Catalase Contents in Cells Determine Sensitivity to the Apoptosis Inducer Gallic Acid

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Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is known to induce apoptosis in cancer cells at lower IC_{50} values compared with values for normal cells. Apoptosis is inhibited completely by the addition of conditioned medium from cultured hepatocytes, whereas it is not prevented by conditioned media from tumor cells. We therefore studied the reason for the different response to GA-induced apoptosis. GA-induced dRLh-84 cell death was completely abolished by the addition of peroxisome or cytosol as well as conditioned medium from primary cultured rat hepatocyte. As GA-induced cell death is known to be mediated by reactive oxygen species (ROS) and intracellular Ca^{2+} , we determined the type of ROS generated by GA and found that GA generated hydrogen peroxide in culture medium. The addition of hydrogen peroxide generated by GA induced cell death in dRLh-84 cells. These results suggest that GA-induced cell death is mediated by hydrogen peroxide. On the other hand, the inhibitory activity of hepatocyte medium on GA-induced cell death was completely abolished by anti-catalase antibody. When the amount of catalase antigen was determined by Western blotting analysis, conditioned medium and the cytoplasm of hepatocytes contained high concentrations of catalase. Conditioned media from various tumor cell lines did not contain catalase, and the cytoplasm contained only low levels of catalase. These results show that GA-sensitive cells, including various tumor cells, produce only small amounts of catalase and secrete little enzyme into media, suggesting a lack of protective machinery against GA. In contrast, GA-insensitive cells, including hepatocytes, produce large amounts of catalase and release it in medium, resulting in the development of insensitivity to GA. In conclusion, catalase contents in cells determine different sensitivity to GA.

Key words gallic acid; apoptosis; hydrogen peroxide; selectivity

Apoptosis is biological cell death for normal tissue homeostasis, cellular differentiation, and development within a multicellular organism and is regulated by the balance between its activators and inhibitors. In addition, apoptosis is also induced in tumor cells by various anticancer therapeutic agents. A common feature of cells undergoing apoptosis is the activation of caspases, a family of aspartate-specific cysteinyl proteases.¹⁾ Many substances such as Fas, tumor necrosis factor (TNF)- α , UV irradiation, and anticancer agents are known to cause caspase activation and result in apoptosis induction.^{2–6)} As intracellular regulators of apoptosis, the two proteins Smac and DIABLO that promote caspase activation by eliminating the inhibitor of apoptosis (IAP), were identified.^{7,8)} Caspase-mediated proteolysis of specific proteins results in an irreversible commitment of cells to undergo apoptosis characterized by specific morphological and biochemical alteration. To date, numerous caspase substrates that contribute to the apoptotic response have been reported.^{9–11)}

On the other hand, various substances have been reported to inhibit apoptosis. For example, interleukin (IL)-10 inhibits apoptosis in infectious T cells, IL-2-starved T cells, and germinal center B cells,^{12–14)} and nitric oxide prevents B cell apoptosis through the maintenance of bcl-2 levels.¹⁵⁾ Furthermore, intracellular inhibitors of the caspases were recently identified in humans, and at least three of the IAP family proteins, MIHA, XIAP, and h-ILP, are able to bind to caspases-3 and -7 and pro-caspase-9 directly^{16,17)} and inhibit apoptosis induced by chemotherapeutic drugs or UV irradiation and expression of Bax or caspases.^{18–20)} In addition, Bcl-2 family proteins including Bcl-2 and Bcl-X_L are major regulators of the mitochondria-initiated caspase activation pathway and prevent the release of cytochrome c in the presence of apop-

totic stimuli.^{21,22)} Conversely, the proapoptotic members of this family, such as Bad, Bax, and Bid, translocate from other cellular compartments to mitochondria in response to proapoptotic stimuli and promote cytochrome c release.²³⁾ Taken together, accumulating evidence indicates that the balance between cytotoxic and cytoprotective activities determines whether cells undergo apoptosis or not.

