Tumor-Promoting Effect of GGN-MRP Extract from the Maillard Reaction Products of Glucose and Glycine in the Presence of Sodium Nitrite in C3H10T1/2 Cells

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GGN-MRP is an extract from the Maillard reaction products of nitrite with glucose and glycine in the Maillard browning system. No genotoxicity of GGN-MRP in culture hepatocyte was found. A two-stage transformation protocol was used to transform chemically mouse embryo fibroblast C3H10T1/2 cells. To initiate transformation, the cells were treated with benzo[*a*]pyrene [B(a)P; 0.1 μg/mL], and GGN-MRP (0.01, 0.1, and 1.0 mg/mL) was employed to subsequently complete the transformation process. Malignant transformed foci were formed in B(a)P-initiated and GGN-MRP-promoted C3H10T1/2 cells after 8 weeks. Cells treated with GGN-MRP alone failed to induce transformation. However, cells initiated with B(a)P and promoted by GGN-MRP demonstrated oncogenic properties. Transformed colonies derived from GGN-MRP-treated cells exhibited enhanced growth rate, anchorage independence, and tumorgenicity in animals relative to parent cells. These results indicated that GGN-MRP contains a tumor promoter and may induce tumor promotion by two-stage oncogenesis.

Keywords: *GGN-MRP; B(a)P initiation; tumor promotion; neoplastic transformation*

INTRODUCTION

Heat treatment of food rich in reducing sugars and amino acids may result in Maillard browning. Maillard browning is one of the most important reactions that occur during the heat processing, storage, and cooking of food and brings about a change in the color, flavor, functional properties, and nutritional value of the food (*1*−*5*). It has been shown that the Maillard reaction of sugars and amino acids produces mutagenic and genotoxic substances (*6*−*12*). However, none of these carcinogenic components generated in the reaction has been identified.

The well-established mutagenicity and carcinogenicity of *N*-nitroso compounds pose potential hazards to human health (*13*, *14*). Many compounds are generated by Maillard reaction, and these compounds can easily undergo nitrosation to form *N*-nitroso compounds. The mutagenicity of these reaction products has been established using several model systems (*15*−*17*). For instance, nonmutagenic Amadori compounds abundant in foodstuff can easily be converted by nitrite into *N*-nitroso compounds with strong mutagenic potential (*18*). In our previous study, we isolated *N*-nitroso-*N*-(3-keto-1,2-butanediol)-3′-nitrotyramine (NO-NTA) from the reaction products of nitrite with tyrosine and glucose in the browning model system (*19*), which proved to be a tumor promoter on mouse C3H10T1/2 cells (*20*) and on mouse skin carcinogenesis (*21*). Similarly, a Maillard reaction of glucose and glycine in an aqueous system could also generate components with DNA strand-breaking activity (*22*). The components with strand-breaking activity thus produced may contribute to the resultant mutagenicity, chromosome aberration, and gene conversion.

Because human food is contaminated with nitrite, the *N*-nitroso compounds formed by treatment of Maillard reaction products (MRPs) are likely to be generated during processing and cooking. Thus, it is necessary to evaluate the genotoxic and tumor-promoting effect of N-nitrosated MRPs. In the present study, the tumor-promoting effect of GGN-MRP (an extract from the Maillard reaction of glucose and glycine in the presence of sodium nitrite) on the two-stage oncogenesis was investigated using cultured C3H10T1/2 cells. The results showed that GGN-MRP is not genotoxic but contains components with tumor promotion potential.

MATERIALS AND METHODS

Chemicals. Glucose, glycine, sodium nitrite, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Percoll, collagenase, EDTA, proteinase K, hydroxyurea, calf thymus DNA, and benzo[*a*]pyrene [B(a)P] were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (MEM), fetal calf serum, trypsin−EDTA, L-glutamine, penicillin and streptomycin mixtures (PSN), basal minimum Eagles medium (BME), and William E were purchased from Gibco BRL (Grand Island, NY). [^{3}H]Thymidine was purchased from Amersham Life Science. Thin-layer plate and other solvents were purchased from Merck (Darmstadt, Germany).

