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Gene expression responses of HeLa cells to chemical species generated by an atmospheric plasma flow

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ABSTRACT

Plasma irradiation generates many factors able to affect the cellular condition, and this feature has been studied for its application in the field of medicine. We previously reported that hydrogen peroxide (H_2O_2) was the major cause of HeLa cell death among the chemical species generated by high level irradiation of a culture medium by atmospheric plasma. To assess the effect of plasma-induced factors on the response of live cells, HeLa cells were exposed to a medium irradiated by a non-lethal plasma flow level, and their gene expression was broadly analyzed by DNA microarray in comparison with that in a corresponding concentration of 51 μM H_2O_2. As a result, though the cell viability was sufficiently maintained at more than 90% in both cases, the plasma-medium had a greater impact on it than the H_2O_2-medium. Hierarchical clustering analysis revealed fundamentally different cellular responses between these two media. A larger population of genes was upregulated in the plasma-medium, whereas genes were downregulated in the H_2O_2-medium. However, a part of the genes that showed prominent differential expression was shared by them, including an immediate early gene ID2. In gene ontology analysis of upregulated genes, the plasma-medium showed more diverse ontologies than the H_2O_2-medium, whereas ontologies such as "response to stimulus" were common, and several genes corresponded to "response to reactive oxygen species." Genes of AP-1 proteins, e.g., JUN and FOS, were detected and notably elevated in the plasma-medium. These results showed that the medium irradiated with a non-lethal level of plasma flow altered various gene expressions of HeLa cells by giving not only common effects with H_2O_2 but also some distinctive actions. This study suggests that in addition to H_2O_2, other chemical species able to affect the cellular responses exist in the plasma-irradiated medium and provide unique features for it, probably increasing the oxidative stress level.

1. Introduction

Plasma produces heat, chemical species, and charged particles, and these factors have been applied to the development of medical equipment and sterilizers, and to cancer research [1–6]. Many studies have reported the effect of plasma irradiation on bacteria [7,8], viruses [9], and cells [10]. Atmospheric plasma flow is characterized by low-temperature. When irradiated to water, it generates a variety of chemical species, including reactive oxygen species (ROS) with only a slight change in temperature [11]. Application of atmospheric plasma flow to cells has been studied due to this feature, and various effects have been observed to date: induction of apoptosis [12,13], cell cycle arrest [14], reduction of tumor size [15], and selective death of melanoma cells [16].

Analyzing the alteration of gene expression is useful to generally understand the response of cells to stimuli. However, limited information is available concerning the differential gene expression of cells by plasma exposure. When a low-temperature plasma jet was percutaneously irradiated to subcutaneous tumors of a mouse, gene expressions involved in organismal injury or abnormalities were altered [15]. Exposure of epithelial cells of human skin to an argon plasma jet increased gene expression of antioxidant enzymes [17].

We previously reported that chemical species generated in cell culture medium by irradiation with an atmospheric plasma flow for 210 s induced inactivation of HeLa cells and that H_2O_2, a representative ROS, was the main cause of this [18]. In order to elucidate the interference mechanism between the plasma flow and cells,

Abbreviation: ROS, reactive oxygen species.

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analysis of the cellular response at the level of gene expression is important. In the present study, we assessed the response of HeLa cells to a culture medium exposed to plasma for 40 s, a much shorter time than in the previous study [18]. Different from our previous study [18], this irradiation level allows the cells to survive without apparent induction of necrosis. The gene expression of HeLa cells was broadly analyzed by a DNA microarray in plasma-treated medium and H_2O_2-added medium as compared with expression in a regular culture medium. This enabled us to separately evaluate the effect of chemical species generated by the plasma and of the H_2O_2 alone.