In our previous study, gallic acid (3,4,5-trihydroxybenzoic acid, GA), a naturally occurring plant phenol that has been reported to show a number of biological activities,^{24–27)} was found to induce apoptosis in tumor cells such as HL-60RG, HeLa, dRLh-84, PLC/PRF/5, and KB cells with higher sensitivity than in normal cells such as rat primary cultured hepatocytes, keratinocytes, macrophages, endothelial cells, and fibroblasts.^{28,29)} The mechanism underlying the difference in sensitivity was partly explained by the difference in the amount of inhibitors produced by hepatocytes and tumor cells.³⁰⁾ In addition, studies of the signaling pathway leading to apoptosis revealed that an increase in intracellular reactive oxygen species (ROS) or Ca^{2+} plays an important role in eliciting an early signal for apoptosis,^{31,32)} and caspase-3 activation and that increases in FKBP12, Pin1, and histone H3.1 followed the increase in ROS or Ca^{2+} (unpublished data). This study was undertaken to clarify the underlying mechanism of the selective cytotoxicity shown by GA and to determine the inhibitor of GA-induced cell death.

MATERIALS AND METHODS

Chemicals GA (Nacalai Tesque Co., Kyoto, Japan) was recrystallized from hot water and used in the following experiments. RPMI-1640 medium and fetal calf serum (FCS) were purchased from Irvine Scientific Co. (Santa Ana, CA,

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U.S.A.). Antibiotics (penicillin and streptomycin) were from Life Technologies Inc. (Grand Island, NY, U.S.A.). Anti-catalase (human erythrocyte) antibody and alkaline phosphatase-conjugated anti-rabbit IgG antibody were obtained from Calbiochem/Novabiochem Co. (San Diego, CA, U.S.A.) and New England Biolabs, Inc. (Beverly, MA, U.S.A.), respectively. Scopoletin and horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture dRLh-84 (rat hepatoma), HeLa (human epithelioid carcinoma), PLC/PRF/5 (human hepatoma), KB (human epidermoid carcinoma), HLE (human hepatoma, nondifferentiated), and HUH-7 (human hepatoma, differentiated) cell lines were provided by the Health Science Research Resources Bank (Sennan, Osaka, Japan). dRLh-84 cells were cultured in MEM-Eagle's salts medium (with nonessential amino acids) supplemented with 20% bovine serum, penicillin 50 U/ml, and streptomycin 50 μ g/ml. PLC/PRF/5 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, lactalbumin hydrolysate 6.5 mg, penicillin 50 U/ml, and streptomycin 50 μ g/ml. HeLa and KB cells were cultured in MEM-Eagle's salts medium (with nonessential amino acids) supplemented with 10% FCS, penicillin 50 U/ml, and streptomycin 50 μ g/ml. HLE and HUH-7 cells were cultured in Dulbecco's MEM medium supplemented with 10% FCS, penicillin 50 U/ml, and streptomycin 50 μ g/ml.

Preparation of the Conditioned Medium of Hepatocytes or Tumor Cells Hepatic parenchymal cells were isolated from male Sprague-Dawley rats (300 g) by the two-step collagenase perfusion method of Seglen.³³ Cell viability was determined by the 0.25% trypan blue dye-exclusion method, and cells with viability greater than $91.3 \pm 1.0\%$ ($n=9$) were used in this study. Hepatocytes were seeded into collagen-coated culture dishes at a density of 5×10^5 cells/well and incubated in William's E medium (pH 7.4), supplemented with 10% FCS, penicillin 100 units/ml, streptomycin 100 μ g/ml, insulin 10^{-7} M and dexamethazone 10^{-7} M. After incubation for 4 h at 37 °C in an atmosphere of 95% air and 5% CO₂, the hormone-containing medium was removed from the wells. The cells were washed twice with phosphate-buffered saline (PBS) and then incubated in William's E medium without FCS for 24 h. The resulting culture-conditioned medium was used in the following study. The culture media derived from dRLh-84, HeLa, PLC/PRF/5, KB, HLE, and HUH-7 cells were prepared as follows. After each cancer cell culture had become confluent, the culture medium was changed to fresh medium without serum, followed by incubation in an atmosphere of 95% air and 5% CO₂ for 24 h. The resulting culture-conditioned medium was used in the following study.

