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Redox regulation of matrix metalloproteinase gene family in small cell lung cancer cells

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Abstract

It has been implicated that reactive oxygen species (ROS) play important roles in modulating tumor progression. However, the mechanisms by which redox-regulated tumor progression are largely unknown. We previously demonstrated that reduced intracellular redox conditions could be achieved in stably transfected small cell lung cancer cells with γ-glutamylcysteine synthetase (γ-GCSh) cDNA which encodes a rate-limiting enzyme in the biosynthesis of glutathione (GSH), a major physiological redox regulator. In the present study, using DNA microarray analyses, we compared the expression profiles between the γ-GCSh-transfected cells and their nontransfected counterpart. We observed downregulation of several matrix metalloproteinases (MMPs), i.e., MMP1 and MMP3, and MMP10 in the transfected cells. Dot blot and Northern blot hybridizations confirmed that, among the 18 MMP gene family members and four tissue inhibitors of matrix metalloprotein family (TIMP) analyzed, the expression levels of these three MMPs were consistently reduced. Transiently increased γ-GCSh expression using tetracycline-inducible γ-GCSh adenoviral expression system also showed down-regulation of MMP3 and MMP10, but not MMP1. Our results demonstrated that redox regulation of MMP1, MMP3 and MMP10 expression depend upon different modes of redox manipulation. These results bear implication that antioxidant modulation of antitumor progression may be contributed at least in part by the downregulation of a subset of metrix metalloproteins.

Keywords: *ROS, matrix metalloproteins, γ-glutamylcysteine synthetase, tumor invasion*

Introduction

Evidence suggests that formation of reactive oxygen species (ROS) and induction of oxidative stress may play an important role in carcinogenesis [1–4]. Under normal physiological conditions, there is a balance between oxidants and antioxidants or an reduction-oxidative (redox) homeostasis. While ROS at sub-micromolar levels may act as second messenger to stimulate cell proliferation, apoptosis and gene expression [4], excess ROS generated endogenously or by adverse extracellular influences has been implicated in neoplastic transformation and tumor progression. This is also consistent with the long-standing concept that antioxidants exhibit antineoplastic transformation and retardation of tumor progression.

One of the important strategies in counteracting excess ROS is the glutathione system. It is present as both reduced form (GSH) and oxidized form (GSSG). Under oxidative stress, GSH is oxidized by GSH peroxidase to GSSG which is eliminated by multidrug resistant protein efflux pump [5] or by

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catalytically reduced back to GSH by the NADPH-dependent GSH reductase. In addition, GSH also regulates the activities and biosynthesis of other redox-regulating enzymes, e.g. superoxidade dismutase, DT-diaphorases. Because of its intracellular abundance (1–10 mM), GSH/GSSG system represents the major redox regulator in the cells.

Biosynthesis of GSH is regulated mainly by the rate-limiting enzyme, γ-glutamylcystein synthetase (γ-GCS). The mammalian γ-GCS is a heterodimer consisting of one 73-kDa heavy or catalytic subunit (γ-GCSh) and one 28 kDa light or regulatory subunit (γ-GCSl). Expression of γ-GCSh can be induced by antitumor agents [6], heavy metals [7], carcinogens [8], and prooxidants [9–12]. All these treatments are associated at various extents, with induction of intracellular ROS imbalance. Furthermore, enhanced levels of ROS are often found in human cancer cells, particularly colorectal cancers, and we have observed that γ-GCSh mRNA is frequently overexpressed in human colon cancers [13]. Overexpression of γ-GCSh (and therefore GSH) feeds back to inhibit the biosynthesis of γ-GCSh mRNA [9] and suppress the enzymatic activities of its encoded protein [14]. Hereditary γ-GCSh deficiency is associated with anemia, jaundice, and neurologic abnormalities; [15] whereas totally γ-GCSh deficient in knockout mice is embryonic lethal [16,17].

We previously demonstrated that it is possible to establish stable cell lines exhibiting constitutive reduced redox conditions by transfecting expression cDNA recombinant encoding γ-GCSh. The present study was initiated to identify genes whose expression may be associated with redox-regulation. We performed DNA microarray analyses by comparing expression profiles between the γ-GCSh-transfected cells and their untransfected counterparts. We found that, among many other changes, a subset of matrix metalloproteinases (MMP) was downregulated in the γ-GCSh-transfected cells.

