Proteomic Identification of Insulin-like Growth Factor-binding Protein-6 Induced by Sublethal H$_2$O$_2$ Stress from Human Diploid Fibroblasts*

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Fibroblasts are the most ubiquitous cell types within our body. They produce various factors to maintain the texture and structure of a particular organ or tissue. To identify protein factors secreted by fibroblasts and alteration of these protein factors upon oxidative stress, HCA$_3$ human skin diploid fibroblasts were exposed to a sublethal dose of H$_2$O$_2$, which induces a prematurely senescent phenotype. Conditioned media from prematurely senescent cells versus control cells were analyzed for proteins using an LC-MS/MS-based proteomic technique. Collagen α1(V), collagen α2(Ⅲ), fibronectin, lumican, and matrix metalloproteinase 2 were among the proteins consistently detected from control and H$_2$O$_2$-treated cells. Insulin-like growth factor-binding protein-6 (IGFBP-6) consistently showed up in the conditioned medium of H$_2$O$_2$-treated cells but not from untreated cells. Increased IGFBP-6 production due to H$_2$O$_2$ treatment was confirmed by RT-PCR and Western blot analyses. While H$_2$O$_2$ induced a dose-dependent elevation of IGFBP-6 mRNA, Western blot analyses detected elevated levels of IGFBP-6 protein in the conditioned medium of H$_2$O$_2$-treated cells. In comparison, fibronectin or matrix metalloproteinase 2 did not show changes at the mRNA level in cell lysates or at the protein level in the conditioned medium by H$_2$O$_2$ treatment. Using several types of toxins at sublethal doses, including cis-platin, hydroxyurea, colchicine, L-mimosine, rhodamine, dithiothreitol, or N-ethylmaleimide, we found that these agents induced increases of IGFBP-6 at mRNA and protein levels. An increased level of IGFBP-6 protein was detected in the plasma of aging mice and of young mice treated with doxorubicin. These data suggest that IGFBP-6 may serve as a sensitive biomarker of cell degeneration or injury in vitro and in vivo. Molecular & Cellular Proteomics 4:1273–1283, 2005.

A large volume of literature suggests that oxidative stress contributes to aging and aging-associated diseases (19–22). Although aging is the highest risk factor for cancer, cardiovascular disease, and neurodegenerative disease, mechanisms underlying the interplay between oxidative stress, aging, and diseases have not been well addressed. Recent experimental evidence supports the hypothesis that induction of the senescent phenotype by oxidants confers a tumor-promoting activity of HFDs (6, 23). Proteins secreted by senescent-like fibroblasts appear to exhibit the ability to promote the growth and colony formation of initiated keratinocytes (23). Uncovering the nature of the proteins secreted by prematurely senescent cells becomes important in understanding the interplay between oxidative stress, aging, and aging-associated diseases.

Recent advancement in available genomic sequence information has provided an infrastructure for the emerging field of proteomics (24–28). Most commonly used proteomic techniques involve separation of a complex mixture of proteins into less complex subgroups, mass spectrometry analysis of peptides derived from the proteins in each subgroup, and data mining using bioinformatic tools. Often two-dimensional gel electrophoresis has been used for protein separation. However, staining two-dimensional gels only detects abun-

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* The abbreviations used are: HDF, human diploid fibroblast; MMP, matrix metalloproteinase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; PDL, population doubling level; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dox, doxorubicin; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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As proteins that are visible, and the efficiency of protein recovery from the polyacrylamide gel is often a rate-limiting step that prohibits detection of proteins with low abundance. A "shotgun" approach based on the separation capacity of liquid chromatography instrumentation becomes possible if the number of proteins is not overwhelmingly large, such as from a defined subproteome (29, 30). Compared with cell lysates, the subproteome of secreted proteins is less complex and allows meaningful identification of proteins using the shotgun approach. Based on the fact that a mass spectrometer measures a molecule based on its abundance given a mixture of different molecules, methods have been developed not only to identify the nature of proteins in a mixture but also to compare relative levels of a protein between different samples (24, 27, 28, 31–33). Protease digestion of the proteins from conditioned media followed by analysis of the resulting peptides using ESI-LC-MS/MS allowed us to measure the alteration of secreted protein factors following oxidative stress.

