Identification of low-dose responsive metabolites in X-irradiated human B lymphoblastoid cells and fibroblasts

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(Received 1 May 2014; revised 31 July 2014; accepted 16 August 2014)

Ionizing radiation (IR) induces cellular stress responses, such as signal transduction, gene expression, protein modification, and metabolite change that affect cellular behavior. We analyzed X-irradiated human Epstein-Barr virus-transformed B lymphoblastoid cells and normal fibroblasts to search for metabolites that would be suitable IR-responsive markers by Liquid Chromatography–Mass spectrometry (LC–MS). Mass spectra, as analyzed with principal component analysis, showed that the proportion of peaks with IR-induced change was relatively small compared with the influence of culture time. Dozens of peaks that had either been upregulated or downregulated by IR were extracted as candidate IR markers. The IR-changed peaks were identified by comparing mock-treated groups to 100 mGy-irradiated groups that had recovered after 10 h, and the results indicated that the metabolites involved in nucleoside synthesis increased and that some acylcarnitine levels decreased in B lymphoblastoids. Some peaks changed by as much as 20 mGy, indicating the presence of an IR-sensitive signal transduction/metabolism control mechanism in these cells. On the other hand, we could not find common IR-changed peaks in fibroblasts of different origin. These data suggest that cell phenotype-specific pathways exist, even in low-dose responses, and could determine cell behavior.

Keywords: low-dose radiation; metabolomics; LC–MS; X-irradiation; fibroblast; B lymphoblastoid cell

INTRODUCTION

Ionizing radiation (IR)-induced phenotypic and molecular changes in biological specimens have been widely and continuously studied, but aspects of the whole picture remain unclear. Forward genetics approaches to analyzing radiation-sensitive disorders, such as the use of ataxia telangiectasia mutated (ATM), have elucidated the radiation-induced signaling mechanisms of kinases and substrates linked to DNA double-strand break repair [1]. Such proteins and protein-modification networks contribute to the major cellular response after ionization. These radiation-induced molecular networks affect a myriad of cellular changes in the epigenetic modification of DNA, transcription, protein levels and modification, and variations in small molecules [2–5]. These alterations could provide biomarkers of IR responses [6].

Small metabolites, such as sugars, organic acids, and amino acids, are ubiquitous molecules in biological organisms. The abundance of these molecules defines the cellular composition, and the cellular environment is produced by gene and protein expression and modification. The role of these molecules as constituents or nutrients (and as signal regulators) remains to be clarified. For example, some amino acids work as second messengers or neurotransmitters. Thus,
analyzing the levels of such metabolites in cellular phenomena is significant because these molecules could be biomarkers of a range of diseases and thus have prognostic implications [7].

Comprehensive analyses of small metabolites in certain tissues or cells are widely applied in the biological field of metabolomics [8]. Two forms of technology are primarily applied in metabolomics: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry [9, 10]. For metabolomics, a mass spectrometer is usually used as a signal detector after the separation of compounds using, for example, gas chromatography (GC) or capillary electrophoresis [11, 12]. Liquid chromatography coupled with mass spectrometry (LC–MS) is most commonly used for analyzing non-volatile hydrophilic compounds [13]. Combination of an electrospray ionization (ESI) ion source and LC–MS allows the detection of polar, fragile biomolecules. LC–MS is now widely used for target analysis of certain disease marker molecules as well as for non-target analysis of markers for phenomena or disease [14]. In particular, the use of a high-resolution mass spectrometer, such as Orbitrap, enables accurate mass acquisition, which allows easier metabolite identification [15]. Additionally, for analyses of polar compounds by LC before MS, hydrophilic interaction liquid chromatography (HILIC) in combination with a high content of organic mobile phase offers an appealing capability of separation based on the retention principle. HILIC is beneficial for ionization efficiency because of good compatibility with ESI using a combination of acetonitrile and volatile salts (such as ammonium formate) [16].

A pioneering series of studies introduced MS-based metabolomics to determination of the radiation response of biological organisms (‘radiation metabolomics’) [17] and defined the patterns of metabolic changes by comparing radiation-exposed with non-exposed cells or animal specimens [18–23]. In these studies, the authors aimed to find markers for the triage of high-dose-irradiated people in large accidentally exposed populations via a minimally invasive method, and they elucidated the metabolomics response to high-dose irradiation to enable biological dosimetry. Other researchers also published a study on the serum metabolomics of irradiated rats using GC–MS [24].