2. Materials and methods

2.1. Plasma generation

A needle electrode was made of a platinum wire 0.3 mm in diameter. A ground electrode of aluminium (60 mm in diameter, and 28 mm thick) had a central hole (10 mm in diameter and 24 mm depth) to hold a microtube. A polypropylene microtube with 1 ml of culture medium was set on the ground electrode, and plasma flow was generated between the needle electrode and the surface of the culture medium (Fig. 1). To prepare plasma-irradiated medium (plasma-medium), a square wave with a voltage of +7.5 kV_{op}, a frequency of 5 kHz, and a duty ratio of 4% was applied to generate plasma, the irradiation time being 40 s. During this period, cells were absent in the medium. The change in pH was −0.1, and the change in temperature was +2.4 °C on average (+8.8 °C at the surface and +0.8 °C at the bottom before mixing of the medium).

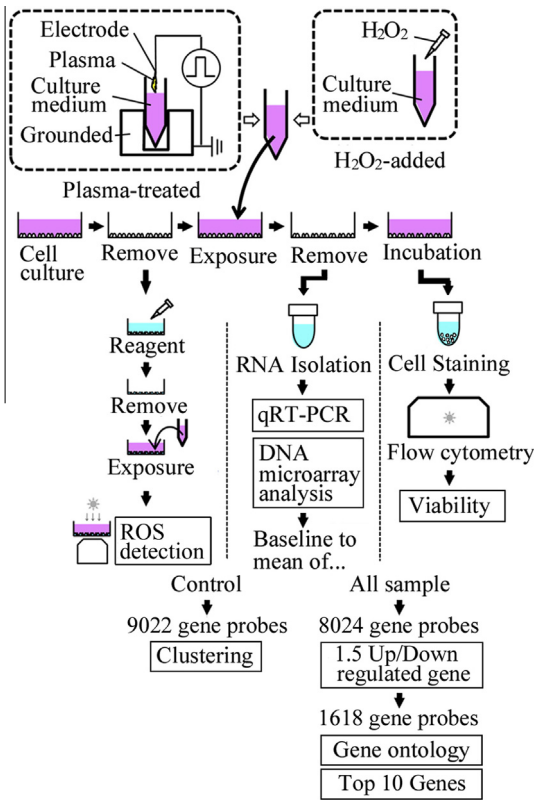

Fig. 1. Experimental setup and procedure. Cells were absent in the medium during the plasma irradiation. The temperature of atmospheric plasma flow was low, which caused a mild increase in medium temperature. Also, change in the medium pH was small by plasma irradiation or H_2O_2.

2.2. Cell culture and exposure of plasma-medium or H_2O_2-medium

HeLa cells (Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University) were cultured with a regular medium consisting of MEM (Sigma Aldrich, St. Louis, MO), 10% FBS (Gibco, Life Technologies, Carlsbad, CA), and 100 units/ml penicillin-100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were then exposed to plasma-medium with the same atmosphere for 1 h, 48 h, or 1 h followed by 48-h incubation with the regular medium. Alternatively, a medium containing 51 μM H_2O_2 was used for exposure (H_2O_2-medium) because when the regular medium was irradiated with plasma flow for 40 s, the concentration of H_2O_2 generated was observed to be 51 μM [18]. This concentration slightly decreased the pH of the medium in the air (−0.5 in average).

2.3. Cell viability assay

After incubation with each medium, the cells were washed with PBS, collected, and then subjected to viability assay by flow cytometry. They were stained with 0.5 x Guava ViaCount Flex Reagent (Merck Millipore, Billerica, MA), which uses two DNA binding dyes to identify viable and dead cells, and the fluorescence signal was measured by a flow cytometer (easyCyte 8HT, Millipore). Data was analyzed by Guava Software (Millipore).

2.4. Cellular reactive oxygen species detection assay

Reactive oxygen species (ROS) in the cells were detected with 5-(and-6)-chloromethyl-2′,7′-dichlorodi-Hydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen, Life Technologies). The cells were transferred to coverglass chambers and incubated in a dark place with 10 μM CM-H_2DCFDA in Hanks' balanced salt solution (Sigma) at 5% CO_2, 37 °C for 5 min. Then they were exposed to plasma-medium or H_2O_2-medium for 1 h. Fluorescence images were photomicrographed with a Carl Zeiss Axio Observer D1.

