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Effects of Allicin on the Gene Expression Profile of Mouse Hepatocytes *in vivo* with DNA Microarray Analysis*

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The major garlic component, Allicin [diallylthiosulfinate, or (*R, S*)-diallyldissulfid-*S*-oxide] is known for its medicinal effects, such as antihypertensive activity, microbicidal activity, and antitumor activity. Allicin and diallyldisulfide, which is a converted form of allicin, inhibited the cholesterol level in hepatocytes, *in vivo* and *in vitro*. The metabolism of allicin reportedly occurs in the microsomes of hepatocytes, predominantly with the contribution of cytochrome P-450. However, little is known about how allicin affects the genes involved in the activity of hepatocytes *in vivo*.

In the present study, we used the short-term intravenous injection of allicin to examine the *in vivo* genetic profile of hepatocytes. Allicin up-regulate ten genes in the hepatocytes. For example, the interferon regulator 1 (IRF-1), the wingless-related MMTV (mouse mammary tumor virus) integration site 4 (wnt-4), and the fatty acid binding protein 1. However, allicin down-regulated three genes: namely, glutathione S-transferase mu6, α-2-HS glycoprotein, and the corticosteroid binding globulin of hepatocytes. The up-regulated wnt-4, IRF-1, and mannose binding lectin genes can enhance the growth factors, cytokines, transcription activators and repressors that are involved in the immune defense mechanism. These primary data, which were generated with the aid of the Atlas Plastic Mouse 5 K Microarray, help to explain the mechanism which enables allicin to act as a therapeutic agent, to enhance immunity, and to prevent cancer. The data suggest that these benefits of allicin are partly caused by the up-regulated or down-regulated gene profiles of hepatocytes. To evaluate the genetic profile in more detail, we need to use a more extensive mouse genome array.

Key Words: Allicin, DNA microarray, Gene expression, Gene profile, Hepatocytes

INTRODUCTION

Garlic (*Allium sativum*) has been used as a spice for kimchi, and many other dishes in Korea and around the world. The mother organosulfur compound allicin [diallylthiosulfinate, or (*R, S*)-diallyldissulfid-*S*-oxide, DADSO], which comes from the crushed garlic cloves, is said to have a broad spectrum of health benefits. For example, it exhibits strong microbicidal actions against bacteria, fungi, and parasites.[1-3] In addition to acting against pathogens, allicin enhances the function of immune cells; for instance, it stimulates lymphocytes and macrophages, which are major effective cells in body defense.[4,5] Allicin has been found to elevate IL-10 and 1L-12 in monocytes,[6] and to inhibit the production of iNOS (inducible nitric oxide synthase) in macrophages resulting in the attenuation of local inflammation.[4] An allicin enriched garlic extract inhibits the nitric oxide metabolism in the microsomes of mammalian cells. Moreover, allicin and diallyldisulfide (DADS), which is one of the major volatile degradative compounds of DADSO, reduce the serum cholesterol level in hyperlipidemic rabbits,[7] and the synthesis of cholesterol in hepatocytes.[8]

The metabolism of allicin and DADS occurs mainly in the microsomes of hepatocytes. The two components cytochrome P-450 monooxygenase (CYP) and flavin-containing monooxygenase are involved in the metabolism. DADS is preferentially metabolized to allicin by CYP2E1 in the human liver.[9,10] These results suggest that allicin can impact on the genes that are involved in the metabolism and other functions of hepatocytes. In a previous study, allicin induced the expression of genes related to the cytokines of peripheral mononuclear cells.[11] However, whether allicin affects the gene profile of the hepatocytes is uncertain. We therefore used a short-term intravenous injection of allicin to analyze the profile of genes from hepatocytes *in vivo*.

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MATERIALS AND METHODS

1. Allicin Preparation

A purified form of allicin was obtained from manufacturer (Nopex Co., England). Because the allicin that is converted from allinin is unstable and easily subject to the variability of other sulfur compounds, it is difficult to make good quality of allicin in a small laboratory. To qualitatively evaluate the gene expression, we used the Atlas™ Plastic Mouse 5 K Microarray and the Atlas ™ Pure Total RNA Labeling System (BD Science, USA).

2. Influence of Allicin *in vivo* Gene Expression

Allicin treatment

Allicin (5 mg per kilogram of body weight) was injected daily for 5 days into the tail vein of ICR female mice (Orient Co., Korea). In the pilot study 5 mg of allicin per kilogram of body weight seemed safe for the mice.[12] The intravenous injection of a higher concentration of 10 mg or 15 mg per kilogram of body weight was fatal. The control (n=10) and experiment (n=10) groups were kept on regular diet.