In the case of the nuclear power plant accident in Fukushima, the residents were exposed to low-dose irradiation (< 25 mSv), as estimated from the Fukushima Health Management Survey [25–27]. In this manuscript, we aimed to elucidate the low-dose acute response of human cells using B lymphoblastoid cell lines and normal fibroblasts using LC–MS-mediated metabolomics, because there is minimal information on this topic to date. Moreover, markers identified by this study could provide primary information for identifying the mechanism by which low-dose exposure affects biological organisms.

MATERIALS AND METHODS

Study design
Figure 1 shows the outline of our analyses. Both human B lymphoblasts and fibroblasts were inoculated and X-irradiated. The cell extracts were analyzed by HILIC–separated mass spectrometry operated in positive and negative ion modes followed by data processing and evaluation. As low-dose irradiation-induced cellular change was predicted to be minimal, experimental procedures were meticulously conducted, and all apparatus were carefully manipulated. For example, several cleaning runs were conducted in a series of analyses to keep the HILIC column and ESI needle clean. If the variability of spectra (especially in total signal intensity and retention time) were high in a series of experiments, LC–MS analyses were repeated.

Reagents
Authentic compounds were purchased from Wako Chemical Co. (Tokyo, Japan), including glutathione, hypoxanthine, xanthine, inosine, uridine, cytidine, guanine, guanosine, and L-ascorbic acid. All solvents were LC–MS grade (Kanto Chemical, Tokyo, Japan).

Cells
Epstein-Barr virus-transformed human B lymphoblastoid cell lines C007, C032 and C035 were obtained from Dr Takahashi (Radiation Effects Research Foundation, Hiroshima) [28]. Heterogeneity of the culture was confirmed by PCR amplification of HLA-DQ loci. Cells were cultured in 10% fetal bovine serum-supplemented RPMI 1640 medium in a humidified CO2 incubator at 37°C. Human fetal lung fibroblasts TIG-1 and TIG-3 [29] were cultured in DMEM supplemented with 10% FCS. These cells could logarithmically proliferate after passage, indicating that the cells had not aged. The population doubling levels (PDLs) used in the study were 39 PDL for TIG-1 and 50 PDL for TIG-3, while the maximum PDLs of TIG-1 and TIG-3 were 50 and 80, respectively.

Radiation exposure
At 16 h before irradiation, B cells cultured on a large scale were collected and re-inoculated at 5 × 10⁶ cells/ml. Cell viability was confirmed to be > 80% before irradiation. Groups of culture flasks (n = 3 for each dose), each with 3 × 10⁶ cells, were moved to an X-ray machine (MBR-1520A, Hitachi Medical, Tokyo, Japan) and irradiated with 20, 100 and 1000 mGy at a dose rate of 0.5 Gy/min (with continuous dose monitoring using a dosimeter probe). Mock-treated cells (indicated as ‘No XRT’ or ‘0 mGy’) were similarly manipulated, except they were not exposed to actual X-irradiation. Control cells (indicated as ‘US’, unstimulated) were not moved from the incubator. Then, cells were cultured...
at 37°C for either 1 or 10 additional hours. Cells were recovered by centrifugation, and cell pellets were washed with 5 ml of phosphate-buffered saline.

In the case of fibroblasts, subconfluent cultures (3 × 10^6 cells/100 mm dish, n = 3) of TIG-1 cells and TIG-3 cells were also irradiated, incubated for 1 or 10 h, and similarly recovered.

**Sample preparation**

For LC–MS, cell pellets were suspended with 200 μl of LC–MS-grade methanol containing 100 μM of L-ascorbic acid. Cell suspensions were sonicated for 5 s using a probe-type sonicator (Sonac-150, Honda Electronics, Aichi, Japan), left on ice for 10 min, and centrifuged at 10 000 g for 5 min at 4° C. The supernatants were recovered and stored at -80°C until analysis.