Preparation of Peroxisomal, Cytosol, and Microsomal Fractions Hepatic parenchymal cells, which were isolated as described above, were homogenized in Tris-HCl 20 mM (pH 8.0)/NaCl 137 mM/10% glycerol/protease inhibitor cocktail tablets (Roche Diagnostics Inc., Penzberg, Germany) at 4 °C and centrifuged at $700 \times g$ for 10 min. The resulting supernatant was centrifuged at $20000 \times g$ for 10 min, and the precipitate was used as the peroxisomal fraction. The supernatant was centrifuged at $100000 \times g$ for 1 h to give the cytosol and microsomal fractions as the supernatant and precipitate, respectively.

Western Blotting Analysis Conditioned medium or the

cytosol fraction of cultured cells (protein 20 μ g) were subjected to 10% SDS-PAGE analysis. After electrophoresis, the separated proteins were transferred to PVDF membranes (Bio Rad Laboratories, Hercules, CA, U.S.A.) using transfer buffer (Tris-HCl 25 mM/glycine 192 mM/0.02% SDS/20% methanol). Membranes were incubated with blocking buffer (5% powdered skimmed milk) in PBS for 2 h and incubated with a 1:2500 dilution of anti-catalase antibody at 4 °C overnight. The membranes were washed in four changes of wash buffer (0.1% Tween-20 in PBS) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody in 1% powdered skimmed milk/0.1% tween-20 in PBS for 1 h at room temperature. Membranes were washed in five changes of wash buffer, and proteins were detected using CDP-StarTM (Roche Diagnostics) as the substrate of alkaline phosphatase.

Determination of Hydrogen Peroxide Various concentrations of GA dissolved in 50 μ l of culture medium were plated into 96-well plates and incubated for 2 h. Scopoletin and horseradish peroxidase were added at concentrations of 24 μ M and 50 μ g/ml, respectively, and incubation followed for 30 min. The assay mixture was fluorometrically measured at 460 nm. Hydrogen peroxide was used as the standard.

Assay of GA-Induced Cell Death dRLh-84 cells (5×10^4 cells/ml) were seeded into a 96-well plates and cultured for 12 h. The conditioned media with or without anti-catalase antibody (20 or 100 μ g) were added to dRLh-84 cell cultures 30 min before the addition of GA 50 μ g/ml. Following 6-h culture in 5% CO₂ in air, cell viability was determined by measuring the number of surviving cells using the 3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.³⁴ In the case of hydrogen peroxide addition, cytotoxic activity was measured using a similar method.

RESULTS

We have previously shown that GA is selectively cytotoxic against cancer cells with lower IC₅₀s values compared with the values for normal cells.²⁸ In addition, GA-induced apoptosis of dRLh-84 cells was completely inhibited by a conditioned medium from cultured hepatocytes, whereas it was not prevented by the conditioned media from cultured cancer cells.³⁰ We therefore studied the reason for the different response to GA-induced cytotoxicity. To determine the localization of the inhibitory activity in cells, we first prepared the peroxisomal, cytosol, and microsomal fractions in addition to the conditioned medium from isolated primary cultured rat hepatocytes as described in Materials and Methods.

As shown in Fig. 1, GA killed rat hepatoma dRLh-84 cells at a concentration of 50 μ g/ml after 6-h incubation. Addition of the hepatocyte medium and the peroxisomal and cytosol fractions to dRLh-84 cells, however, inhibited GA-induced cell death completely.

Our previous study demonstrated that GA-induced cell death was mediated by ROS and intracellular Ca²⁺, as evidenced by the finding that catalase, N-acetyl cysteine, and intracellular Ca²⁺ chelator effectively abolished it.^{29,31,32} Therefore we next examined whether GA generated hydrogen peroxide in the culture medium during incubation. As GA was decomposed in culture medium during 3-h incubation (data not shown), the generation of hydrogen peroxide was mea-

Fig. 1. Effect of Medium from Cultured Hepatocytes, and Peroxisome, Cytosol, and Microsome Fractions of the Cells on GA-Induced Cell Death of dRLh-84 Cells

Hepatocyte conditioned medium, and peroxisomal, cytosol, and microsomal fractions, which were prepared as described in Materials and Methods, were added at a concentration of protein 50 $\mu\text{g}/\text{ml}$ to dRLh-84 cells 30 min before the addition of GA 50 $\mu\text{g}/\text{ml}$. After 6-h incubation, cell viability was determined by the MTT method. Each value represents the mean $\pm$ S.E. of 6 wells.