MATERIALS AND METHODS

Chemicals and Reagents—Chemicals were purchased from Sigma unless otherwise indicated. Stabilized H$_2$O$_2$ (H-1009, Sigma) was used, and the concentration of the stock was verified by absorbency at 240 nm.

Maintenance of Cell Culture—HCA$_3$ human dermal fibroblasts at the population doubling level (PDL) 20 were obtained from Dr. Olivia Periera-Smith. These cells typically reach replicative senescence after PDL 80 and were used for this study at PDL 26–40. HCA$_3$ cells were subcultured weekly in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at a seeding density of 1 × 10$^5$ cells/100-mm Falcon dish. Under these conditions, the cells reached confluence 6–7 days after subculture.

Treatment with H$_2$O$_2$ and Various Toxicants—HCA$_3$ cells were seeded at a density of 2 × 10$^5$/100-mm dish 5 days before treatment. At the time of H$_2$O$_2$ treatment, the cells had reached confluence, and the density of cells was 10.48 ± 0.85 × 10$^5$. Confluent cells were treated with 600 μM H$_2$O$_2$ in a 100-mm dish containing 10 ml of medium. This dose is equivalent to ~0.6 pmol of H$_2$O$_2$/cell. The dose less than 0.85 pmol/cell has been shown to be non-lethal and induce premature senescence in early passage HDFs (13). For the dose-response experiments, cells were treated with H$_2$O$_2$ from 150 to 600 μM. After 2-h incubation in the presence of H$_2$O$_2$, cells were placed in fresh DMEM containing 10% (v/v) FBS and were allowed to develop a stable phenotype in 3 days.

cis-Platin (50 μM), hydroxyurea (50 μM), colchicine (100 μM), l-mimosine (750 μM), rhodamine (1 μM), dithiothreitol (1 mM), N-ethylmaleimide (8 μM), and H$_2$O$_2$ (250 μM) were used to treat confluent cultures of HCA$_3$ cells for 4 h. The cells were then placed in fresh DMEM containing 10% (v/v) FBS for 3 days of culture. For treatment with retinoic acid (1 μM), confluent cells were incubated in the medium containing this drug for 3 days without medium change.

Preparation of Conditioned Media—To collect conditioned media of HDFs for proteomic analysis, culture media for HCA$_3$ cells in 100-mm dishes were removed 3 days after H$_2$O$_2$ or other toxicant treatment. The cells were rinsed twice in DMEM and were placed in 6 ml of fresh DMEM containing 0% FBS for 3 days of culture. The serum-free conditioned media were collected, filtered through a 0.45-μm filter to remove cell debris, dialyzed against 0.01 N NH$_4$HCO$_3$, and concentrated 100 times down in volume using a speed vacuum concentrator. Protein concentration in the concentrated medium was determined by the Bradford method according to the manufacturer’s instructions (Bio-Rad).

LC-MS/MS Analysis—The concentrated media were digested overnight with trypsin at a 50:1 ratio, i.e. 1 μg of trypsin/50 μg of protein (34). The resulting peptides were analyzed by a ThermoFinnigan (San Jose, CA) LCQ Classic quadrupole ion trap mass spectrometer equipped with a Microm (Auburn, CA) MAGIC2002 HPLC instrument and a nanospray ion source (University of Washington). A mixture of peptides equivalent to 7 μg of proteins was loaded onto a 10-cm-long capillary column with a diameter of 365 μm (outer diameter) or 100 μm (inner diameter). The capillary column was generated using a P2000 capillary puller (Sutter Instrument Co., Novato, CA) and was packed with 5–6 cm of Vydac C$_18$ material. Samples were eluted at a flow rate of 200–300 nl/min into a mass spectrometer using reversed phase solvent conditions. Tandem MS spectra of peptides were analyzed with the Turbo SEQUEST software that as peptidase sequences to the spectra (28). The software was used to search for known human proteins in the non-redundant database from the National Center for Biotechnology Information (NCBI).