**LC–MS data acquisition**

Cell extracts of each cell line were serially analyzed by LC–MS in order of elapsed time in the same session (to exclude instrumental and environmental variability). A 10 μl methanol extract was separated on a HILIC column (TSKgel Amide-80, 2.0 × 150 mm, 5 μm, TOSOH, Tokyo, Japan) [30] connected to an LC (LC20, Shimadzu, Kyoto, Japan, or UltiMate 3000, Thermo Scientific/Dionex, CA USA). The LC gradient using mobile phase A and B (A: 10% MiliQ water, 90% Acetonitrile 10 mM ammonium formate, B: 10 mM ammonium formate in MiliQ) was as follows: 0–3 min, 100% A; 3–16 min, 0–50% B; 16–35 min, 50% A, 50% B; 35–40 min, 100% A. Elute with separated molecules from the column was ionized with an ESI source heated to 250°C, which works in positive ion mode at 4000 V or negative ion mode at 3500 V. Sheath and auxiliary gas were set to 50 and 15 (arbitary units), respectively. The voltage configuration of several electrodes and lenses from the entrance to the ion trap was manually decided to obtain maximum signal strength of m/z 182 for positive ion mode and of m/z 180 for negative ion mode (L-tyrosine). The mass spectra were measured by full scan mode from m/z 50 to m/z 600 using an Orbitrap Velos Pro or an Orbitrap Elite mass spectrometer (Thermo Scientific, MA, USA). No apparent difference between the data obtained by 2 LC-MS systems was observed (data not shown). In order to retain mass accuracy of the spectra, the ‘Lock Mass’ function of Tune Plus software (Thermo Scientific, MA, USA) was used.
Scientific) was enabled, detecting continuous peaks of environmental origin: for positive ion mode, \( m/z \) 391.2843 of bis (2-ethylhexyl)phthalate \([M+H]^+\) or \( m/z \) 198.1852, an unidentifed peak, were used, and for negative ion mode, \( m/z \) 255.2330 of palmitic acid \([M-H]^-\) and \( m/z \) 283.2643 of stearic acid \([M-H]^-\) were used.

**Data analysis**

Parameters of LC–MS spectra, including ion intensity, mass accuracy, and retention time, were first evaluated using the RawMeat software (VAST Scientific, Cambridge, MA, USA) and analyzed using MZmine 2 [31] coupled with MSFileReader (Thermo Scientific). Ion peaks were extracted at every scan time, and ion chromatograms were built and deconvoluted to obtain peak lists with \( m/z \) and retention time. Then, peak lists of different samples were aligned by \( m/z \) and retention time. Aligned peak lists were exported to a comma-separated values (CSV) format file and further analyzed using Microsoft Excel. Before peak comparison, the sum of peak areas for every spectrum was calculated (total peak area). When the relative standard deviation of the total peak area of spectra in a series of experiments exceeded 10%, peak area normalization was performed by calculating scaling factors of total peak area compared with that of a non-irradiated cell spectrum. The value of each peak area was multiplied by the scaling factors. Then, normalized peak areas of three samples were used for multivariate analyses and quantitative comparison between mock-treated and irradiated cells. The aligned peak lists were also used for the principal component analysis (PCA) tool built into MZmine 2. All of the deconvoluted and aligned ion peaks for every sample were selected and analyzed. The PCA figures obtained were redrawn as black-and-white presentations. Peak increase or decrease was evaluated by the Student’s \( t \)-test in Microsoft Excel, with significance determined by \( P \)-values < 0.05.

**Molecular ion identification**

Extracted ions that differed among samples were further used for molecular identification. The accurate mass value of a peak was used to list candidate formulae within 3 ppm mass tolerance using QualBrowser of Xcalibur software (Thermo Scientific). The human metabolome database was used to identify molecules with candidate formulae [32, 33]. Further confirmation was performed using parent mass-directed automatic MS/MS analyses, MS/MS spectra of peaks were compared with the MS/MS data of candidate molecules registered in the MassBank database [34, 35]. Some candidate molecules were confirmed by comparing LC retention time and MS/MS spectra for authentic compounds.