The matrix metalloproteinase (MMPs) multigene family which degrades all the main protein components of the extracellular matrix (ECM) and basement membrane, have been demonstrated to play important roles in tumor progression. MMPs can be classified into four major groups: (i) the collagenases (MMP1, MMP8, and MMP13, MMPL1) which cleave triple helical collagen into two fragments; (ii) gelatinases (MMP2 and MMP9) which degrade denatured collagen; and (iii) stromelysines (MMP3 and MMP7, MMP10, and MMP11) which degrade a broad range of molecules, including aggrecan, fibronectin, laminin and procollagens; and (iv) MT-MMPs (MMP14, MMP15, MMP16 and MMP17) which are similar to other MMPs except for the addition of a transmembrane domain and a cytoplasmic tail at the C terminus [18]. MMPs are frequently overexpressed in virtually all human and animal tumors [19]. Moreover, it has been reported that stages of tumor progression are positively correlated with the expression of MMP family members (MMP1, 2, 3, 7, 9, 11 and 14) [20] and changing MMP expression levels could dramatically affect their invasiveness in animal models [19]. Additional data support for the involvement of MMPs in tumor progression came from studies of their endogenous tissue inhibitors (TIMPs). Several studies showed that overexpression of TIMPs reduced experimental metastasis; [21–23] and inhibition of TIMP expression promoted tumor growth [24].

Material and methods

Cell lines

SR3A cells were derived from a small cell lung cancer (SCLC) cell line which were established from the bone marrow of a SCLC patient [9]. SR3A-13, SR3A-14, and SR3A-15 were three independently established cell lines by transfecting expression recombinant γ-GCSh cDNA into SR3A cells under the transcriptional control of the CMV promotor and the neomycin resistance marker for G418 selection. The transfected cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum containing 400 μg/ml G418 at 37°C in 5% CO_2 atmosphere.

Micro array analysis

cDNA microarray was carried out using the CG11 pathway array designed by the Core laboratory of M. D. Anderson Cancer Center according to the protocols described [25]. This pathway array consists of a total of 1800 distinct oligo gene fragments. While many differentially expressed genes were observed, however, only those showing a threshold exceeding 3.0 as determined based on the *t*-score (computed from *t*-statistics) were noted.

Dot-blot array analysis of MMP1 and TIMP gene family

mRNA was obtained from both the parent and transfected cells using the kit from Qiagen (Valencia, CA). ^{32}P-labeled cDNA was synthesized using Superscript Reversed transcriptase (purchased from Invitrogen, Carlsbad, CA). This labeled cDNA was used to hybridize with nylon membrane (purchased from SuperArray, Frederick, MD), which contains a panel of 18 different MMP and 4 different TIMP DNA sequence as well as positive control (β-actin, GAPDH) and negative control (pUC18 plasmid DNA) under the hybridization conditions specified by the vendor. After 24 h of hybridization, membrane was washed and signal intensity was recorded using phosphoimager.

Northernblot analysis

Total cellular RNA was isolated from both parental and transfected cells by RNA Stat-60 according to vendor's specifications (Friendwood, TX), electrophoresed and transferred onto a GeneScreen membrane (New Res. Product, Boston, MA) and hybridized with ^{32}P labeled γ-GCSh, or various MMPs, TIMPs cDNA probes. The membrane was washed, and signal intensity was recorded by phosphoimager. β actin cDNA probe was used as a control for the loadings of RNA.

Western blot analysis

Cell lysate was prepared as per our previous publication [11]. Briefy, 1×10^6 cells were lysed with buffer containing 10 mM Tris-HCl, pH7.4, 100 mM NaCl, 1 mM EDTA, 1mM EGTA, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF and protease inhibitor cocktail from Sigma (St. Louis, MO), centrifuged at 14,000 × *g* and the supernatant was used for immunoblot. The polyclonal antibody for γ-GCSh was described previously [11]. Anti-MMP1 and anti-MMP3 antibodies were purchased from Triple Point Biologic. Inc. (Forest Grove, OR). The immunoblots was detected by chemoiluminescence.