Western Blot Analysis—Proteins from conditioned media were separated by SDS-polyacrylamide gel electrophoresis using a mini-Protein II electrophoresis apparatus (Bio-Rad) run at 90 V. The separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA) by electrophoresis. The membrane was incubated with antibodies against IGFBP-6 (1:200 dilution; polyclonal, H-70, Santa Cruz Biotechnology, Inc.), fibronectin (1:2000 dilution; polyclonal, F-3648, Sigma), or matrix metalloproteinase 2 (MMP-2) (1:2000 dilution; polyclonal, AB19015, Chemicon International). Antibody was detected using a secondary antibody conjugated with horseradish peroxidase (1:8000; Zymed Laboratories Inc.) for an ECL reaction.

RNA Isolation and Semiquantitative RT-PCR—Total RNA was extracted from cells with TRIzol (Invitrogen) and was used as template for reverse transcription. The resulting cDNA was used for PCR amplification using the primers for IGFBP-6 (35). The PCR products were analyzed with the Turbo SEQUEST software that assigns peptide sequences to the spectra (28).

RESULTS

Identification of Proteins in Conditioned Media of HDFs by LC-MS/MS—Early passage HCA$_3$ human skin fibroblasts were treated with H$_2$O$_2$ and were allowed to develop a stable senescence-like phenotype. To determine the effect of H$_2$O$_2$...
FIG. 1. Total ion current chromatogram of conditioned media from control and H$_2$O$_2$-treated HDFs. HCA$_3$ fibroblasts (PDL 26–40) were treated with 600 μM (~0.6 pmol/cell) H$_2$O$_2$ for 2 h. Cells were allowed to develop a senescent morphology for 3 days before changing medium for conditioned medium collection as described under “Materials and Methods.” Concentrated conditioned media were used for overnight tryptic digestion, and an equivalent of 7 μg of proteins were injected into the LC-MS/MS instrument. Each peak represents one parent peptide ion detected by the mass analyzer at each particular time point. The peak height reflects the abundance of the peptide. A, control (Ctr); B, H$_2$O$_2$ treatment.
treatment on protein factors secreted, we compared the profiles of proteins in the conditioned medium from control versus H2O2-treated cells. Serum-free conditioned media were collected for concentration and protease digestion. The resulting peptide mixtures were injected into the ESI-LC-MS/MS instrument. A representative total ion current chromatogram from the conditioned medium of control or H2O2-treated cells is shown in Fig. 1. The mass spectrometer was operated in a data-dependent MS to MS/MS switching mode so that precursor peptide ions detected in a MS survey scan trigger an ion fragmentation for obtaining MS/MS spectra for each of the precursor peptide ions. MS/MS spectra indicate primarily fragment ions originating from either the C terminus (y ion series) or N terminus (b ion series) of a peptide and were searched against a human protein sequence database using the Turbo SEQUEST software. This software searches the entries against all peptide sequences in the database and assigns correlation scores for the probability of matches. The judgment of a confident match is largely based on two parameters: “Xcorr” and “Ions.” Xcorr represents the cross-

| Control fibroblasts | H2O2-treated fibroblasts |
|---------------------|--------------------------|
| Collagen α1(I) chain | Collagen α1(I) chain |
| Collagen α1(VI) chain | Collagen α1(VI) chain |
| Collagen α2(I) chain | Collagen α2(I) chain |
| Collagen α2(V) chain | Fibronectin |
| Fibronectin | Lumican |
| Fibulin 1 | MMP-1 |
| MMP-2 | TIMP-1 |
| TIMP-1 | IGFBP-6 |
| Quiescin Q6 | Quiescin Q6 |
| Superoxide dismutase 3 | Clathrin |
| Tetrancetin (plasminogen-binding protein) | Plasminogen activator inhibitor |
| Human complement C1s protease | Procollagen C-endopeptidase enhancer |
| Zinc finger protein 335 | Decorin isoform a preproprotein |
| Decorin isoform a preproprotein | Complement component 1, s subcomponent |
| TIMP-1 | Vimentin |