Preparation of GGN-MRP. D-Glucose (25 mmol), 5 mmol of glycine, and 5 mmol of sodium nitrite were dissolved in SSC buffer (0.015 sodium chloride, 0.0015 M sodium citrate, pH 7.0), and the total volume was adjusted to 50 mL. The pH value of the solution was adjusted to 3. The mixture was heated at 37 °C for 24 h in a water bath to obtain nitroso-MRP. The

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mixtures were extracted with dichloromethane and dried by a liquid nitrogen stream. Thin-layer chromatography (TLC) was performed on a silica gel plate with a developing solvent of *n*-hexane/glacial acetic acid/ethyl acetate (40:5:20, v/v). The fraction ($R_f = 0.74$) was accumulated from preparative TLC and eluted with chloroform/methanol (9:1, v/v). The collective fraction ($R_f = 0.74$) was defined as GGN-MRP.

Cell Culture. Cells and transformed cells were incubated at 37 °C in 5% CO_2. The culture medium was BME, supplemented with 10% heat-inactivated fetal calf serum and PSN antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin).

Preparation of S-9 Mixture. Liver supernatant (S-9) was prepared from 8−10-week-old Sprage−Dawley rats, which were treated with 1500 mg/kg of Arclor 1254. An S-9 mixture solution, containing 1 mL of S-9, 0.2 mL of $MgCl_2$−KCl salts, 0.05 mL of 1 M glucose 6-phosphate, 0.4 mL of 0.1 M NADP, 5 mL of 0.2 M phosphate buffer (pH 7.4), and 3.35 mL of deionized H_2O, was prepared for this experiment.

Cytotoxicity Assays. The cytotoxicity assay was performed according to the MTT colorimetric assay (*23*). The C3H10T1/2 cells were seeded and treated with GGN-MRP at 0.001−2.0 mg/mL concentration for 24 h at 37 °C with or without activation system (S-9 mixture). After the exposure period, media from all treatments were withdrawn, and the cells were washed with PBS solution. Thereafter, the cells were transferred and incubated with 20 μL of MTT (5 mg/mL) for 4 h. The viable cell number/dish is directly proportional to the production of formazan, which, following solubilization with 2-propanol, can be measured spectrophotometrically at 563 nm.

Genotoxicity Assay. Hepatocytes were isolated from adult female Sprague−Dawey rats (Taichung Veterans General Hospital, Taiwan; 150−200 g fed ad libitum) according to the collagenase perfusion method (*24*). They were plated at a cell density of 5×10^5 cells/mL in 4 mL of William E medium supplemented with 10% calf bovine serum, 1% PSN antibiotic mixture, and nicotinamide (1 mg/L). The medium was renewed 3 h after plating to remove nonadherent cells. In addition, the culture was maintained for the time indicated. For treatment of culture with test agent, stock solutions of chemical in dimethyl sulfoxide (DMSO) were added to the cultures so that the final concentration of DMSO was <0.02%.

The genotoxicity of GGN-MRP on DNA repair synthesis was measured by determining the amount of [*methyl*-^{3}H]thymidine incorporated into nuclear DNA in the presence of hydroxyurea (15 mM) (*25*). Hepatocyte culture (5×10^5 cells) was treated with 0.005, 0.01, 0.1, and 1.0 mg/mL GGN-MRP following preincubation with hydroxyurea for 1 h. A 1 μCi/mL aliquot of [^{3}H]thymidine was added to culture medium for an additional 18 h. The cell monolayer was washed twice with PBS and harvested at the end of culturing. Following harvesting, the cells were loaded onto a 25 mm, 2 μm pore size PC filter holder to lyses the cells with 10 mL of lysing buffer containing 2% SDS, 0.025 M EDTA, 0.5 mg/mL thymidine, and 0.1 M glycine (pH 10) plus 0.15 mg/mL proteinase K and washed with 3 mL of the same buffer without proteinase K. The filter was carefully transferred to a scintillation vial. A 1 mL aliquot of 0.5 N $HClO_4$ was added to the vial and heated at 60 °C in a water bath for 1 h. An aliquot was taken for the determination of radioactivity by liquid scintillation counter (LSC-900 Aloka) and DNA content, expressed as dpm per microgram of DNA. DNA was measured in a cell lysate using the DABA method (*26*).