**RESULTS**

**PCA of mass spectra**

The peak profiles obtained from mass spectra were further analyzed using multivariate data analysis with the use of MZmine2-attached PCA. First, the characters of every spectrum were evaluated by PCA score plot. The spectra of 100-mGy-irradiated and non-irradiated cells in positive and negative ion modes are shown in Fig. 2a and b, respectively. Score plots show cell-dependent spatial separation of the groups (coarsely dotted circles), indicating that cellular spectra have cell-specific characters. Moreover, spectra of B lymphoblastoids and fibroblasts formed cell-type-dependent
clusters (finely dotted circles). On the other hand, spectra of irradiated cells did not produce independent groups in every cell cluster. These data meant that mass spectra contained cell line- and cell phenotype-specific characters, and the radiation-induced peak change was relatively small.

Then, in order to clarify the effects of IR, spectra of cells exposed to different doses of irradiation were also included in PCA. All cell spectra were analyzed, including non-irradiated and 20-, 100- and 1000-mGy-irradiated cells obtained in positive or negative ion modes (Fig. 3). In

Fig. 3. Principal component analysis of LC–MS spectra comparing radiation dose with culture time. B lymphoblastoid cells and fibroblasts were irradiated at 0 (sham-treated, indicated as ‘No XRT’) 20, 100 or 1000 mGy and cultured for 1 h or 10 h. PCA score plots of positive ion mode (a) and negative ion mode (b) are indicated. US (circles with checkered pattern), 20 mGy (blank circles), 100 mGy (hatched circles), and 1000 mGy (black circles) are plotted with culture times. Numbers indicated beside the circles are culture times. Spectra of 0-, 1- and 10-h cultures are separately enclosed in dotted circles.
### Table 1. Differential peaks of C007 and TIG-3 by comparing sham-treated and 100-mGy-irradiated cells

#### C007 B lymphoblastoid cells

| m/z     | Retention time (min) | Change | P-value | Candidate formula | Name                              | MS/MS |
|---------|----------------------|--------|---------|-------------------|-----------------------------------|-------|
| 276.12  | 12.98                | 2.15   | 0.004   | [C10H17N3O6+H]+   | Gamma-Glutamyl glutamine          | ○     |
| 138.05  | 4.26                 | 1.77   | 0.038   | [C5H4N4O+H+13C]+  | Hypoxanthine                      | ○     |
| 137.05  | 4.26                 | 1.68   | 0.022   | [C5H4N4O+H]+      | Hypoxanthine                      | ○     |
| 152.06  | 7.22                 | 1.67   | 0.035   | [C5H5N5O+H]+      | Guanosine fragment                | ○     |
| 489.11  | 15.75                | 1.57   | 0.001   | [C14H26N4O11P2+H]+| CDP-choline                       | ○     |
| 175.00  | 4.26                 | 1.5    | 0.039   | [C5H4N4O+K]+      | Hypoxanthine                      | ○     |
| 325.04  | 13.31                | 1.43   | 0.032   | [C9H13N2O9P+H]+   | UMP                               | ○     |
| 162.11  | 12.64                | 1.29   | 0.048   | [C7H15N3O3+H]+    | L-Carnitine                       | ○     |
| 494.32  | 8.31                 | 1.28   | 0.012   | [C24H48N10P7H]+   | LysoPC(16:1)                      | ○     |
| 348.07  | 12.74                | 0.26   | 0.011   | [C10H14N5O7P+H]+  | AMP                               | ○     |
| 260.11  | 13.03                | 0.47   | 0.014   | [C11H17N6O6H+]    | Fumaryl carnitine                 | ○     |
| 426.36  | 5.3                  | 0.52   | 0.008   | [C25H47N4O4+H]+   | Oleoylcarnitine                   | ○     |
| 259.11  | 13.04                | 0.55   | 0.017   | [C8H20N6P+H+13C]+ | sn-glycero-3-Phosphocholine       | ○     |
| 258.11  | 13.02                | 0.56   | 0.016   | [C8H20N6P+H]+     | sn-glycero-3-Phosphocholine       | ○     |
| 398.33  | 6.06                 | 0.57   | 0.002   | [C23H43N4O4+H]+   | 9-Hexadecenoylcarnitine           | ○     |
| 524.37  | 8.28                 | 0.7    | 0.045   | [C26H54N10P7H]    | LysoPC(18:0)                      | ○     |
| 400.34  | 6.83                 | 0.7    | 0.001   | [C23H45N4O4+H]    | Palmitoylcarnitine                | ○     |
| 232.15  | 9.42                 | 0.74   | 0.003   | [C11H21N4O4+H]    | Isobutyryl-L-carnitine            | ○     |
| 216.06  | 12.28                | 0.74   | 0.047   | [C5H14N6O6P+H+]   | sn-glycero-3-Phosphoethanolamine  | ○     |
| 372.31  | 6.82                 | 0.76   | 0.008   | [C21H41N4O4+H]+   | Tetradecanoylcarnitine            | ○     |