Construction of the replication-deficient recombinant adenovirus with tetracycline-regulatable γ-GCSh

The human *γ-GCSh* cDNA sequence from 10 bp preceding the translation initiation codon to 54 bp downstream from the stop codon was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from total human liver RNA. The tetracycline (tet) regulatable γ-GCSh adenovirus was constructed according to the procedure described previously [26]. In brief, the cDNA was blunt-ended and inserted into the *Hind*III site of shuttle vector pΔE1.tTA which retains the Ad5 packaging signal sequence. The resultant recombinant shuttle plasmid, pΔE1.tTA. γ-GCSh and the Ad5 master plasmid pBHG10 were co-transfected into human embryonic kidney-derived cell line HEK293 cells in 24-well tissue culture plates by the $CaCl_2$ precipitation method. pBHG10 contains the backbone of the Ad5 genome with partial deletion of both E1 and E3 sequences. Identification, purification and propagation of recombinant adenovirus, designated as AdΔE1.tTA. γ-GCSh, followed the procedures previously described [26].

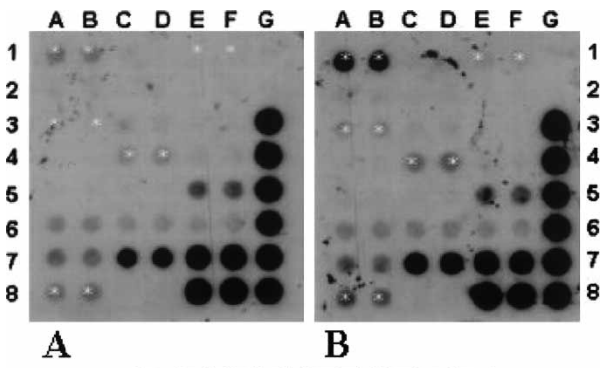
Measurements of GSH and ROI

Methods of determination of GSH + GSSG contents were described previously [9]. Relative levels of reactive oxygen intermediate (ROI) were measured by using DHR123 (Sigma, St. Louis, MO), an uncharged and nonfluorescent dye, which passively diffuses across most cell membranes and reacts with ROI resulting in the formation of cationic fluorescent R123. R123 fluorescence intensity was measured by a FACScan flow cytometer (Becton Dickson, Mountain View, CA) with the excitation at 488 nm and detection between 515 and 550 nm according to the procedure described previously described [9].

Results

We previously established several stable γ-GCSh-transfected SCLC cell lines, including SR3A-13. SR3A-14 and SR3A-15, for investigation. Using the RNA protection assay, we previously documented that these cell lines exhibited 1.9-, 2.8- and 2.2- fold, respectively, increased levels of γ-GCSh mRNA as compared with those in the parental cell line SR3A. Correspondingly, cellular GSH levels were increased 3.0-, 3.96- and 2.78-fold. Moreover, using ROI sensitive marker DHR123 in flow cytometry, we reported that these γ-GCSh-transfected exhibited reduced redox conditions in reference to the parental cells SR3A or empty vector-transfected SR3A cells [9]. In this study, we chose SR3A-14 which expresses the highest amount of γ-GCSh mRNA and γ-GCSh protein for DNA microarray analyses. Our results revealed several upregulated and downregulated genes in the SR3A-14 cells as compared with those in the SR-3A cells. Among the downregulated candidates, we are particularly interested in the three sequences in the MMP gene family, MMP1(X54952), MMP3(J03209), and MMP10(X07820) for their potential roles in regulating tumor progression.

To confirm these results and to extend the investigation to other MMPs and TIMPs in response to redox alterations, we performed dot blot hybridizations as described in the method section using duplicated filters containing a panel of 18 different MMP and four different TIMP DNA sequences, each in duplication. Positive control (β-actin and glyceraldehyde phosphate dehydrogenase, GAPDH) and negative control (pUC18 plasmid DNA) sequences were also included in the filters. One filter was used for hybridization with cDNA probe prepared from SR3A mRNA and the other from SR3A-14 mRNA. The hybridization signals were quantified by densitometry. The signal intensities between the duplicated dots for the given gene sequences were very similar; and signals for the positive control DNA hybridized by the two different probes, SR3A (panel B) and using SR3A-14 (panel A) cDNA probes, were within ±10% variations. These results suggested that the analyses reliably reflected the abundance of mRNA levels between two cell lines. Several conclusions could then be made from the results of this experiment (Figure 1): (i) Densitometric analyses showed that the ratios of