Transformation and Initiation—Promotion Experiments. The initiation−promotion experiments were performed as described by Mondal et al. and Frazelle et al. (*27*, *28*). Initially, 2000 cells (mouse embryo fibroblast C3H10T1/2 cells, passage 5) were seeded per 60 mm Petri dish. After 24 h, the cells were treated for another 24 h with B(a)P (0.1 μg/mL) as initiator. The culture was then treated with GGN-MRP (0.01, 0.1, or 1.0 mg/mL) for a period of 5 days. The medium was changed twice a week. After 8 weeks, transformed foci were identified by microscopic examination according to the procedure of Reznikoff et al. (*29*) and stained with 5% Giemsa solution to count the foci. The cytotoxicity of the GGN-MRP was measured by determining of the plating efficiency according to a previous method (*29*).

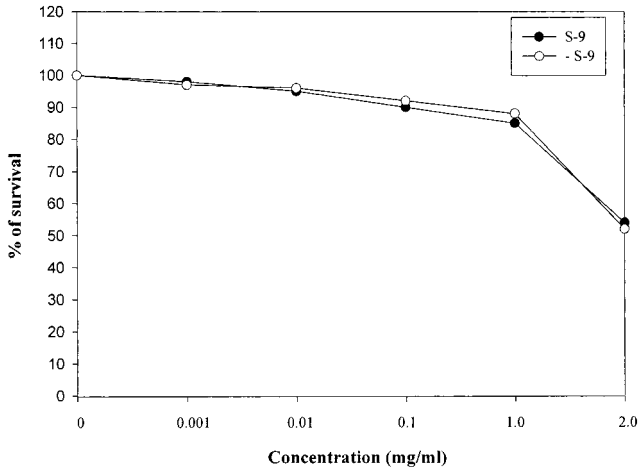

Figure 1. Percentage survival of C3H10T1/2 cells treated with GGN-MRP using MTT method. The values are percent of control.

Establishment of GGN-MRP-Transformed Cells and Growth Assay. GGN-MRP-transformed cells were established by B(a)P as initiator and treated with GGN-MRP. The transformed foci (type III) were removed by trypsinization using the ring cloning technique. Isolated cells were mixed and suspended in medium and seeded at a low density, and morphologically transformed foci were recloned 10 times. These cells (0.5×10^5 cells/60 mm Petri dish) were incubated at 37 °C in 5% CO_2, supplemented with 10% fetal calf serum for 14 days, and counted with a hemocytometer every other day.

Colony Formation in Soft Agarose. BME for agarose experiments was supplemented with 10% fetal calf serum, 0.1% sodium bicarbonate, and 1% antibiotic solution. The cells were suspended in 0.5% agarose/BME. The medium supplemented with 10% heat-activated fetal calf serum was replenished every 6 days. The colonies were scored after 28 days (*30*, *31*).

Tumorgenicity Testing. Cells in late logarithmic growth with various degrees of neoplastic transformation were washed with serum-free medium and suspended in warm PBS before subcutaneous intrascapular (10^7 cells in 0.2 mL) administration to CD-1 mice. The animals were fed ad libitum thereafter and inspected for the appearance of tumors at least once a week.

Statistical Analysis. The results were reported as means (standard deviations from individual magnitudes). Statistical differences were analyzed according to Student's *t* test; differences were considered to be significant at $P < 0.05$.

RESULTS

Cytotoxicity of GGN-MRP. The cytotoxicity of GGN-MRP was determined with an MTT assay. Figure 1 shows the survival percentage of C3H10T1/2 cells after treatment with GGN-MRP at different concentrations (0.001−2 mg/mL) with or without the S-9 activation system. Cell survival was observed to be inversely proportional to the concentration of GGN-MRP with an IC_{50} value of >2 mg/mL.

Genotoxicity of GGN-MRP. The genotoxicity of GGN-MRP was determined with an unscheduled DNA synthesis (UDS) assay. Experimental results indicated that GGN-MRP did not induce DNA repair synthesis in various concentrations of GGN-MRP (Table 1). This result reveals that GGN-MRP is not genotoxic in primary culture hepatocyte.