Tissue extraction

As soon as the livers were excised from the control and experiment group, the livers were cut into two parts, and frozen swiftly in liquid nitrogen. Each liver was frozen in a cryovial (Nalgen Co., USA) and stored in a deep freezer at -70 ℃.

Extraction of Total RNA

The total RNA was extracted in accordance with the instructions of the Atlas™ Pure Total RNA Labeling System (BD Science, USA). Before pouring it into a 50 mL polypropylene centrifuge tube (Nalgen Co.) containing 6 mL of a denaturing solution, we powdered the liver with a pestle in mortar. After mixing the powder well with the solution, the solution was left in ice for 10 minutes. To remove the tissue debris, we pelleted it at 12,000 rpm for 5 minutes at 4 ℃, and then transferred the supernatant to a new tube. We added 12 mL of saturated phenol to the tube, and incubated in ice for 5 minutes. In addition, it was added and vigorously mixed with 3.6 mL of chloroform in room temperature. After further incubation in ice for 5 minute, it was centrifuged at 12,000 rpm for 10 minutes at 4 ℃. After moving the supernatant to a new tube, the process was repeated twice. We then added 12 mL of isopropanol to the supernatant for the final stage of refining. The solution was left in ice for 10 minutes and then centrifuged for 10 minutes at 12,000 rpm at 4 ℃. After twice washing the precipitated RNA with 6 mL of 80% ethanol, we dissolved the concentrated RNA in 2 μg/μl with RNase-free water.

We added DNase I (1U/μl) for 30 minutes at 37 ℃ in order to remove the genomic DNA from the purified total RNA, and we eluted the RNA through the purification of phenol-chloroform. The final process was carried out to remove residual salt in the eluted total RNA and to reduce the genomic DNA fragment to less than 0.0001 percent with the application of NucleoSpin RNA kit (BD Science).

Poly A^+ purification and cDNA probe synthesis

The poly A^+ purification and cDNA probe synthesis of the extracted total RNA was processed with the aid of the Atlas™ Pure Total RNA Labeling System (BD Science). We added 45 μl of RNase-free water and 1 μl of biotinylated oligo (dT) to 20 μg of the total RNA, that had been denatured for 2 minutes at 70 ℃ and left for 10 minutes at room temperature. After adding 15 μl of streptavidin beads to the total RNA, we mixed the mixture with a shaker for 30 minutes at room temperature. The beads were dissolved with 2 μl of RNase-free water after the washing processes.

After adding 1 μl of mixture of random primer and cDNA synthesis control to poly A^+ RNA, we heated the mixture for 3 minutes at 94 ℃ and maintained the temperature at 42 ℃ for 2 minutes. We then added the following components in order: a 5× powerscript reaction buffer, a 10×dNTP mix, [α-^{33}P] dATP (10 μCi/μl, Amersham Biosciences, USA), DTT (100 mM), and powerscript reverse transcriptase. The reaction of these components for 30 minutes at 42 ℃ enabled us to synthesize the cDNA, and we stopped the reaction by adding 2 μl of the 10× termination mix. Finally, the synthesized cDNA was purified with the aid of column chromatography.

Hybridization

The microarray plates (Sigma Co., USA) were soaked with RNase-free water at 60 ℃ for 30 minutes in a 60 ℃ incubator. After discarding the water, we added a 60 ℃ hybridization solution to the plates, and we processed the pre-rinsing for 30 minutes at 60 ℃. Moreover, the final eluted cDNA probe was denatured for 2 minutes at 94 ℃. After mixing the denatured cDNA and 15 mL of the hybridization solution at 60 ℃, we put the mixture into the hybridization box for 20 hours at 60 ℃. The next day, we washed array plates twice with a high salt wash liquid (2×SSC, 0.1% SDS) and low salt wash liquid (0.1×SSC, 0.1% SDS) at 60 ℃. Finally the dried microarray plate was exposed to a phosphoimager screen for 48 hours. A difference in the degree of gene expression between the two groups was observed after 48 hours.

Analysis

To evaluate the effects of allicin on expression of genes, we used software called AtlasImage (BD Science, version 2.7) to analyze the difference in the degree of expression between two groups. The gene expression was normalized with glyceraldehy-3-phospate dehydrogenase (G3PDH), and we removed the variation of array spot by the application of calibration standard (calibration standard #3110700), which was provided by the BD science to correct any error in the comparison of the two groups. The gene classification was assorted in accordance with the classification table of Atlas™ Gene List version 5.0, which was provided by the BD Science. The results qualitatively, but not quantitatively, show the expression level of the genes from the hepatocytes.