#### Negative ion mode

| m/z     | Retention time (min) | Change | P-value | Candidate formula | Name                              | MS/MS |
|---------|----------------------|--------|---------|-------------------|-----------------------------------|-------|
| 150.04  | 7.2                  | 1.73   | 0.007   | [C5H5N5O-H]−      | Guanosine fragment                | ○     |
| 111.02  | 2.44                 | 1.61   | 0.02    | [C4H4N2O2-H]−     | Uracil                            | ○     |
| 136.03  | 4.2                  | 1.6    | 0.012   | [C5H4N4O-H+13C]−  | Hypoxanthine                      | ○     |
| 151.03  | 5.16                 | 1.57   | 0.022   | [C5H4N4O2-H]−     | Xanthine                          | ○     |
| 135.03  | 4.21                 | 1.53   | 0.013   | [C5H4N4O-H]−      | Hypoxanthine                      | ○     |
| 171.01  | 4.2                  | 1.49   | 0.018   | [C5H4N4O+CL−]−    | Hypoxanthine                      | ○     |
| 267.07  | 6.6                  | 1.43   | 0.039   | [C10H12N4O5-H]−   | Inosine                           | ○     |
| 329.25  | 1.1                  | 1.33   | 0.028   | [C22H34O2-H]−     | Docosapentaenoic acid             | ○     |
| 281.25  | 1.11                 | 1.2    | 0.026   | [C18H34O2-H]−     | Octadecenoic acid                 | ○     |
| 302.1   | 12.48                | 0.57   | 0.035   | [C8H20N6P+HCOO−]− | sn-glycero-3-Phosphocholine       | ○     |
| 171     | 12.5                 | 0.71   | 0.046   | [C3H9O6P-H]−      | sn-Glycerol 3-phosphate           | ○     |
| 229.01  | 12.64                | 0.71   | 0.044   | [C5H11O8P-H]−     | D-Ribose 5-phosphate              | ○     |

Continued
| m/z  | Retention time | Change | P-value | Candidate formula | Name                                      | MS/MS |
|------|----------------|--------|---------|-------------------|-------------------------------------------|-------|
| 214.05 | 12.09          | 0.75   | 0.008   | [C5H14NO6P-H]−    | sn-glycero-3-Phosphoethanolamine          |       |
| 243.06 | 7.66           | 0.79   | 0.038   | [C9H12N2O6-H]−    | Uridine                                   |       |