Table 1. Genotoxicity of GGN-MRP in Primary Culture Hepatocyte by UDS Assay

treatment	concn	dpm/μg of DNA[a]
normal	hydroxyurea	26.4 ± 0.4
normal		14.9 ± 0.6
control	0.02% DMSO	15.0 ± 1.0
GGN-MRP	0.05 mg/mL	15.3 ± 0.8
	0.01 mg/mL	16.6 ± 1.4
	0.1 mg/mL	17.2 ± 1.5
	1 mg/mL	13.8 ± 0.3

[a] Hepatocytes (5×10^5/60 mm Petri dish) were treated with GGN-MRP for 24 h and DNA repair was detected by UDS assay. The values are mean ± SD, $n = 3$.

Transformation and Initiation—Promotion Experiments. The C3H10T1/2 cells were used in initiation−promotion experiments. At the end of the 8-week experiments, the morphologically transformed foci were identified by light microscopy after staining with 5% Giemsa solution. We found that while foci formation was absent in cells treated with 0.02% DMSO only, a large number of transformed foci were formed in cells treated with B(a)P as initiator and with GGN-MRP for 5 days. The morphological characteristics of various foci have been described by Reznikoff (*29*). Type II and type III foci were found in GGN-MRP-promoted C3H10T1/2 cells. Table 2 summarizes the tumor-promoting effect of GGN-MRP on B(a)P-initiated C3H10T1/2 cells. No transformed foci were found in cells treated with 0.02% DMSO or GGN-MRP only for a period of 5 days. A significant increase ($P < 0.001$) in transformation was found upon treatment with both B(a)P and GGN-MRP after confluence had been reached, as compared to the cells that had not been further treated. The relationship between the concentration of GGN-MRP and transformation frequency (TF) was determined. Results showed that TF was proportional to GGN-MRP concentration (Table 2).

Growth of GGN-MRP-Promoting Transformed Cells. At the end of the transformation assay, the transformed foci were removed by trypsinization using the ring cloning technique and the cells were recloned 10 times. These cells (5×10^4 cells/60 mm Petri dish) were incubated for 14 days and were counted every other day with hematocytomer. Figure 2 depicts the untransformed C3H10T1/2 cells (group A), which reach confluence on the fourth day. However, the GGN-MRP-transformed cells, initiated by B(a)P and treated subsequently with GGN-MRP, exhibited a longer lag phase and a higher growth rate and exceeded the saturation density of the normal cells.

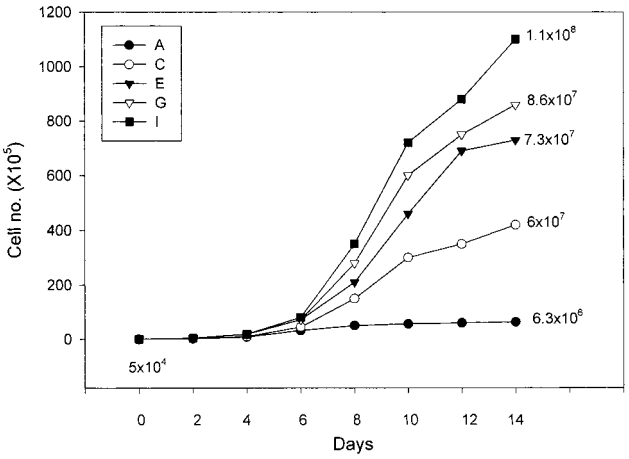

Figure 2. Growth curve of C3H10T1/2 cells and GGN-MRP-transformed cells: (A) C3H10T1/2 cells; (C) 0.1 μg of B(a)P/mL; (E) 0.1 μg of B(a)P/mL + 0.01 mg of GGN-MRP/mL; (G) 0.1 μg of B(a)P/mL + 0.1 mg of GGN-MRP/mL; (I) 0.1 μg pf B(a)P/mL + 1.0 mg of GGN-MRP/mL.