2.5. RNA isolation

RNA was extracted by NucleoSpin RNA II (Takara Bio, Otsu, Japan) from the cells cultured with plasma-medium and from those cultured with H_2O_2-medium for 1 h. The integrity of RNA was checked with Agilent 2100 Bioanalyzer, and samples with RNA integrity number 9.40–10.0 were subjected to DNA microarray and quantitative real time PCR.

2.6. DNA microarray

A microarray chip of SurePrint G3 Human Gene Expression 8 × 60 K Microarray Kit (Agilent, Santa Clara, CA) was used for DNA microarray. After cRNA synthesis and Cy3 labeling with Low Input Quick Amp Labeling Kit One-Color (Agilent), hybridization was carried out overnight with Gene Expression Hybridization Kit (Agilent), followed by washings with Gene Expression Wash Buffer (Agilent). Data were scanned by Agilent DNA microarray scanner. Four technical replicates were evaluated in each comparison of plasma-medium vs regular medium (control), and H_2O_2-medium vs control.

2.7. Microarray data analysis

Microarray data were analyzed with GeneSpring GX12.1 software (Agilent). Normalization of data was carried out with an unpaired *t*-test ($p < 0.05$) and multiple testing correction by Benjamini-Hochberg False Discovery Rate (FDR). These algorithms

extracted 8024 gene probes, and their expression levels in plasma-medium and those in H_2O_2-medium were compared with those in regular medium (control). Gene probes which showed fold change $\geqslant 1.5$ or $\leqslant -1.5$ (≈ 0.67) (1.5 up and 1.5 down, respectively) were subjected to gene ontology analysis.

As for hierarchical clustering analysis, data of plasma-medium and H_2O_2-medium were corrected with control, and then imported to GeneSpring GX12.1 software. Clustering was done with 9022 gene probes extracted by unpaired *t*-test ($p < 0.05$) and subjected to multiple testing correction by Benjamini-Hochberg FDR.

2.8. Real-time PCR

Gene expression levels of antioxidant enzymes SOD1, CAT and PRDX6 were analyzed by quantitative real-time PCR with a housekeeping gene HPRT1 as the normalizer. A specific primer set for each gene was purchased from Takara Bio. RNA concentration was adjusted to 100 ng/μl with TE Buffer Solution, pH 8.0 (Nacalai tesque), and then cDNA was synthesized from 700 ng RNA with PrimeScript® RT reagent Kit and gDNA Eraser (Takara Bio) by using MJ Mini thermal cycler (Bio-Rad, Hercules, CA). Then mixture of Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany), DNase/RNase free water, forward and reverse primers (0.5 μM each), and 44 ng cDNA was subjected to real-time PCR by using a Qiagen Cycler Rotor Gene. The reaction condition consisted of 95 °C for 5 min and 30 cycles of 95 °C for 5 s and 60 °C for 10 s.

3. Results and discussion

The viability of the HeLa cells in the plasma-medium and the H_2O_2-medium was estimated by flow cytometry in each exposure condition of 1 h, 48 h, and 1 h followed by 48 h-culture (Fig. 1). The viability was more than 90% in the two media in all conditions (Fig. 2A). This finding showed that the cell toxicity was not high enough in these media to induce discernible damages. The viability was not significantly different between the two media in 1 h exposure, but was lower in the plasma-medium in 48 h exposure, and even in 1 h exposure followed by 48 h-culture. Some effect by the plasma-medium was feasibly responsible for the difference of viability, though no morphological difference was apparent by phase contrast microscopy (data not shown). To assess how the cells responded in the early phase, the condition of 1 h exposure was used in the following experiments.