	A,B	C,D	E,F	G
1	MMP-1	MMP-2	MMP-3	pUC 18
2	MMP-7	MMP-8	MMP-9	pUC 18
3	MMP-10	MMP-11	MMP-12	β-actin
4	MMP-13	MMP-14	MMP-15	β-actin
5	MMP-16	MMP-17	MMP-19	GAPDH
6	MMP-20	MMP-24	MMP-26	GAPDH
7	MMPL1	TIMP-1	TIMP-2	GAPDH
8	TIMP-3	TIMP-4	GAPDH	GAPDH

C

Figure 1. Array hybridization of MMP1 and TIMP gene families. ^{32}P-labeled cDNA probes prepared from SR3A-14 (panel A) and SR3A (Panel B) mRNA by reverse transcriptase were used to hybridize cDNA sequences of various MMP and TIMP genes and GAPDH and actin (purchased from Super Array, Bethesda, MD). Panel C shows the coordinates of various gene fragments. *indicates differences in hybridization signals between the two probes.

expression levels between SR 3A and SR3A-14 for MMP1 (coordinate 1-A,B), MMP3 (1-E,F), MMP10 (3-A,B), MMP14 (4-C,D), and TIMP3 (8-A,B) mRNA were 3.3, 1.7, 2.8, 2.1, and 1.36, respectively. These results confirm that expression levels of MMP1, MMP3 and MMP10 were reduced in SR3A-14 cells as consistent with the DNA microarray results. (ii) Moreover, levels of MMP14 and TIMP3 mRNA were reduced in SR3A-14 cells. (iii) Levels of MMP19, 20, 24, 26 and L1 mRNA were not different between these two cell sources. (iii) Levels of MMP-2, 7, 8, 9, 11, 12, 13, 15, 16, and 17 mRNA were too low to be detectable in both cell lines. (These results suggest that the expression of a subset of MMP genes are modulated by the redox conditions).

These results were obtained using a single pair of cell lines. To verify these initial results and also to investigate the generality of the results, we performed the following two approaches: First, we performed Northern blot hybridization to analyze levels of the relevant MMP and TIMP mRNA in SR3A-14 and two additional γ-GCSh-transfected SR3A lines, i.e. SR3A-13, and SR3A-15 cells. In comparison with those in the SR3A cells, levels of MMP1, MMP3 and MMP10 mRNA in all three transfected cell lines remained reduced (Figure 2A). Moreover, levels of reduction are generally inversely correlated with the levels of γ-GCSh mRNA levels among the three transfected cell lines. For example, levels of MMP1, MMP3 and MMP10 mRNA in SR3A-14 and SR3A-15 were correspondingly lower than those in SR3A-13 cells, which expressed the lowest levels of γ-GCSh mRNA. There is a minor exception: the expression of MMP1 in SR3A-15 was lower than that in the SR3A-14 whereas higher expression levels of γ-GCSh mRNA were found in SR3A-14 cells. Northern blot hybridization shown in Figure 2A also demonstrated that the expression levels of MMP14, TIMP1 and TIMP3 mRNA were variable that did not correlate with the corresponding γ-GCSh mRNA levels among the three transfected cells.

We also carried out western blot analysis to determine MMP protein levels using commercially available anti-MMP1 and anti-MMP3 antibodies. Levels of γ-GCSh were also determined using polyclonal antibody against γ-GCSh [11]. As shown in Figure 2B, all the transfected cells express the exogenous γ-GCSh as a smaller molecule (indicated by *). The observation of the slightly increased in mobility also observed in the γ-GCSh-transfected colorectal cancer cell lines [11]. While the reason for the altered mobilities remains to be elucidated, nonetheless, the intensities of γ-GCSh signal in