Growth Properties in Soft Agarose. We found that the transformed cells grew well in soft agarose, whereas untransformed cells failed to do so. Anchorage-independent growth in soft agarose correlates with the tumorgenicity of cell lines (*32*). Accordingly, we tested the ability of nontumorgenic C3H10T1/2 cells and the GGN-MRP-transformed cells for growth in soft agarose. The results are summarized in Table 3. Nontransformed cells failed to grow in soft agarose, and colony formation was absent. On the other hand, GGN-MRP-transformed cells formed large (3−4 mm in diameter) and well-defined colonies. A significant increase in colony formation in GGN-MRP-transformed cells was observed compared to untreated cells. The ability to form colonies in GGN-MRP-transformed cells was 39-, 87-, and 120-fold higher than that in B(a)P-transformed cells, respectively.

Tumorgenicity of GGN-MRP-Transformed Cells. Because GGN-MRP-transformed cells displayed alterations in growth properties and were anchorage-independent, we studied their tumorgenicity by injecting 1×10^7 cells into CD-1 mice and monitoring tumor appearance and growth. These cells were tumorgenic

Table 2. Transformation of C3H10T1/2 Cells Produced by B(a)P and GGN-MRP Treatment

			foci					
group	treatment[a]	PE[b] (%)	II	III	foci/dish	TF[c]	*P* value[d]	
A	normal	19.5	0	0	0	0		
B	DMSO (0.02%)	19.0	0	0	0	0		
C	B(a)P (0.1 μg/mL)	18.8	2	0	0.17 ± 0.25[e]	0.01		
D	GGN-MRP (0.01 mg/mL)	18.5	0	0	0	0		
E	C + D	18.1	92	64	13.00 ± 2.15	0.72	<0.001	E vs C
							<0.001	E vs D
F	GGN-MRP (0.10 mg/mL)	18.7	0	0	0	0		
G	C + F	17.9	138	75	17.75 ± 3.12	0.99	<0.001	G vs C
							<0.001	G vs E
							<0.001	G vs F
H	GGN-MRP (1.0 mg/mL)	18.2	0	0	0	0.01		
I	C + H	17.4	167	81	20.67 ± 3.59	1.19	<0.001	I vs C
							<0.001	I vs G
							<0.001	I vs H

[a] Three independent experiments were performed and had similar results; a representative one is shown here. [b] PE (plating efficiency) = (colonies/seed cell no.) × 100. [c] TF (transformation frequency) = foci no./PE. [d] Statistical significance was determined by *t*-test analysis. [e] Mean ± SD, $n = 12$.

Table 3. Anchorage-Independent Growth of GGN-MRP-Promoting Transformed Cells

group	colonized cells[a]	colonies in soft agarose	colonies[c,d]
A	C3H10T1/2	0/6	0.00 ± 0.00
C	B(a)P (0.1)[b]	8/6	1.33 ± 0.51
E	B(a)P + GGN-MRP (0.01)	312/6	52.00 ± 6.84*
G	B(a)P + GGN-MRP (0.10)	695/6	115.80 ± 10.24*
I	B(a)P + GGN-MRP (1.00)	957/6	

[a] Cell lines were transformed with B(a)P in the presence or absence of GGN-MRP-transformed colonies. Transformation of C3H10T1/2 cells by B(a)P and GGN-MRP is described under Materials and Methods. [b] Numbers in parentheses, concentration: B(a)P, μg/mL; GGN-MRP, mg/mL. [c] Mean ± SD, $n = 6$. [d] *, $P < 0.0001$, compared with the B(a)P alone treated group (C group).

Table 4. Tumorgenicity of GGN-MRP-Promoting Transformed Cells

cell cloned[a]	tumorgenicity[b] (tumors/injections)	tumor appearance[c] (weeks)
C3H10T1/2	0/12	
B(a)P (0.1)[d]	0/12	
B(a)P plus		
GGN-MRP (0.01)	3/12	3−4
GGN-MRP (0.10)	6/12	2−4
GGN-MRP (1.00)	7/12	2−3

[a] Same as footnote *a* of Table 3. [b] Tumor formation (≥2 mm in the largest diameter) by 28 days postinjection of CD-1 mice inoculated with 1×10^7 cells per injection. [c] Approximate time interval during which visually apparent, palpable tumors arose. [d] Numbers in parentheses, concentration: B(a)P, μg/mL; GGN-MRP, mg/mL.

and induced tumor formation. The time of tumor appearance varied slightly with different transformed cells but was ~2−4 weeks postinjection (Table 4). A majority of the tumors attained a diameter of at least 10 mm after 6 weeks and continued to grow. In contrast, control cells showed a lower incidence of tumor formation under these conditions. These observations demonstrate that GGN-MRP-transformed cells potentiate the tumorgenicity of C3H10T1/2 cells.