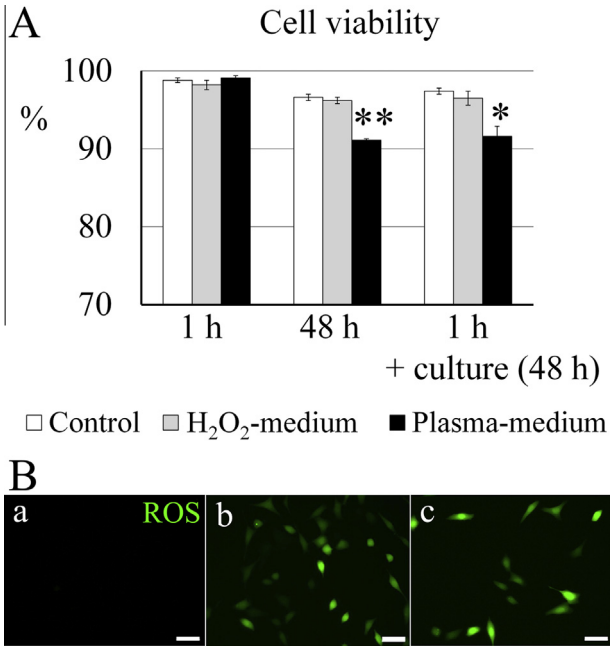

Fig. 2. Cell viability and ROS detection. (A) Cell viability in each medium with three exposure conditions. The data were percent of total cells applied (Mean ± S.D., $n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control or H_2O_2-medium. (B) Detection of intracellular ROS in control (a), H_2O_2-medium (b), and plasma-medium (c) in the exposure condition of 1 h. Fluorescence was emitted by CM-H_2DCFDA, a cell-permeant indicator for ROS, upon oxidation within the cell. Scale bars = 50 μm.

Although cellular toxicity was assessed to be low, ROS likely existed in the cells because H_2O_2 was contained even in the plasma-medium. As expected, cellular ROS were detected as the fluorescence of an indicator in both the plasma-medium and the H_2O_2-medium (Fig. 2B). This means that H_2O_2 permeated the cellular membrane and was transferred into the cells. Nonetheless, real-time PCR analysis of antioxidant enzymes revealed that the expression levels of SOD1, CAT and PRDX6 in the two types of media were not significantly different from those in the regular medium (Table 1). It is conceivable that the ROS represented by H_2O_2 were transferred into the cells, but the response time of 1 h was too short to achieve discernible induction of these antioxidant enzymes.

Accordingly, DNA microarray was performed in order to clarify what response occurred in gene expression by the plasma-medium and the H_2O_2-medium. Each of the two media was compared with the regular medium (control), so that the effect of chemical species generated by plasma was separately examined from that of H_2O_2 alone. The microarray data were then subjected to clustering analysis and gene ontology analysis.

The clustering analysis was carried out with 9022 gene probes. Samples with similar gene expression patterns are closely clustered in this analysis. The results of the plasma-medium and the H_2O_2-medium (4 replicates in each) were clustered separately (Fig. 3A), which showed that the alteration pattern of gene expression was different in these two media. Next, the number of 1.5 up/down genes was compared between the two media among 8024 gene probes selected by algorithms (Fig. 3B). The number of 1.5 up and that of 1.5 down were 457 and 30 in the plasma-medium, whereas they were 224 and 907 in the H_2O_2-medium, respectively. Thus, a larger population of genes was upregulated in the plasma-medium, but down-regulated in the H_2O_2-medium. This fact also confirmed the difference in gene expression pattern in the two media.

The top 10 genes of differential expression were partly shared by the two media (Tables 2 and 3). Five genes, i.e., ID2, RNU4ATAC, PGF, ARC and FOSB, were common in the upregulated genes (Table 2), and CXCL1 and TNF were common in the downregulated genes (Table 3). ID2 has been known as the immediate early gene which is rapidly induced in response to various stimuli [19]. Its protein, DNA-binding protein inhibitor ID-2, plays a role in regulation of cell cycle and differentiation [20,21]. FOSB is also an immediate early gene together with JUN (Table 2), and they encode components of transcription factor complex AP-1, which regulates gene expression responding to a wide variety of cellular stimuli [22]. PGF is a VEGF-related protein, and reported to be induced by oxidative stress [23]. Notably, elevation of the immediate early genes ID2 and FOSB and other common genes was greater in the plasma-medium, suggesting that some factors other than H_2O_2 account for this difference because an equivalent concentration of H_2O_2 was contained in the both media. In other words, H_2O_2 possibly contributed, at least in part, to the expression change of the common genes in the plasma-medium.