**Table I**

List of proteins identified from all three experiments

The conditioned medium was prepared as described in the legend of Fig. 1 for LC-MS/MS analysis. The proteins listed here meet the selection criteria of Xcorr ≥ 1.8 for +1 ions, Xcorr ≥ 2.5 for +2 ions, and Xcorr ≥ 3.5 for +3 ions. The value of ions exceeds 50% in all cases as analyzed by the Turbo SEQUEST software. TIMP, tissue inhibitor of metalloproteinases.
correlation value computed from the experimental MS/MS spectrum when compared with the theoretical candidate peptide MS/MS spectrum, while Ions stands for the number of matched ions in the experimental MS/MS spectrum with the total number of possible sequence ions theoretically predicted for the peptide sequence. Based on the recommendation of the SEQUEST software, selection criteria for a confident protein identification include Xcorr/H11350 1.8 for +1 ions, Xcorr/H11001 2.5 for +2 ions, and Xcorr/H11002 3.5 for +3 ions. In all cases, the value of Ions must be greater than 50%.

Table I lists proteins identified by the criteria described above in the conditioned media collected from three independent experiments. The reproducibility of the analytical method is 80–90% between different runs with the same sample. Several proteins consistently showed up in all three experiments in both control and H2O2-treated groups (Table I). These proteins include collagen α1(IV) chain, collagen α2(I) chain, lumican, fibronectin, and MMP-2 (gelatinase A or 72-kDa type IV collagenase). Table II summarizes the scores of Xcorr and Ions and the number of peptides identified for these proteins. IGFBP-6 appeared in the conditioned medium of H2O2-treated cells from all three experiments (Table II). The MS/MS spectra and SEQUEST Flicka protein information output on IGFBP-6 identified from three experiments are shown in Fig. 2, A–D. Each MS/MS spectrum has a high ion-matching ratio. Despite the fact that only one peptide was identified in each experiment for IGFBP-6, the MS/MS spectra and Xcorr and Ions scores all provide high confidence in the detection. This suggests that IGFBP-6 may appear only in the conditioned medium of H2O2-treated cells or that H2O2-treated cells produce more IGFBP-6 than control untreated cells.