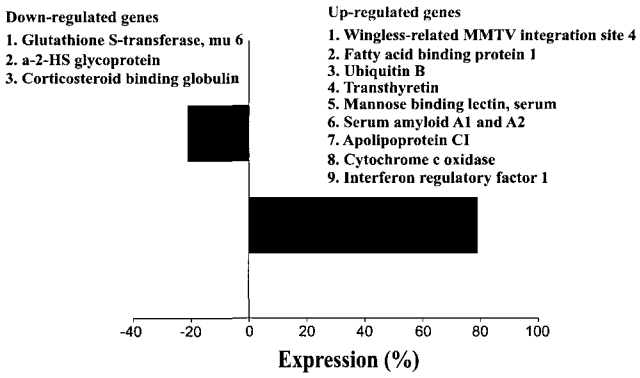

Fig. 1 Global gene expression analysis. The control and experiment group were analyzed for gene expression using data generated from an Atlas Plastic Mouse 5 K Microarray (BD Science, USA). The microarray results show the up-regulated genes on right side and down-regulated genes on left side.

RESULT AND DISCUSSION

1. Role of Allicin in Regulating the Expression of 13 Genes in Mouse Hepatocytes *in vivo*

Allicin regulated expression of 13 genes (0.2 percent) out of 5043 genes in the hepatocytes *in vivo* with the aid of the Atlas Plastic Mouse 5 K microarray. With *in vivo* treatment, Allicin up-regulated ten genes (10/13, 79 percent), but down-regulated three genes (3/13, 21 percent) (Fig. 1 and Table 1). We classified the group of genes with the different aspect of expression by the function of genes (Table 2). The classification consequently contains genes that are related to cell transportation, trafficking, signal transduction, protein synthesis, immunity, and metabolism, all of which are important in function of hepatocytes.

It is difficult to compare these results with others results due to the lack of article dealing with the genetic profile of hepatocytes after allicin treatment. Knowles

Table 1. Comparison of gene expression between the control and the experimental group

Gene name	Allicin		Ratio	Protein
	Unstim.	Stim.		
GStm 6	124	34	0.27	Glutathione S-transferase, mu 6
Ahsg	593	262	0.44	α-2-HS glycoprotein
Sepina 6	87	39	0.44	Corticosteroid binding globulin
Wnt-4	34	68	2.00	Wingless-related MMTV integration site 4
Fabp 1	36	74	2.05	Fatty acid binding protein 1
Ubiquitin B	34	74	2.16	Ubiquitin B
Prealbumin	369	867	2.35	Transthyretin
Mlb 2	26	63	2.42	Mannose binding lectin, serum
Saa 1	137	332	2.42	Serum amyloid A1 and A2
Saa 2	83	467	5.30	
Apoc 1	642	1578	2.46	Apolipoprotein CI
Cox4i 1	33	83	2.52	Cytochrome c oxidase
IRF 1	19	55	2.89	Interferon regulatory factor 1

Table 2. Classification of genes based on their expression and function

Gene expression	Genes	Function
Down-regulation	Glutathione S-transferase, mu 6	Not classified
	α-2-HS glycoprotein	Extracellular transport/carrier proteins
		Trafficking/targeting proteins-endocytosis
	Corticosteroid binding globulin	Extracellular transport/carrier proteins
Up-regulation	Wingless-related MMTV integration site 4	Growth factors, cytokine, and chemokines
	Fatty acid binding protein 1	Not classified
	Ubiquitin B	Protein turnover, Stress response proteins
	Transthyretin	Extracellular transport/carrier proteins
	Mannose binding lectin, serum	Extracellular transport/carrier proteins
		Immune system proteins
	Serum amyloid A1 and A2	Extracellular transport/carrier proteins and not classified
	Apolipoprotein CI	Extracellular transport/carrier proteins
	Cytochrome c oxidase	Metabolism
	Interferon regulatory factor 1	Transcription activators and repressors, Intracellular transducers/ effectors/ modulators

and Milner[13] reported that DADS, one of the derivatives of allicin, up-regulated that a group of genes related to growth factors and receptors; for example the interferon-gamma receptor, and the platelet-derived growth factor receptor in HCT-15 human colon tumor cell lines.[13] Even though their results came from tumor cell lines *in vitro*, they correlate well with our results for the up-regulation of IRF-1 gene and wingless-related MMTV integration site 4 (wnt-4). Wnt-4 gene which is located at chromosome 6 in mouse regulates the growth hormone releasing factor receptor in the pituitary gland.[14] The up-regulation of cytochrome c oxidase gene in this experiment correlated well with that of metabolism of allicin, furthermore, production of DADS depends on the amount of cytochrome P-450 monooxygenase and flavin-containing monooxygense in the liver microsome.[9,10]