Gene Ontology analysis is useful to evaluate the microarray data via categorization of genes based on their attributes and the properties of their products. In 1.5 up genes, the "biological process" domain had the largest number of genes out of the three domains (Fig. 4A). As shown in the figure, 221 genes were extracted from the plasma-medium, whereas 103 genes were

Table 1
Gene expression of antioxidant enzymes in each medium analyzed by real-time PCR and microarray.

Gene symbol	Fold change[1]			
	Real-time PCR		Microarray	
	Plasma-medium	H_2O_2-medium	Plasma-medium	H_2O_2-medium
SOD1	1.04 ± 0.07	0.96 ± 0.07	1.19	1.07
CAT	0.89 ± 0.15	0.93 ± 0.11	1.08	1.17
PRDX6	0.93 ± 0.08	0.88 ± 0.16	1.07	1.07

Data in real-time PCR are shown as mean ± S.D. ($n = 4$).
[1] The expression ratio to the regular medium.

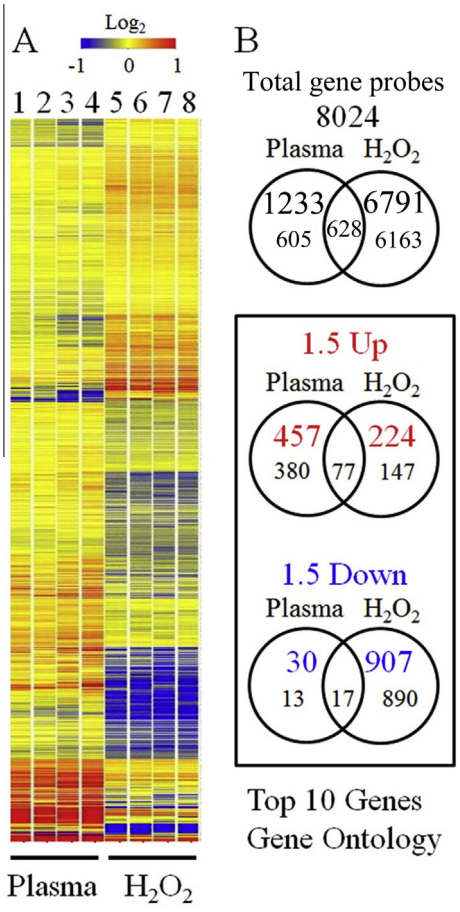

Fig. 3. Hierarchical cluster analysis and gene counting on DNA microarray data. (A) The hierarchical cluster was created from 9022 gene probes and four comparisons of each medium with control based on the Euclidean as distance metric (definition of distance). 1–4: plasma-medium, 5–8: H_2O_2-medium. Color bar indicates the expression level of genes with Log_2 Fold change. (B) Number of probes of total entities, and 1.5 up and 1.5 down genes. The 1.5 up/down gene probes were used for extracting the top 10 genes and for gene ontology analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracted from the H_2O_2-medium. In the next layer of this domain, 5 ontologies with a major population of genes, such as "response to stimulus," were comparable in both media, whereas several ontologies were unique for the plasma-medium (Fig. 4B). The number of genes in "signaling" was far greater in the plasma-medium, which also seemed to be a feature of this medium (Fig. 4B). When lower layers of "response to stimulus" and its branch "response to stress" were evaluated (Fig. 4C), several genes were found to correspond to "response to reactive oxygen species" in the two media (Table 4). Immediate early genes were included there, e.g., JUN, FOS, FOSL1

Table 2
Top 10 up-regulated genes in each medium.

Probe name	Gene symbol	Fold change
Plasma-medium vs control (/457 gene probes)		
A_32_P69368	ID2*	35.71
A_23_P143143	ID2*	24.23
A_33_P3839897	RNU4ATAC*	14.62
A_33_P3276693	PGF*	10.54
A_23_P53370	RND1	10.04
A_23_P365738	ARC*	9.67
A_32_P74409	C11orf96	7.02
A_33_P3246513	DIO3	6.52
A_23_P429998	FOSB*	6.51
A_33_P3323298	JUN	6.44
H_2O_2-medium vs control (/224 gene probes)		
A_32_P69368	ID2*	10.41
A_23_P42386	CGA	9.66
A_23_P143143	ID2*	5.75
A_33_P3839897	RNU4ATAC*	4.46
A_23_P365738	ARC*	4.10
A_24_P68908	LOC344887	3.61
A_33_P3276693	PGF*	3.31
A_23_P429998	FOSB*	3.28
A_23_P61317	GKN2	3.15
A_32_P196193	PAQR9	2.85

Ten probes are listed irrespective of duplication of genes.
* Gene common to the two media.