### Table II

| Name               | Exp. | Group | Xcorr | Ions | No. of peptides |
|--------------------|------|-------|-------|------|-----------------|
| Proteins appearing in both control and H2O2 groups |      |       |       |      |                 |
| Collagen α1(IV) chain | 1    | Ctr   | 2.66  | 67.7 | 1               |
| H2O2               | 2    |       | 2.64  | 50.0 |                 |
| 2                  | Ctr  | 3.04 ± 0.51 | 56.9 ± 6.8 | 5 |
| H2O2               | 3    | 4.30 ± 1.09 | 60.1 ± 1.5 | 3 |
| 3                  | Ctr  | 3.21 ± 0.57 | 59.4 ± 7.4 | 3 |
| H2O2               |      | 4.19 ± 0.88 | 68.1 ± 9.7 | 3 |
| Collagen α2(I) chain | 1    | Ctr   | 4.01 ± 0.80 | 63.7 ± 7.7 | 41  |
| H2O2               | 2    | 3.63 ± 0.40 | 61.2 ± 8.9 | 10 |
| 2                  | Ctr  | 3.54 ± 0.82 | 56.7 ± 4.2 | 14 |
| H2O2               | 3    | 3.66 ± 0.91 | 56.7 ± 8.2 | 7  |
| 3                  | Ctr  | 3.38 ± 0.51 | 58.3 ± 6.3 | 6  |
| H2O2               |      | 3.72 ± 0.82 | 57.2 ± 5.7 | 12 |
| Fibronectin        | 1    | Ctr   | 3.77  | 57.14| 1               |
| H2O2               | 2    | 3.24 ± 0.63 | 61.5 ± 11.8 | 3  |
| 2                  | Ctr  | 3.07 ± 0.44 | 60.0 ± 10.9 | 5  |
| H2O2               | 3    | 3.53 ± 0.31 | 63.1 ± 15.1 | 5  |
| 3                  | Ctr  | 3.30 ± 0.51 | 61.7 ± 8.1 | 7  |
| H2O2               |      | 3.17 ± 0.18 | 61.2 ± 10.0 | 9  |
| Lumican            | 1    | Ctr   | 3.17 ± 0.12 | 71.4 ± 19.3 | 2  |
| H2O2               | 2    | 3.25  | 72.0  | 1   |
| 2                  | Ctr  | 3.61 ± 0.86 | 67.1 ± 12.1 | 5  |
| H2O2               | 3    | 3.49 ± 0.70 | 66.0 ± 9.4 | 4   |
| 3                  | Ctr  | 3.70 ± 1.03 | 72.0 ± 8.5 | 3   |
| H2O2               |      | 3.49 ± 0.73 | 72.5 ± 9.0 | 5   |
| MMP-2              | 1    | Ctr   | 3.17  | 52.8 | 1               |
| H2O2               | 2    | 2.97 ± 0.10 | 69.0 ± 19.0 | 2   |
| 2                  | Ctr  | 3.37 ± 0.65 | 67.9 ± 9.5 | 3   |
| H2O2               | 3    | 3.17 ± 0.57 | 62.8 ± 4.8 | 3   |
| 3                  | Ctr  | 3.54 ± 0.67 | 65.0 ± 7.4 | 5   |
| H2O2               |      | 3.17 ± 0.34 | 66.1 ± 10.6 | 8  |
| Proteins appearing in H2O2 group alone |      |       |       |      |                 |
| IGFBP-6            | 1    | H2O2  | 2.75  | 94   | 1               |
| 2                  | H2O2 | 5.13  | 79   | 1               |
| 3                  | H2O2 | 4.63  | 75   | 1               |
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**Fig. 2.** MS/MS spectra of IGFBP-6 peptide and SEQUEST Flicka output for detected IGFBP-6 peptides. MS/MS spectra of the IGFBP-6 peptide detected in three independent experiments are shown in A–C. The bold letters indicate the detected b and y ions matching the predicted ion mass in the database. In D, the SEQUEST Flicka protein information page for IGFBP-6 shows the fragments detected.
teins, including fibronectin and MMP-2, from both control and H₂O₂-treated cells. To verify these results, we performed Western blot analyses using conditioned media collected from control or H₂O₂-treated cells. The data indicate that there is no significant difference in the level of MMP-2 and fibronectin in the conditioned media between control and H₂O₂-treated cells (Fig. 3). With cell lysates, a minor elevation of fibronectin was detected with H₂O₂ treatment. MMP-2 was not detected with cell lysates, suggesting that MMP-2 is a secreted protein.

IGFBP-6 is known to be O-glycosylated at 5 amino acid residues (Thr¹²⁶, Ser¹⁴⁴, Thr¹⁴⁵, Thr¹⁴⁶, and Ser¹⁵²) (36, 37). Western blot analyses of conditioned media showed two bands of IGFBP-6 (Fig. 3). Presumably the lower molecular weight band represents the non-glycosylated form, and the higher molecular weight band represents the glycosylated form of IGFBP-6. With either form, IGFBP-6 protein showed an elevation in the conditioned medium of H₂O₂-treated cells (Fig. 3). IGFBP-6 protein from cell lysates showed a molecular weight between the two forms present in the conditioned medium (Fig. 3), suggesting that the protein is partially glycosylated. The level of IGFBP-6 protein from cell lysates did not show a dramatic increase with H₂O₂ treatment. These data demonstrate that we are able to verify the data on IGFBP-6 obtained by LC-MS/MS with Western blot analyses.
To characterize the induction of IGFBP-6 by H$_2$O$_2$ treatment, we performed dose-response studies to measure levels of IGFBP-6 mRNA in cell lysates and to determine levels of IGFBP-6 protein in the conditioned medium. HCA$_3$ cells were treated with 0, 150, 300, 450, or 600 µM H$_2$O$_2$. RNA and conditioned media were collected in parallel from the same set of samples. Western blot analyses showed a dose-dependent increase of IGFBP-6 protein, both glycosylated and non-glycosylated forms, in the conditioned medium of H$_2$O$_2$-treated cells (Fig. 4A). In comparison, no significant changes of fibronectin and MMP-2 at the protein level were detected in the conditioned media of HCA$_3$ cells treated with various doses of H$_2$O$_2$. Consistent with the protein measurement data, semiquantitative RT-PCR showed a dose-dependent increase of IGFBP-6 mRNA with H$_2$O$_2$ treatment (Fig. 4B).