**TIG-3 cells**

| m/z  | Retention time | Change | P-value | Candidate formula | Name                                      | MS/MS |
|------|----------------|--------|---------|-------------------|-------------------------------------------|-------|
| 170.03 | 12.1           | 2.38   | 0.004   | [C4H9N3O2+K]+     | Creatine                                   |       |
| 446.33 | 4.9            | 2.03   | 0.011   | [C25H45NO4+Na]+   | Linoleylcarnitine                          |       |
| 301.29 | 1.6            | 1.78   | 0.004   | [C18H37NO2+H+13C]+| Sphingosine                                |       |
| 525.37 | 7.5            | 1.76   | 0.046   | [C26H54NO7P+H+13C]+| LysoPC (18:0)                             |       |
| 300.29 | 1.4            | 1.69   | 0.003   | [C18H37NO2+H]+    | Sphingosine                                |       |
| 522.35 | 7.6            | 1.56   | 0.012   | [C26H52NO7P+H]+   | LysoPC (18:1)                             |       |
| 302.3  | 1.6            | 1.53   | 0.007   | [C18H39NO2+H]+    | Sphinganine                                |       |
| 346.05 | 13.2           | 1.5    | 0.0001  | [C10H17N3O6S+K]+  | Glutathione                                |       |
| 524.37 | 7.5            | 1.5    | 0.044   | [C26H54NO7P+H]+   | LysoPC (18:0)                             |       |
| 448.34 | 4.7            | 1.45   | 0.049   | [C25H47NO4+Na]+   | Oleoylcarnitine                            |       |
| 133.08 | 13             | 1.4    | 0.008   | [C4H9N3O2+H+13C]+| Creatine                                   |       |
| 216.06 | 13.2           | 1.38   | 0.001   | [C5H14NO6P+H]+    | sn-glycero-3-Phosphoethanolamine          |       |
| 426.36 | 4.9            | 1.37   | 0.02    | [C25H47NO4+H]+    | Oleoylcarnitine                            |       |
| 219.14 | 11.6           | 1.35   | 0.039   | [C10H19NO4+H+13C]+| Propanoylcarnitine                        |       |
| 520.34 | 7.7            | 1.35   | 0.047   | [C26H50NO7P+H]+   | LysoPC (18:2)                             |       |
| 76.04  | 12.5           | 1.33   | 0.001   | [C2H5NO2+H]+      | Glycine                                    |       |
| 90.06  | 12.1           | 1.29   | 0.007   | [C3H7NO2+H]+      | beta-Alanine                               |       |
| 454.29 | 6.7            | 1.28   | 0.014   | [C21H44NO7P+H]+   | LysoPE(16:0)                              |       |
| 116.07 | 13.8           | 1.27   | 0.01    | [C5H9NO2+H]+      | Proline                                    |       |
| 133.06 | 13.5           | 1.27   | 0.001   | [C4H8N2O3+H]+     | Asparagine                                 |       |
| 147.12 | 15.9           | 1.27   | 0.02    | [C7H15NO2+H+13C]  | 3-Dehydroxycarnitine                      |       |
| 218.14 | 11.6           | 1.27   | 0.018   | [C10H19NO4+H]+    | Propanoylcarnitine                        |       |
| 510.35 | 7.6            | 1.27   | 0.02    | [C25H52NO7P+H]+   | LysoPE (20:0)                             |       |
| 120.07 | 12.3           | 1.26   | 0.005   | [C4H9N3O3+H]+     | Threonine                                  |       |
| 132.08 | 13.2           | 1.26   | 0.012   | [C4H9N3O2+H]+     | Creatine                                   |       |
| 126.02 | 10.3           | 1.25   | 0.009   | [C2H7N3O3S+H]+    | Taurine                                    |       |
| 110.03 | 11.9           | 1.21   | 0.003   | [C2H7NO2S+H]+     | Hypotaurine                                |       |
| 233.16 | 10.4           | 1.21   | 0.046   | [C11H21NO4+H+13C]+| Isobutyryl-L-carnitine                    |       |
| 146.12 | 15.9           | 1.2    | 0.008   | [C7H15NO2+H]+     | 3-Dehydroxycarnitine                      |       |
| 466.33 | 5              | 1.2    | 0.004   | [C25H49NO4+K]+    | Stearoylcarnitine                         |       |

Continued
positive ion mode, spectra produced coarse and overlapped groups with culture time but not with radiation dose in score plots of each cell. In negative ion mode, C032 and C035 cells did not show culture-time clustered plot profiles. These score plot data indicate weak correlation with culture time in positive ion mode and also indicate that change in peaks in irradiated cells was relatively small compared with culture time, and culture time-dependent changes were not significant. Much less spectral change was induced by culture time and irradiation in negative ion mode compared with that in positive ion mode.

### Identification of different peaks

To define the IR-changed peaks, a Student’s *t*-test was used to compare mock-treated with irradiated groups recovered at corresponding times. Table 1 shows candidate molecules that commonly changed in 100-mGy-treated 10 h-cultured B lymphoblastoid cell line, C007. Dozens of peaks increased and decreased. Similar change was also observed in other B cell lines, C032 and C035 (Supplementary Table 1). The numbers of peaks increased (up) or decreased (down) were, C007 (+ion: 14 up, 27 down; –ion: 19 up, 9 down), C032 (+ion: 24 up, 22 down; –ion: 12 up, 3 down), C035 (+ion:
24 up, 12 down; +ion: 20 up, 10 down), TIG-1 (+ion: 25 up; −ion: 39 up, 23 down), TIG-3 (+ion: 86 up, 68 down; −ion: 17 up, 11 down). Common prominent changes in molecules included an increase in nucleic acid metabolites (such as hypoxantine) and a decrease in acylcarnitines (carboxylic acid-bound carnitines, such as oleylcarnitine: oleic acid-bound carnitine).