Table 3
Top 10 down-regulated genes in each medium.

Probe name	Gene symbol	Fold change
Plasma-medium vs control (/30 gene probes)		
A_23_P319050	ZNF493	−2.61
A_23_P7144	CXCL1*	−2.50
A_23_P376488	TNF*	−2.28
A_33_P3385453	BRCC3	−2.17
A_23_P28012	ZNF682	−2.00
A_33_P3317048	PLEKHM3	−1.96
A_33_P3259865	C1orf220	−1.87
A_23_P318904	SERTAD4	−1.87
A_33_P3287879	HIST1H3H	−1.86
A_33_P3328360	MIRLET7BHG	−1.83
H_2O_2-medium vs control (/907 gene probes)		
A_23_P7144	CXCL1*	−3.63
A_33_P3407013	IL6	−3.59
A_23_P376488	TNF*	−3.48
A_33_P3333992	LOC100132495	−3.36
A_33_P3416142	LOC100131234	−3.30
A_32_P42018	LOC100500938	−2.97
A_33_P3390107	RN18S1	−2.86
A_33_P3381117	LOC100128563	−2.81
A_23_P146325	ASAP1-IT1	−2.81
A_33_P3350343	MLL	−2.77

* Gene common to the two media.

and DUSP1. FOS and FOSL1 encode proteins of AP-1 as well. DUSP1 in the plasma-medium and shared HMOX1 are also known as oxidative stress genes [24,25]. The gene expression of AP-1 proteins was noticeably elevated in the plasma-medium as compared with that in the H_2O_2-medium. Though microarray data confirmed that the expression of the aforementioned antioxidant enzymes was not significantly changed, consistent with the real-time PCR data (Table 1), candidate genes involved in the response to ROS in the early phase of 1 h exposure were detected here. Regarding 1.5 down genes, on the other hand, statistically significant ontology was not extracted in either the plasma-medium or the H_2O_2 medium. These findings suggest that a cellular response similar to that of the H_2O_2-medium occurred in the plasma-medium, especially by the upregulated genes, including the response to chemical species, oxidative stress and ROS. Besides these analogous responses,