The up-regulation of fatty acid binding protein 1 gene, ubiquitin B, and apolipoprotein C1 genes reflect the enhanced metabolism of lipids and cholesterol in the hepatocytes following the allicin treatment. The increased expression of these genes strongly supports the research that indicates the role of allicin in reducing serum cholesterol,[7] in producing cholesterol from hepatocytes,[8,15] and in reducing yellow streaks in the hyperlipidemic rabbit aorta.[7]

2. Importance of wnt-4, IRF-1, and MBL-4 in Immune Defense and Cell Signals

The up-expression of wnt-4 increases production of various growth factors, cytokines, and chemokines, which are the key molecules in the cell-to-cell work of the immune system. This phenomenon correlates well with our previous results in which allicin treatment in humans increased the expression of cytokine from peripheral mononuclear cells.[11] The increased expression of IRF-1 and mannose binding lectin 2 (MBL 2) show the effect of allicin on the immune function. The up-regulation of MBL 2 expression is important for facilitating the clearance of microorganism by neutrophils, macrophages, and professional phagocytes in the primary defense against pathogens. It also shows how allicin affects the activation of cells involved in the innate immunity *in vivo*. Shadkchan *et al.*[12] demonstrated that allicin inhibits the growth of *aspergillus spp. in vitro* and *in vivo*. Our results show that antimicrobial activity of allicin relies on the genetic evidence of the increase of MBL 2.

The transcriptional factor IRF-1, which binds to *cis* element of type 1 interferon (interferon-α and interferon-β), is important in modulating the IFN-γ signaling in hepatocytes. Disruption of the IRF-1 gene, a downstream target of IFN-gamma/STAT1, abolishes ConA-induced liver injury and suppresses leukocyte infiltration into the liver.[16] Furthermore, a deficiency of IRF-1 exhibited a markedly low number of Natural Killer cell, NK-T cells, and CD-8 T cells in mice.[17]

It is well known that garlic compounds minimize intracellular oxidative stress and inhibit nuclear factor-kB.[18] However, there is no explanation as to how garlic suppresses oxidative stress, such as hydrogen peroxide and nitric oxide, in the cell. Allicin works as a heme enzyme inhibitor that blocks nitric oxide (NO) metabolism in a microsome.[19] The increased expression of cytochrome c oxidase can produce cytochrome c (III) to inhibit the NO metabolism. Another factor that can inhibit oxidative stress is the level of intracellular glutathione (GSH) in an endothelial cell.[18] In addition, allicin down-regulates glutathione S-transferase gene, which is an enzyme that induces conjugation of the metabolites in GSH, and the decreased level of glutathione S-transferase can affect the level of GSH. Allicin therefore enhance both the innate and adaptive immunity.

3. Anti-tumor Effect of Allicin

The IRF-1 mediated IFN gamma reportedly induces apoptosis of ovarian cancer cells.[20] The two antioncogenic transcription factors IRF-1 and p53 converge to regulate the expression of the p21 gene and to inhibit the formation of tumors.[21] These reports explicate the genetic mechanism by which allicin exhibits anti-tumor effects. In this context, increased expression of IRF-1 in hepatocytes enhances the antitumor effect of allicin. In addition to the function of allicin at the transcription level in tumor, it has been reported that DADS directly down-regulate oncogenes, the cell-division protein kinase 6, and other growth factors and receptors in the colon tumor cell line.[13]

4. Other Genes Regulated by Allicin

Allicin down-regulates the α-2-HS glycoprotein gene and the corticosteroid binding globulin gene, both of which work as extracellular transport-carrier proteins. Up-regulated genes such as transthyretin, mannose binding lectin, and serum amyloid A1 and A2 also function as extracellular transport-carrier proteins. The specific function of these genes is not clear at present.

CONCLUSIONS

We used short-term intravenous injection of allicin to examine the *in vivo* genetic profile of hepatocytes. Allicin up-regulates ten genes in hepatocytes; for instance, IFR-1, wnt-4, and the fatty acid binding protein 1. It also down-regulates three genes; namely, glutathione S-transferase mu6, α-2-HS glycoprotein, and the corticosteroid binding protein of hepatocytes. The up-regulated wnt-4, and the IRF-1, and the mannose binding

lectin genes can enhance the growth factors, cytokines, transcription activators and repressors involved in the immune defense mechanism. The primary data that we generated with aid of the Atlas Plastic Mouse 5 K Microarray suggest the mechanism of allicin that enables it to act as a therapeutic agent, to enhance immunity, and to prevent cancer is partially the result of the up-regulated or down-regulated gene profiles of hepatocytes. However, to evaluate genetic profile in more detail, we need to use a more extensive mouse genome array.

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