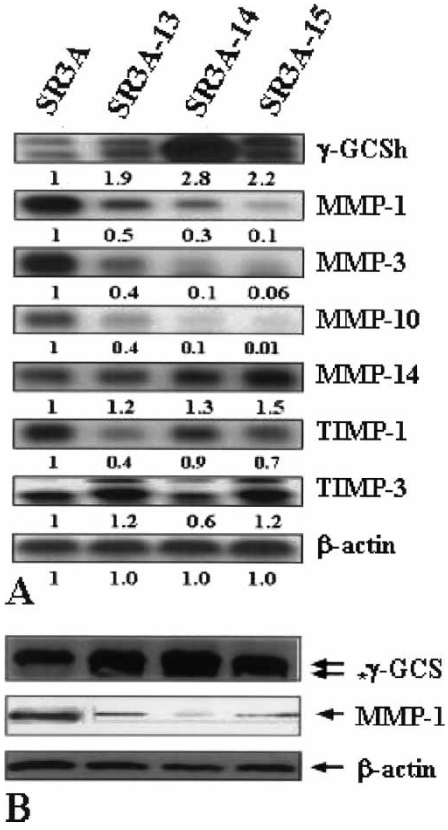


Figure 2. (A) Northern blot hybridization analyses of the steady-state mRNA levels of γ-GCSh, and various MMPs, TIMPs and β-actin in SR3A cell line and in its γ-GCSh transfected cell lines SR3A-13, SR3A-14, SR3A-15. (B) Western blot analyses of γ-GCSh and MMP1 expression in the stable SR3A transfectants SR3A-13, SR3A-14 and SR3A-15. Note that the transfected cell lines also contain a faster migrating signal γ-GCSh denoted by asterisk (*), in addition to the major band (arrow).

these transfectants are in general agreement with the mRNA levels respectively (Figure 2A). Levels of MMP1 were reduced in the γ-GCSh transfected cells (Figure 2B). No signal for MMP3 proteins were detected. The inability of detecting MMP3 protein by the Western is likely due to the low expression levels of MMP3 as suggested in the dot-blot hybridization result (Figure 1) and/or the low sensitivity of the commercially available antibody against MMP3. Since the expression levels of MMP-14 were not consistently reduced in all the transfected cell lines as well as in the adenoviral vector which carrying γ-GCSh gene (see below), no western blot for MMP-14 was carried out. No antibody for MMP-10 is commercially available. Nonetheless, these results, taken together, not only confirmed the dot blot data as presented in Figure 1 but also demonstrated the reduced expression of MMP1, MMP3 and MMP10 in the three γ-GCSh-transfected cells.

Second, we constructed an inducible human γ-GCSh expression system using recombinant adveno-viral vector, AdE1.tTA.γ-GCSh. This recombinant adenoviral vector contains two expression cassetts: one constitutively expresses tetracycline-regulatable transactivator (tTA) and the other contains a γ-GCSh expression cassette whose expression is under the control of tTA [26]. In the absence of tet, the constitutively expressed tTA binds to the promoter of γ-GCSh and activates the transcription of γ-GCSh from the recombinant adenoviral genome (tet-on). Addition of tet to the culture medium allows its binding to tTA. This prevents the rTA's transactiva-tion activity, and consequently switches-off (tet-off) the expression of γ-GCSh. The ability to turn-off the expression in the presence of tet, and to turn-on in its absence provides an controllable system for regulating γ-GCSh expression.

SR3A cells were transduced with AdE1.tTA. γ-GCSh either in the presence or absence of tet. At different time intervals, cells were harvested and the expression of γ-GCSh were determined by western blotting. Recombinant adenovirus encoding bacterial β -galactosidase was used as control. Figures 3A and B show that γ-GCSh expression under tet on conditions (no tet in the medium) increased more than 10-fold 24 h after transduction and reached about 80-fold 96 h thereafter. These results indicated that the recombi-nant adenoviral virus inducible system expressed high levels of γ-GCSh. Levels of GSH were increased and reached 2.69-fold 48-h after induction but declined thereafter (Figure 3B). The rise and fall profile of GSH while continuing elevated expression of γ-GCSh was also observed in cultured cells treated with oxidative stress-induced agents [6,10]. The reduction of GSH levels at the late phase of adenoviral infection was because the elevated production of GSH feedbacks to inhibit the biosynthesis and enzymatic activities of γ-GCSh as mentioned above [6,9,10] (also see below).