Using IGFBP-6 as an in Vitro and in Vivo Biomarker of Cellular Injury—We extended our study by asking whether or not IGFBP-6 can serve as a biomarker of oxidative injury. HCA$_3$ cells were treated with a variety of chemicals, including a DNA-damaging agent (cis-platin), DNA polymerase inhibitor (hydroxyurea), microtubule disruptor (colchicine), amino acid analogue (mimosine), mitochondrial uncoupler (rhodamine), and reducing agents (dithiothreitol and N-ethylmaleimide). The nuclear receptor agonist retinoic acid, which has been reported to induce IGFBP-6 expression (38, 39), was included as a comparison. Cells were treated with a sublethal dose of toxins and were allowed to recover for 3 days before collecting the conditioned medium and RNA as described under “Materials and Methods.” Western blot analyses indicated that the IGFBP-6 protein in the conditioned media increased to various degrees due to the treatment with different chemicals (Fig. 5A). Judging from the molecular weight markers, the increased IGFBP-6 from various chemical treatments is mainly the glycosylated form. In contrast, levels of fibronectin or MMP-2 proteins did not appear to change (Fig. 5A). RT-PCR results also indicated up-regulated IGFBP-6 mRNA levels in cells treated with these chemicals (Fig. 5B). These data suggest that a variety of chemical stresses can induce IGFBP-6.

Based on the fact that IGFBP-6 is a secreted protein that increases its expression when cells encounter damaging agents, we reason that this protein may serve as a biomarker of cell injury if the increase can be detected in the plasma of individuals. Two animal models were used to test this hypothesis: aging and Dox treatment. There is much evidence in support of the fact that oxidative stress contributes to cell degeneration during the process of aging. In comparison, Dox, an antineoplastic drug that is known to produce reactive oxygen species, can induce cardiomyopathy and other types of tissue injury. Plasma was collected from young (5–6 weeks) or old (16 months) mice for Western blot analysis to measure the level of IGFBP-6. The results show that old mice, male or female, exhibited an elevated level of IGFBP-6 in the plasma (Fig. 6A). The protocol of administering Dox, as described under “Materials and Methods,” has been shown to induce cardiomyopathy (35). The plasma of Dox-treated mice showed an elevation of IGFBP-6 (Fig. 6B). With serum samples from mice, IGFBP-6 appeared to show in one broad band. It is not known whether this band represents the non-glycosylated, glycosylated, or partially glycosylated form. Regardless our data...
In this study, a ThermoFinnigan LCQ Classic quadrupole ion trap mass spectrometer, detects peptides at a sensitivity of 200 fmol. With this conventional ion trap, considerable amounts of potentially valuable information are missed when several peptides co-elute, and the instrument is unable to fragment them all efficiently. Much of the emphasis in the proteomic industry involves improving mass spectrometers in sensitivity, speed, selectivity, dynamic range, and mass accuracy. A linear ion trap is able to collect much more information due to its much faster scanning time. One experiment using a demo model of the ThermoFinnigan linear ion trap LC-MS/MS instrument identified 71 proteins from the conditioned medium of control HDFs. This is a 3–7-fold enhancement in the capacity of protein identification. Two-dimensional linear trap mass spectrometers have recently been developed that offer an increased ion trapping capacity, increased detection sensitivity, and better quality of tandem mass spectra. This type of capillary multidimensional liquid chromatography produces a better separation of complex peptide mixtures with two or more series of orthogonal nano-HPLC columns, allowing the characterization of an entire proteome from cell lysates, which contain 200,000–300,000 proteins (30, 44). The improvement in instrumentation will enhance our capacity to profile secreted proteins toward the level that truly reflects the actual number of secreted proteins from a particular cell type.