Table 1 also shows candidate molecules commonly changed in 100-mGy-treated, 10-h cultures of TIG-3 fibroblasts. Some of the changed peaks in B lymphoblasts (such as those for carnitines and nucleic acid metabolites) showed opposite responses. TIG-1 cells showed a different pattern of response from TIG-3 cells, despite these two cells both being categorized as ‘fetal lung fibroblasts’ (Supplementary Table 1).

These B lymphoblast and fibroblast data indicate that low-dose IR could affect nucleic acid metabolism in cytoplasm and carnitine metabolism in mitochondria, even at low doses.

**MS/MS identification**

Changed peaks were identified by their accurate mass and accurate mass-directed MS/MS fragmentation analyses. If MS/MS spectra were obtained, they were compared to the records registered in MassBank or HMDB. Additionally, purchased authentic compounds were used to confirm the MS/MS spectra and chromatographic retention time. Figure 4 shows some of the molecules identified by MS/MS analysis, and putative MS/MS fragments are indicated in the formulae.

**Dose- and time-dependent change**

Dose- and time-dependent changes of some metabolites in irradiated C007 cells are shown in Fig. 5.
irradiation, nucleic acid metabolites (except for inosine) increased with culture time when comparing US 0 with US 10. These US points used cells without any manipulation, indicating time-dependent change. Sham treatment (taking the culture to the X-ray generator and taking it back to the incubator) showed some change in guanine and the carnitines, indicating that these metabolites might alter their levels as a result of mechanical stress or temperature change. In order to compare the radiation-induced metabolic alteration, we compared the 0-Gy- and 100-mGy-treated cells. Nucleic acid metabolites (hypoxanthine, IMP, guanine, and xanthine) clearly increased (even after 1 h) as a result of 100-mGy irradiation, and the level of increase was similar in 1000-mGy-irradiated cells (Fig. 5a). These metabolites had accumulated more by 10 h, indicating time-dependent change was enhanced by irradiation. On the other hand, in the case of the acylcarnitines, culture time-dependent decrease was observed in untreated cells. Irradiation induced a slight decrease in 20-mGy-irradiated cells recovered after 1 h, whereas cells treated with more than 100 mGy showed a more prominent decrease (Fig. 5b).

These molecules, however, showed the opposite change in TIG-3 cells (acylcarnitines: Fig. 6a; nucleic acid metabolites: Fig. 6b). The acylcarnitines showed a time-dependent increase in US cells, whereas the increase was cancelled in mock-treated cells. Irradiation recovered the time-dependent increase that is prominent in 100-mGy-treated cells. On the contrary, hypoxanthine and inosine showed a time-dependent decrease, which was cancelled by sham treatment. IR failed to maintain the levels of these compounds induced by sham treatment.

Increase and decrease in these metabolites, even due to sham treatment and to low doses of irradiation, suggests the presence of some cellular machinery that is controlled by mechanical stress, temperature change or IR. The various forms of this machinery may interact with each other, and could correlate cellular responses such as cell cycle arrest and apoptosis.

**DISCUSSION**

The biological significance of the metabolite changes after low doses of irradiation as revealed by this study is still unknown. However, the increase in nucleic acid metabolites in high-dose-irradiated animals has already been reported in previous ‘radiation metabolomics’ studies [19–23]. These
Fig. 5. Metabolites in C007 cells altered by irradiation. C007 cells were irradiated at 0 (sham-treated), 20, 100 and 1000 mGy, cultured for 1 or 10 h, and recovered. ‘US’ indicates untreated cells. The values of peak areas of indicated metabolites in mass spectra obtained in positive ion mode are plotted. An asterisk indicates significant difference ($P<0.05$): (a) metabolites increased by X-irradiation; (b) metabolites decreased by X-irradiation.