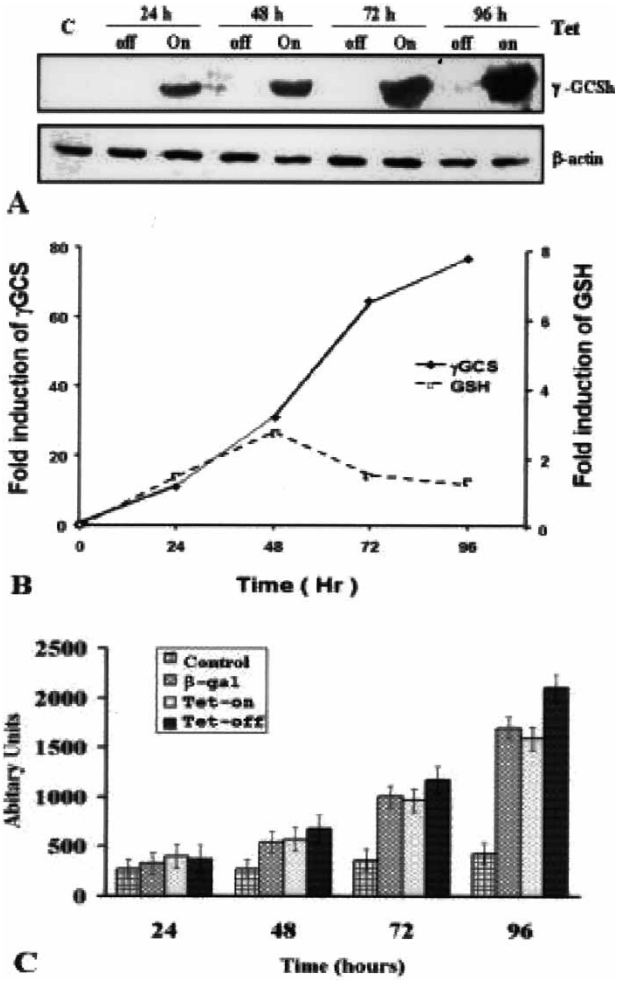


Figure 3. Analyses of γ-GCSh expression, GSH production, and reactive oxygen intermediate levels in the SR3A cells transduced with recombinant adenovirus encoding a tetracycline-inducible promoter. The cells were treated with recombinant adenovirus Ad.E1.tTA.γ-GCSh at 50 MOI for different lengths of time as indicated either in the presence (+) or absence of (−) of 1 μg/ml of tet. Total cell lysates were prepared and probed using anti-γ-GCSh and anti-β-actin antibodies (A) and fold of induction as determined by densitometry of the western blotting is shown in (B). Panel B also shows GSH levels in the corresponding samples. (C) ROI levels in the adenovirus-infected cells. The experiments were reproduced at least two times, and the results were similar.

Alternatively, it could be due to the enhanced GSH consumption at the later phase of adenoviral infection. Despite the reduction of GSH in the late phase of infection, Figure 3C shows that induction of ROI levels by adenovirus infection through out the entire 96 h. Importantly, levels of ROI produced under tet-off conditions where γ-GCSh expression was turn off were higher than those under the tet-on conditions where high levels of γ-GCSh protein were produced. These results collectively suggest that the oxidative stress induced by the adenoviral infection could be suppressed by the expression of γ-GCSh, and such suppression did not require continuing biosynthesis of GSH at later stage of infection.

To investigate the effects of γ-GCSh on selective MMP and TIMP expression, we performed northern blot hybridization analyses to determine mRNA levels of their respective mRNA in SR3A cells treated with AdE1.tTA.γ-GCSh under tet-on and tet-off conditions. Tetracycline at the concentration used (1 μg/ml) did not induce γ-GCSh expression (not shown). Under tet-on conditions, levels of γ-GCSh mRNA were increased approximately 285-fold and 366-fold 48 and 96 h, respectively, whereas under tet-off conditions, 2.3- and 10.7-fold, respectively. The observed γ-GCSh expression under tet-off conditions could be due to leakage of tet-control system and/or adenovirus-induced expression of endogenous γ-GCSh expression due to the associated oxidative stress.

Figure 4 shows that while levels of MMP1 mRNA were marginally changed under either tet-on or tet-off conditions, levels of MMP3 and MMP10 mRNA were nonetheless increased 2.4- and 2.0-fold 48 and 96 h after transduction under tet-off conditions. These results suggest that expression of MMP3 and MMP10 could be induced by oxidative stress due to adenoviral infection. At given time points, levels of MMP3 and MMP10 mRNA were reduced under tet-on conditions as compared with those under tet-off conditions. These results demonstrated that expression of MMP3 and MMP10 were redox-regulated in this assay system.