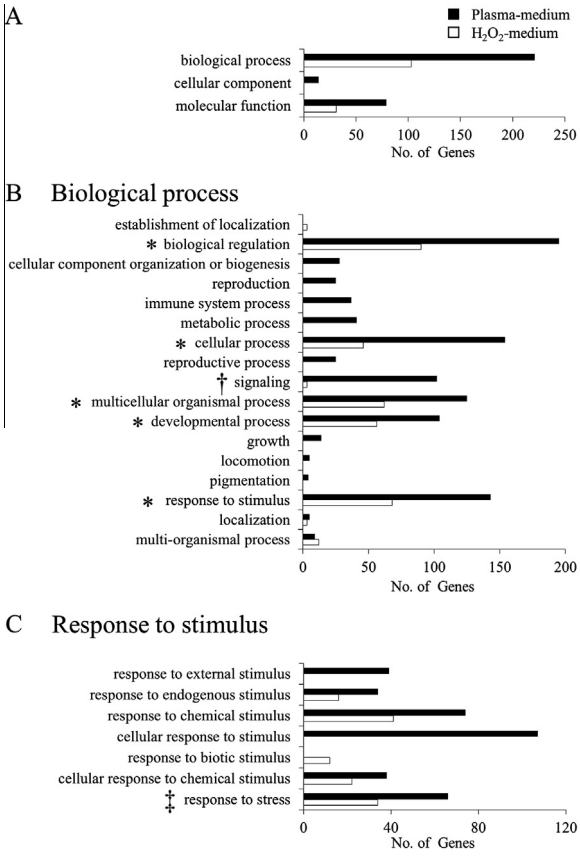

Fig. 4. Gene ontology analysis of 1.5 up genes. (A) Three domains comprise this analysis: cellular component, biological process, and molecular function. (B) Ontologies in the next layer of biological process. Five common ontologies were comparable in the two media (*). "Signaling" was also common in the plasma-medium but not in the H_2O_2-medium (†). (C) Ontologies in the next layer of response to stimulus. "Response to stress" (‡) includes "response to oxidative stress" and its branch "response to reactive oxygen species".

Table 4
Genes corresponded to "response to reactive oxygen species" by gene ontology analysis.

Gene symbol	Gene name	Fold change
Plasma-medium vs control		
JUN*	jun proto-oncogene	6.44
FOS	FBJ murine osteosarcoma viral oncogene homolog	5.11
KLF2	Kruppel-like factor 2 (lung)	3.23
HBA2*	Hemoglobin, alpha 2	2.13 (2.11**)
DDIT3	DNA-damage-inducible transcript 3	2.12
HMOX1*	Heme oxygenase (decycling) 1	2.08
APOE	Apolipoprotein E	1.91
DUSP1	Dual specificity phosphatase 1	1.61
H_2O_2-medium vs control		
HMOX1*	Heme oxygenase (decycling) 1	2.36
JUN*	jun proto-oncogene	1.90
FOSL1	FOS-like antigen 1	1.73
AQP1	Aquaporin 1 (Colton blood group)	1.72
HBA2*	Hemoglobin, alpha 2	1.68 (1.54**)
LCN2	Lipocalin 2	1.53

* Gene common to the two media.
** Another probe.

the plasma-medium appeared to induce a variety of unique responses of the cells.

An interesting finding was that the gene expression pattern of HeLa cells was generally different between the plasma-medium and the H_2O_2-medium, as shown by the clustering analysis and gene counting (Fig. 3), while the principal effects on the cellular response were partly shared by the two media as judged from the overlap of the top 10 genes and the gene ontology analysis (Tables 2–4, Fig. 4). These findings seem to be due to the difference in the oxidative stress levels and factors. In addition to H_2O_2, chemical species generated by the plasma irradiation, including ROS, are able to influence cellular response. We have reported that nitrogen oxides and ozone were generated when the culture medium was exposed to the plasma [11]. Thus, the oxidative stress level is considered to be higher in the plasma-medium than in the H_2O_2-medium. In our previous study with high level irradiation of plasma, the cell inactivation effect of formed H_2O_2 was predominant [18]. Once it penetrates cells, H_2O_2 produces hydroxyl radicals (·HO), which are a key factor of oxidative stress. Moreover, H_2O_2 damages DNA because it reaches the nucleus due to its stability [26]. In contrast, at the non-lethal level of 40 s irradiation employed here, HeLa cells sufficiently survived and showed a wide variety of gene expression responses, and the influence of ROS other than H_2O_2 appears to more significantly affect the responses of viable cells. It is likely that these factors contribute to the unique effects of the plasma-medium on cells, modifying the effects of the H_2O_2 alone. In fact, the cell viability was more greatly affected, and immediate early genes were generally more upregulated in the plasma-medium. DUSP1 (MKP-1) protein inhibits p38 MAPK and JNK and has been reported to be involved in cellular defense against oxidative stress-induced apoptosis with the MAPK-AP-1 pathway [24]. Upregulation of this gene in the plasma-medium may reflect self-defense against higher oxidative stress indicated by the elevated gene expression of AP-1 proteins. Further study of the effect of chemical species generated by plasma will lead to control of the oxidative stress levels and cellular responses.

In conclusion, in culture medium exposed to plasma for 40 s, change in various gene expressions of HeLa cells was induced and there were intrinsic effects on the cell responses in addition to the common effects with H_2O_2 alone. This study provides a base to clarify and control the multiple stimuli by plasma to cells.

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