Despite the fact that LC-MS/MS analysis can generate data reproducibly for certain proteins, such as fibronectin, MMP-2, and IGFBP-6, we have observed considerable variations in the proteins identified (Table I). Several caveats for the LC-MS/MS-based method may explain the observed variations.

1) The principle of the LC-MS/MS instrument analysis involves...
detection of ions that are most likely abundant. 2) Classical LC-MS/MS is operated under the assumption of “first come and first serve.” Because our current instrument does not have detailed ion separation capacity, the detection is somewhat a process of randomization. 3) The optimal detection range of the mass spectrometer is 700–3000 Da for a peptide. Depending on the completion of protease cleavage of each protein, a peptide within this mass range may not always appear in a large abundance. 4) The data depicting specific protein identifications are largely dependent on the selection criteria of SEQUEST software. The current setting is quite stringent and therefore filters out the peptide ions that may not meet the level of high confidence. 5) Variations may exist between cells from different passages. Because our three experiments were performed with different passages of cells in culture and HDFs are known to progress toward replicative senescence with each subculture, culture conditions and age of the cells may possibly contribute to variations of proteins in the conditioned medium. Regardless of these caveats, the finding that oxidants and multiple toxicants increase the expression and secretion of IGFBP-6 presents a novel biomarker for diagnosing sublethal cell injury. The data from our animal models of aging and Dox treatment support this biomarker argument. With plasma samples in human studies, a gradual increase in the level of IGFBP-6 up to 2-fold has been documented with increasing age (45). Consistent with this finding, we detected an increased level of IGFBP-6 in the plasma of aged mice.

IGFBP-6 belongs to a family that contains six well characterized members. These members share sequence homologies, contain abundant cysteine residues, and have been found in a variety of biological fluids, including the plasma (46, 47). IGFBP-6 undergoes glycosylation during the process of secretion (36, 37). Our data show two broad bands of IGFBP-6 in Western blots, suggesting non-glycosylated and glycosylated forms, both of which showed elevation in the conditioned medium of H2O2-treated cells. However, an in vivo study showed only one band of IGFBP-6. It is not known whether the band represents the glycosylated or non-glycosylated form because the glycosylated band seems to be dominant as evidenced by in vitro studies, but the molecular weight of the band from animal serum is close to what is presumably the non-glycosylated form. Species differences also contribute to the complication. Mouse IGFBP-6 appears to have a slightly lower molecular weight than rat IGFBP-6 (36). Regardless, glycosylation appears to play a role in IGFBP-6 protein stability (36, 48). This feature may contribute in part to the increased level of IGFBP-6 protein in addition to transcriptional activation of the IGFBP-6 gene by H2O2. Several nuclear receptor ligands, such as retinoids, vitamin D, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (39, 49, 50), have been shown to induce the expression of IGFBP-6. In contrast, transforming growth factor β and agents that elevate intracellular cAMP concentration cause decreases in IGFBP-6 expression (51). Our finding points to a novel pathway regulating the expression of IGFBP-6 by oxidants and toxicants.

The biological function of IGFBP-6 may vary depending on experimental systems. IGFBP-6 preferentially binds to insulin-like growth factor (IGF)-II rather than to IGF-I. Although IGFBPs can modulate the activity of IGFs through high affinity binding, IGFBPs may also regulate biological processes, such as cell proliferation or growth arrest in an IGF-independent manner, for example through direct cell association. Glycosylation does not appear to affect IGF-II binding but appears to inhibit direct cell association of IGFBP-6 (36, 48). Overexpression of IGFBP-6 arbitrarily in non-small cell lung cancer cells activates programmed cell death (52). With TCDD-induced expression of IGFBP-6, a high degree of IGFBP-6 elevation appeared to enhance apoptosis, whereas a reduction of IGFBP-6 inhibited TCDD from inducing apoptosis in thymoma cells (53). Inactivating the expression of IGFBP-6 in colon cancer cells resulted in a gain of cell proliferation, suggesting that IGFBP-6 may be inhibitory for cell growth (43). Whether IGFBP-6 mediates growth arrest or apoptosis induced by H2O2 treatment remains to be determined.

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