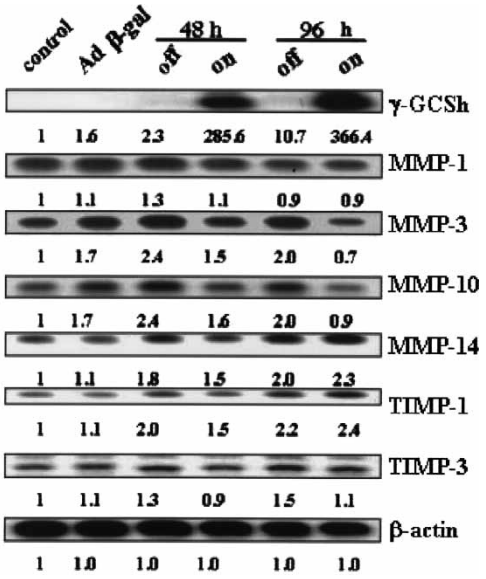


Figure 4. Northern blot hybridization analyses of several MMP and TIMP mRNA levels in SR3A cells transduced with recombinant adenoviral vector carrying tet-inducible γ-GCSh expression. 1×10^6 cells each in a 60-mm dish were infected for 1.5×10^9 viral particles for various lengths of times as indicated either in the presence (tet-off) or absence (tet-on) of 1 μg/ml of tetracycline inducer. Total RNA were prepared and hybridized with cDNA probes for the given MMP or TIMP gene family member as indicated.

Discussion

Regulation of MMP1, MMP3 and MMP10 expression under different redox regulation modes

In this study we sought to evaluate the spectra of MMP gene and TIMP gene families that are regulated by redox homeostases. Since SCLC is known to rapidly metastasize, we have studied MMP expression in SCLC-derived cell lines. Using gene array approach, we first identified that MMP1, MMP3 and MMP10 were downregulated in a γ-GCSh-transfected cell line which expressed *constitutively* reduced redox conditions. Dot-blot hybridization approach not only confirmed this result but also extended the analyses of 18 MMP genes and 4 TIMP genes. And the dot-blot results were in turn confirmed by the northern blot hybridization which included two additional γ-GCSh transfected cell lines. Finally, we took advantage of a tet-off system to *transiently* regulate γ-GCSh expression and demonstrated that MMP3 and MMP10 but not MMP1 gene that are redox regulated under these vigorous assay approaches.

The stable γ-GCSh transfection cell lines provided persistently elevated GSH levels in a panel of isogenic cell lines. This approach did not involve treatments of cytotoxic agents that are known to induce endogenous γ-GCSh expression. This approach allowed us to investigate the expression of MMP/TIMP gene family under a non-stressed, reduced redox environment. The use of recombinant adenovirus with tet-regulatory expression system for γ-GCSh represents a novel approach for the analyses of redox-regulation of gene expression. Because adenoviral infection *per se* induces oxidative stress which upregulates endogenous γ-GCSh expression and elevated GSH levels, this system offers the investigation of stress-induced gene expression. But because the recombinant adenovirus encodes a γ-GCSh gene, this system also provides a means for investigating the effects of elevated γ-GCSh expression on gene under continue stress conditions. Unlike the stably transfected cell lines, induction of elevated GSH levels in the latter system is transient. The decline of GSH in the later phase of transduction is due to the feedback inhibition of GSH because the γ-glutamyl moiety of GSH competes the occupation of the glutamate-binding site of γ-GCSh [27,28]. Moreover, it has been suggested that competition of GSH binding to the heavy subunit of γ-GCSh is reduced on the formation of holoenzyme between γ-GCSh and γ-GCSl [29]. In the present study, transduction of recombinant adenoviral vector of γ-GCSh is used. Overexpression of γ-GCSh alone without cotransduction provide an open target of the feedback effect. The precise reasons why the overexpressed GSH in the stably transfected cell lines did not feedback to inhibit the biosynthesis of GSH are not clear, but explanations

can be offer: (i) In the stably transfected cell line, the increased γ-GCSh mRNA were <3-fold, and GSH, <4-fold. These levels of increase may not reach the critical threshold that could exert its feedback reaction. This explanation is consistent with the observation that in the feedback inhibition only observed when high levels of γ-GCSh/GSH were expression, i.e. late phase of recombinant AdE1.tTA. γ-GCSh-traansduction under tet-on conditions. (ii) Because stable γ-GCSh-transfected cell lines had gone through a clonal selection process, it remains possible that an adaptive elevated γ-GCSh/GSH mechanism has been developed during this process.