Fig. 2. Determination of H₂O₂ Generation in the Medium after Addition of GA

GA was added at the indicated concentrations to the culture medium. After 2-h incubation, H₂O₂ generation was determined by the scopoletin method described in Materials and Methods.

sured after 2-h incubation using the scopoletin method. GA was found to generate hydrogen peroxide in a dose-dependent manner (Fig. 2). The concentrations of hydrogen peroxide generated by GA and that which induced cell death in dRLh-84 cells are nearly comparable, as shown in Fig. 3. These results strongly suggest that GA induces apoptosis by generating hydrogen peroxide. This was supported by the finding that the addition of anti-catalase antibody in culture abolished the inhibitory activity in hepatocyte conditioned medium (Fig. 4).

The contents of catalase in conditioned media and cytoplasm were measured by Western blotting analysis. Of the conditioned media prepared from various cell cultures, only the medium from hepatocytes contained easily detectable catalase, and the media from other tumor cells appeared unlikely to contain catalase (Fig. 5A). Similarly, when the catalase contents in the cytoplasm of various cells were determined, hepatocytes showed higher levels of catalase than the other tumor cells, including epithelioid carcinoma and epidermoid carcinoma, except for hepatoma (Fig. 5B). This indicates that tumor cells with low contents of catalase, especially intracellular catalase, become sensitive to GA and that they may not be able to secrete catalase differently from he-

Fig. 3. Cytotoxicity of H₂O₂ against dRLh-84 Cells

H₂O₂ at the indicated concentrations was added to dRLh-84 cells (5×10^4 cells/ml). After 6-h incubation, cell viability was determined by the MTT method. Each value represents the mean $\pm$ S.E. of 6 wells.

Fig. 4. Effect of Anti-catalase Antibody on the Inhibitory Activity of Medium from Cultured Hepatocytes against GA-Induced Cell Death of dRLh-84 Cells

Hepatocyte conditioned media (100 $\mu\text{g}/\text{ml}$) were treated with 20 or 100 μg of anti-catalase antibody at 37 $^{\circ}\text{C}$ for 30 min. Thereafter, the immune complex was removed by incubating with protein G Sepharose for 30 min, followed by centrifugation at $1000 \times g$ for 5 min. The resulting supernatant was added to dRLh-84 cells before the addition of 50 $\mu\text{g}/\text{ml}$ of GA and cell viability was determined by the MTT method. Lane 1, no hepatocyte conditioned medium (HCM); lane 2, HCM; 3, HCM absorbed with 20 μg of anti-catalase antibody; 4, HCM absorbed with 100 μg of anti-catalase antibody.

Fig. 5. Determination of Catalase in Culture-Conditioned Media (A) and Cytoplasm (B) of Various Cells by Western Blotting Analysis

Conditioned media (20 μg) and cytoplasm (20 μg) prepared from hepatocytes or cancer cells were subjected to SDS-PAGE. Catalase was detected by Western blotting analysis using anti-catalase antibody.

patocytes.

DISCUSSION

ROS are known to be involved in various diseases including carcinogenesis, atherosclerosis, and inflammatory disorders, and aging. In addition, ROS are highly cytotoxic and produce alterations in lipids, proteins, and cellular structures,

causing a variety of pathologies. Therefore free radical research has so far focused on disease treatment with radical scavengers. On the other hand, to prevent damage by ROS, cells have developed several antioxidant systems. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) in mammalian cells. The two predominant systems for decomposition of endogenously produced hydrogen peroxide in the liver are catalase and GPx.³⁵⁾ The relative contributions of catalase and GPx in the decomposition of endogenously generated hydrogen peroxide are determined largely by the subcellular localizations of the enzymes in liver cells.³⁶⁾