Fig. 6. Metabolites in TIG-3 cells altered by irradiation. TIG-3 cells were irradiated at 0 (sham-treated), 20, 100 and 1000 mGy, cultured for 1 or 10 h, and recovered. ‘US’ indicates untreated cells. The values of peak areas of indicated metabolites in mass spectra obtained in positive ion mode are plotted. An asterisk indicates significant difference ($P<0.05$): (a) metabolites increased by X-irradiation; (b) metabolites decreased by X-irradiation.
papers propose that quantitative evaluation of these molecules could facilitate their use as markers for biological dosimetry and suggest that a dose-responsive mechanism may exist. Our data indicated that the mechanism also functioned in low-dose ranges (as low as 20 mGy). We should further analyze the response of these nucleosides for evaluation of the effects of low-dose irradiation.

The significance of changes in nucleotide metabolites is usually discussed in the context of DNA repair. In our study, a time-dependent increase was also observed without irradiation. Recent reports concerning ATM kinase function indicate that the pentose phosphate pathway is upregulated by ATM under oxidative stress, which results in the accumulation of nucleotide metabolites [36]. The time-dependent increase might be induced by a similar stress mechanism, because continuous culture depletes the medium content and increases waste products (such as lactate); this should be determined by further analysis.

In this study, we used two types of cells: EB lymphoblast cell lines and fetal lung fibroblasts. Differences in radiation-induced cellular metabolomic change were observed between these types of cells, indicating cell-type-specific radiation response pathways. On the other hand, metabolic differences occurred even within cell types. Many parameters might affect cell response, such as genetic background and PDLs in fibroblasts. These differences indicate the difficulties of clarifying low-dose responses. Previous reports have indicated that the response to IR differs among cell types in cell cycle profile, gene expression and bystander effect [37–39]. Our data indicate a possibility that the metabolomics differences may affect cellular response. Indeed, Lall et al. reported that low-dose-radiation-induced metabolic change controlled by HIF1α might regulate cellular adaptive responses observed in fibroblasts, lymphoblasts and mouse models [40]. Their data, showing the cancellation of the low-dose response by N-acetylcysteine treatment, indicates the existence of low-dose sensitive radiation-responsive machinery mediated by superoxide. Further experiments are required to prove the relationship between the low-dose response and superoxide production.

Additionally, the decrease in some acylcarnitines reveals a possible mechanism for mitochondrial disturbance caused by low-dose radiation in our data. Acylcarnitine is a chaperone of fatty acids from the mitochondrial intermembrane space through the mitochondrial inner membrane to the matrix. Production of radicals by radiation exposure might trigger fatty acid metabolism around mitochondria. Especially in low doses, radical signals would be dominant because DNA strand breaks might be at low frequencies. These low-dose-responsive molecules could provide information regarding radical-induced signals, and this needs to be clarified further.

In a high-dose experiment, long-term differential changes in mouse intestines were analyzed by GC–MS-based metabolomics [41]. That study showed that many pathways, including nucleotide metabolism, are perturbed, even 2 months post-irradiation, and even though organs such as the small intestine are comprised of highly regenerative tissue. Consequently, we should investigate the long-term effects of low-dose irradiation in future studies.

In conclusion, our data clearly indicate that radiation-induced metabolic change occurred in low-dose-irradiated cells, and the responses varied among different cell types according to LC–MS. The application of this method to many kinds of biological samples, such as the tissues and body fluids of low-dose-irradiated specimens, is likely to produce a detailed picture of radiation response in living organisms.

SUPPLEMENTARY DATA

Supplementary data are available at the Journal of Radiation Research online.

ACKNOWLEDGEMENTS

The operation of Orbitrap Velos Pro in QBIC RIKEN was kindly approved and supported by Dr Tsutomu Masujima. And the operation of Orbitrap Elite in Fukushima Medical University was kindly supported by Dr Yoshimi Homma, Dr Toshiyuki Suzuki and Dr Satoshi Waguri.

FUNDING

This work was partly supported by a Grant-in-Aid for Scientific Research (C), No. 24619004, from the Japan Society for the Promotion of Science, and by the Fukushima Prefecture Radiation Medicine Research Development Fund from the Japanese Ministry of Education, Culture, Sports, Science, and Technology. Funding to pay the Open Access publication charges for this article was also provided by a Grant-in-Aid for Scientific Research (C) described before.

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