The down-regulation of MMP-1 in the stable γ-GCSh-transfected cell lines but not in the AdE1.tTA. γ-GCSh infected cell lines as described in this communication may reflect the differences in responses to these different redox-regulated modes.

Regulation mechanisms of MMP1, MMP3 and MMP10 expression

The mechanisms of redox-induced downregulation of MMP3 and MMP10 have not been investigated. It has been reported that both transcriptional and post-transcriptional mechanisms are involved in the regulation of MMP3. Transcriptional regulation of MMP3 is mediated by the transcriptional factor AP-1 and Nuclear factor kappa B (NF-κB) [30]. Because these transcription factors are sensitive to redox conditions and we have found that in the γ-GCSh-transfected cells, AP-1 and NF-κB factors are downregulated (data not shown), our present finding is consistent with the concept that down-regulation of MMP3 under redox conditions is mediated by these transcription factors. Moreover, post-transcriptional regulation through the p38a MAPK pathway by enhancing MMP3 mRNA stabilities has been reported in TNF-α-treated normal human skin cells [31] and it is known that the p38 signaling is also redox-regulated [32]. While the mechanisms underlying the regulation of MMP10 remains to be studied, given that its coordinated expression with that of MMP3, it is likely the they may share the same control mechanism of expression.

While redox-regulation of MMP1 may depend upon the modulating conditions as described above, it has been shown that transcriptional factors AP-1 and NF-κB as well as post-transcriptional mechanism elicited by p38 signaling also regulated expression of MMP1 [33–35]. It is likely that MMP1, MMP3 and MMP10 may have a common mechanism in response to the environmental changes in redox homeostasis. However, given the genomic structural differences among these three genes, it is anticipated that quantitative and/or qualitative differences in their responses to different redox environments are anticipated.

Redox regulation of MMP expression and tumor development

Because MMP1, MMP3 and MMP10 are known to degrade extracellular matrix proteins and their expression levels are considered to play a role in tumor cell invasion, our results have important implication of redox-regulation of tumor progression. Although there are *in vitro* assay systems for determining whether overexpression MMP genes are correlated with cell migration, however, these assay systems did not always truly reflect the abilities *in vivo*. Perhaps this is not surprising because multiple layers of control mechanisms are involved in the regulation of MMP's proteolytic activities, including transcription and post-transcriptional regulation (mRNA stability), translational and post-translational controls such as proenzyme activation and inhibition, enzymatic stability and secretion, and shedding and oligomerization, etc [36,37]. Moreover, redox regulations of other yet-to-be identified genes may complicate the overall analysis of cell invasiveness. Given these complications, it remains critically to determine whether redox-regulated MMP1, MMP3 and MMP10 observed here could result in retarding cell migration ability.

The concepts correlating MMP expression and tumor progression have been evolved for some time. As such, several clinical trials have been undertaken to evaluate the feasibility of suppressing tumor progression through modulating MMP expression. Several MMP inhibitors (MMPIs) have been developed and so far significant benefits remain to be conclusively demonstrated [38,39]. Most of these inhibitors are small molecules and targeting one or a few species of MMPs. The present results showing that it is possible to simultaneously down-regulate multiple MMP genes by overexpressing γ-GCSh may provide a new thinking of using redox modulators for the development of antioxidant-based tumor treatment.

Finally, the demonstration that AdE1.tTA. γ-GCSh recombinant produces high levels of γ-GCSh may have application for studying the complex mechanism(s) involved in redox regulation and tumor progression in gene therapy. Outside the cancer prevention/progression field, this recombinant adenovirus may be of value in the treatments of various redox-related diseases, including cholestasis-induced liver fibrosis [40], alcohol-induced liver injury [41], and oxidant stress-induced pancreatic islets [42], in addition to the prevention of tumor spread in certain cancers which are known to metastasize due to overexpression of MMPs. In so doing, newly developed recombinant adenoviral

vectors in which several major viral genes were removed should be considered because of their reduced toxicities to the host [43].

Acknowledgements

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