the U937 cell line and that the intracellular Ca\(^{2+}\) increase plays a crucial role in apoptosis induced by hyperthermia. Taken together with these reports, we suggest that GLA plus hyperthermia activates superoxide generation by carcinoma cells due to an increase in the intracellular Ca\(^{2+}\) level. The mechanisms of the hyperthermic enhancement of GLA-induced generation of superoxide are unclear. More detailed studies are in progress to clarify the action of GLA combined with hyperthermia and to investigate more effective combinations of poly-unsaturated fatty acids with hyperthermia for cancer therapy.

References

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温熱療法は、ラット肝臓癌細胞に対するγ-リノレン酸の
フリーラジカル依存性殺細胞作用を増強する

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要　旨：不飽和脂肪酸が、フリーラジカル反応を惹起することにより、癌の発育を抑制することが、
in vitro, in vivo で報告されている。また、近年、われわれは、不飽和脂肪酸を前投与することで、温熱療法の抗癌作用が増強することを明らかとし報告した。本検討では、不飽和脂肪酸、特にγ-リノレン酸のスーパーオキシド産生能、温度依存性に増強し、そのことが、殺細胞効果の増強を導いていることを明らかとした。不飽和脂肪酸の中では、2重結合の数の多いほど、また、2重結合が同数の3つのα-リノレン酸とγ-リノレン酸では、γ-リノレン酸のほうが、スーパーオキシド産生能が高く、殺細胞作用も強かった。
以上より、不飽和脂肪酸 (とくにγ-リノレン酸) 併用温熱療法の増感作用機序に、スーパーオキシドが関与していることが示唆された。