DISCUSSION

The occurrence and formation of known carcinogens in a variety of food have been extensively discussed in the literature. Previous papers have reported that the Maillard reaction of glucose/glycine in an aqueous system produced a substance with DNA strand-breaking activity (*12*, *22*). The sugar−amino acid mixtures are well-known to be easily nitrosated (*33*), and such nitrosated products in addition to other *N*-nitrosamines are possibly formed upon the heating of cured meats. (*34*) Our previous study showed that the reaction of tyrosine and glucose with nitrite generates a nitroso product, NO-NTA (*19*). It was found that NO-NTA was strongly tumor-promoting in mouse embryo fibroblast cells (*20*) and mouse skin (*21*) by two-stage oncogenesis.

Two-stage morphologic transformation in vitro using mouse embryo fibroblast C3H10T1/2 cells has been previously discussed (*27*, *28*). These methods have been extensively used by many investigators to study the transformation process and identify new tumor promoters (*31*, *35*, *36*). Upon treating C3H10T1/2 cells with GGN-MRP alone, we found no foci formation. Also, treatment with B(a)P alone resulted in very few foci formation. However, the number of transformed foci increased significantly when C3H10T1/2 cells were pretreated with B(a)P, as initiator, and subsequently with GGN-MRP. These results imply that GGN-MRP is a tumor promoter.

A two-stage transformation assay has been useful for confirming some novel tumor promoters, such as diethylstilbestrol (*36*), styrene (*31*) and Roussi's red (*35*). The transformed cells thus obtained failed to be verified as cancer cells but were identified as potential tumor-promoting cells. When type III foci were harvested, they invariably resulted in oncogenically transformed cells irrespective of the transformation stage, but type II foci were unable to induce cancer (*29*). Hence, we select type III foci for transformation in this study. We further tested their growth rate and anchorage-independent characteristics. Transformed cell lines from GGN-MRP-transformed colonies had higher/enhanced growth rates and anchorage independence relative to control cells. Thus, GGN-MRP has a property for promoting transformation in cultures of C3H10T1/2 cells.

The tumor-promoting GGN-MRP compound produced in the nitrite treatment of MRPs can be a nitroso compound. Most *N*-nitroso compounds are carcinogenic in laboratory animals, so *N*-nitrosoglycosylamines and *N*-nitroso Amadori compounds, which may either be present in human diet or formed in the stomach from the corresponding amino precursors and nitrosating species (e.g., nitrite and nitrogen oxides), may constitute a hazard to human health. Humans are exposed to dietary *N*-nitroglycosylamines and *N*-nitroso Amadori compounds or to their precursors, which may undergo endogenous nitrosation, a reaction that has been shown definitely to occur in humans (*37*). Here, we have shown that GGN-MRP has promoting activity in a two-stage transformation in C3H10T1/2 cells. Further studies should follow to identify GGN-MRP and evaluate whether GGN-MRP poses a significant human risk, taking into account its presence in food dietary food choices and cooking practices.

In conclusion, the new tumor promoter, GGN-MRP, has the potential to transform C3H10T1/2 cells but only in the presence of B(a)P. The GGN-MRP-transformed cells exhibited high cell density, ability to grow in soft agarose, and tumorgenicity in animals. Taken together, these results imply that GGN-MRP is a tumor promoter and thus could be an important index in the evaluation of the health value of food.

ABBREVIATIONS USED

MRPs, Maillard reaction products; B(a)P, benzo[*a*]-pyrene; PE, plating efficiency; TF, transformation frequency; UDS, unscheduled DNA synthesis; GGN-MRP, an extract from the Maillard reaction of glucose and glycine in the presence sodium nitrite; NO-NTA, *N*-nitroso-*N*-(3-keto-1,2-butanediol)-3′-nitrotyramine; MTT, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide; PSN, penicillin and streptomycin mixtures; DMSO, dimethyl sulfoxide.

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