Catalase plays only a minor role in hydrogen disposal at low rates of hydrogen peroxide generation in the endoplasmic reticulum, but its function increases as the rate of hydrogen peroxide production is enhanced. In erythrocytes, it was reported that GPx and catalase play a principal role in hydrogen peroxide metabolism at low and high hydrogen peroxide generation rates, respectively.^{37,38)} In addition, it was reported that MCF-7 breast cancer cells, which develop resistance to hydrogen peroxide, show elevated catalase activity, but not GPx, explained by stabilization of catalase mRNA³⁹⁾; a variant line of Chinese hamster ovary cells become resistant to hydrogen peroxide with increased catalase activity⁴⁰⁾; and overexpression of catalase in the cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury.⁴¹⁾ Furthermore, MCF-7 cells transfected with the cDNA of human GPx are found to be relatively resistant to hydrogen peroxide, but not to radiation or doxorubicin.⁴²⁾

These results indicate that catalase plays an important role in hydrogen peroxide disposal in cells as well as GPx. However, the antioxidant system in hepatocellular carcinoma cells is known to be severely impaired, when compared to that in an appropriate normal cell control.^{43–45)} Catalase is markedly reduced by the lower catalase gene transcription rate resulting from increased methylation of the catalase gene⁴⁶⁾ and binding of the negative regulator to the silencer element present in hepatoma cells.⁴⁷⁾

In the present study, we confirmed that GA generates hydrogen peroxide, which is a causal substance in the induction of apoptosis, *via* superoxide anion stemming from oxidation of GA itself. In addition, the selectivity of GA, which kills tumor cells at much lower IC₅₀ values than normal cells, is reflected in the contents of catalase in target cells or their conditioned medium. Catalases in rat hepatocytes exist in peroxisomes and cytosol,⁴⁸⁾ of which the subcellular fractions efficiently inhibited GA-induced cell death in tumor cells. Furthermore, hepatocyte conditioned medium effectively suppressed GA-induced cell death, and its inhibitory activity was completely abolished by the addition of anti-catalase antibody.

The entire nucleotide sequence of rat catalase cDNA revealed that the molecular weight is 59,758 from the predicted amino acid sequence; the NH₂-terminal residue of rat liver catalase is a blocked alanine at position 2; and rat catalase shows about 90% homology with bovine liver catalase.¹⁾ Analysis of the cDNA sequence also demonstrated that rat liver catalase did not have signal peptides for translocation into peroxisomes or secretion and appeared not to be secreted from cells. However, we identified catalase in the medium from hepatocyte culture by immunoprecipitation

and determined the molecular weight and its N-terminal sequence (data not shown). The molecular weight was about 60 kDa, which was slightly larger than bovine catalase and the N-terminal amino acid sequence was not determined, because the N-terminal residue of rat liver catalase is blocked at alanine position 2, as previously reported.¹⁾ Thus the catalase in the medium from hepatocyte culture does not appear to be different from intracellular catalase, which is thought not to be secreted.⁴⁹⁾ However, human T-cell line CCRF-CEM secreted catalase to prevent apoptosis from hydrogen peroxide present in the extracellular milieu, although the amount was only 3% of total catalase activity and the nature of secreted catalase was not determined.⁵⁰⁾ Further study is required to clarify how catalase is released from cells and the differences among the catalases present in conditioned medium, peroxisome, and cytosol.

The following results from three reports suggest the usefulness of GA in hepatic cancer therapy. Diallyl sulfide decreases liver catalase protein levels without significant change in catalase activity in the kidney, lung, and brain⁵¹⁾; local generation of superoxide anion by hypoxanthine and xanthine oxidase was effective in reducing tumor size in an animal model⁵²⁾; and oral administration of GA results in a relatively high concentration of GA in the blood, especially the portal vein.⁵³⁾ Taken together, the results suggest that if it were possible to decrease the catalase outside of hepatocytes with a catalase inhibitor such as diallyl sulfide, GA should be able to attack hepatic tumor cells with depressed catalase activity effectively without any effect on hepatocytes rich in catalase activity.

Accumulating evidence prompts us to speculate that the cytotoxicity of ROS can cause various diseases, but also may serve as a powerful treatment to destroy harmful agents including tumor cells. In conclusion, studies of the differences in biological characteristics between tumor and normal cells and of the mechanism of action of therapeutic agents will pave the way for developing new therapeutic agents against cancer.

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