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Reduction-Oxidation (Redox) State Regulation of Extracellular Matrix Metalloproteinases and Tissue Inhibitors in Cardiac Normal and Transformed Fibroblast Cells

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Abstract Latent matrix metalloproteinases (MMPs) in normal myocardium are activated in end-stage heart failure. In vitro oxidized glutathione (GSSG) activates myocardial MMPs which contains a cysteine residue. In vivo GSSG induce the collagen lysis and cardiac dilatation. To assess whether thiol and non-thiol reducing agents have direct effect on the interstitial human heart fibroblast (HHF) proliferation and MMP expression, HHF and polyoma virus transformed fibroblast cells were cultured with or without the thiol-containing reduced (GSH) or oxidized (GSSG) glutathiones, pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC), and non-thiol ascorbic acid. After 100 μg/ml (~0.3 mM) GSH or PDTC treatment the proliferative (synthetic) phenotype of transformed fibroblast cells was changed to quiescent (contractile) phenotype. Also, after GSH, PDTC, and ascorbic acid treatment the medium was then analyzed for MMP activity by zymography. The results indicate reduction in MMP expression in transformed fibroblast cells after GSH and PDTC treatments and no effect after ascorbic acid treatment. Based on reverse zymography, we observed the level of tissue inhibitor of metalloproteinase (TIMP) at a decreased level in transformed cells. The effect of the reducing agent at the gene transcription was measured by estimating mRNA (Northern blot analysis) of MMP and of TIMP in the cells that were cultured in medium in the presence and absence of GSH. These results indicate that GSH induces MMP-2 and MMP-1 expression in normal HHF and that GSH reduces MMP-2 and MMP-1 in transformed fibroblast cells. After the treatment, the TIMP-2 level was repressed in normal HHF and TIMP-2 level increased in transformed fibroblast cells. These events are dependent on the nuclear transcription factor activity on the collagenase promoter in normal HHF cells. On the other hand, in polyoma transform fibroblast cells these events are not dependent on this collagenase promoter. These results suggest that oxidative environment induces normal HHF cell proliferation, and the reducing agent decreases normal HHF cell proliferation by inducing MMP and repressing TIMP gene transcription. In transformed cells reducing agents inhibit MMP expression and increase TIMP levels, which suggests a role of antioxidants in preventing tumorigenesis.

Key words: Oxidative stress, redox-state, antioxidants, extracellular matrix metalloproteinase, tissue inhibitor of metalloproteinase, fibroblast, polyoma virus transformation, tumor, gene expression

In response to ischemic injury, activated leukocytes damage the endothelium and infiltrate the interstitium [Springer, 1994]. Particularly, this is the case in microvessels where there is no media and no smooth muscle cells. Activated leukocytes such as monocytes, lymphocytes, or neutrophils are recruited at the site of injury and the leukocytes release cytokines, proteinases, and oxidants [Rossen et al., 1988; Pinckard et al., 1983; McManus et al., 1983]. Activated neutrophils are toxic to cardiac myocytes and cause myocyte necrosis [Kabour et al., 1994]. Most of the intracellular glutathione is in the reduced form (GSH) and maintains the redox-

Abbreviations: MMPs, matrix metalloproteinases; ECM, extracellular matrix; HHF, human heart fibroblast; GSSG, oxidized glutathione; GSH, reduced glutathione; TIMP, tissue inhibitor of metalloproteinase; PDGF, platelet derived growth factor; FGF, fibroblast growth factor; EGF, epithelial growth factor; MEM, minimum essential medium; FCS, fetal calf serum; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; Il-1, interleukin-1; NF-k-B, nuclear factor-k-B; IGF, insulin growth factor; PBS, phosphate buffered saline; PMSF, phenyl methyl sulfonyl fluoride; PDTC, pyrrolidine dithiocarbamate; NAC, N-acetylcysteine.

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state of the living cells. After cell damage released glutathione rapidly gets oxidized to GSSG. A high level of GSSG is generated at the site of tissue injury. Also, the hypoxic, acute coronary occlusion and ischemic/reperfusion conditions increase oxidized glutathione in cardiac interstitium [Park et al., 1991; Romero et al., 1987; Curello et al., 1985] which leads to the change in the redox-state of interstitium which surrounds the fibroblast cells. We hypothesize that the change in the oxidative environment around the fibroblast induces extracellular matrix (ECM) proteinases and induces inhibitor gene expression. Also, oxidative stress may play an important role in the transformation of normal fibroblast to malignant phenotype.

In vitro GSSG activates myocardial MMPs which contain a mixed-function oxidative cysteine residue. We have shown that oxidized glutathione activated myocardial latent MMP [Tyagi et al., 1993a], probably through cysteine switch mechanism [Springman et al., 1990]. The neutrophil elastase degrades tissue inhibitor of metalloproteinase (TIMP) in MMP/TIMP complex [Tyagi et al., 1993a]. Therefore, this degradation leads to MMP activation [Tyagi et al., 1995a]. In vivo GSSG induces the cellular proliferation, the collagen lysis, and the cardiac dilatation [Janicki et al., 1994; Janiszewski et al., 1994].

Transformation of fibroblast cells by the environment or by the oncogenic viruses profoundly alter their morphology and growth characteristics [Alexander and Werb, 1989; Liotta et al., 1991]. To maintain the metastatic nature of these cells, transformed cells produce high levels of matrix metalloproteinase (MMP) and reduced expression of tissue inhibitor of metalloproteinase (TIMP) [Alexander and Werb, 1989; Liotta et al., 1991]. Oxidative stress proliferate transformed cells [Burdon et al., 1989]. Using c-fos-deficient mice generated through gene knockout, Hu et al. [1994] isolated fibroblast cells from primary embryonic fibroblast cell culture. These cells which are implicated in tumor invasiveness grow normally; however, mRNA for MMP which are usually induced by oncogenes and growth factors were not induced by PDGF and EGF in these c-fos deficient cells. In contrast, transformation of these mutant cells with polyoma virus middle T oncogene essentially restores wild-type levels of MMPs expression. These studies clearly demonstrate that the MMP gene expression in T antigen transformed cells involves other mechanisms than oncogenes. We tested the hypothesis that polyoma selectively induces MMP gene expression through an antioxidant sensitive pathway in fibroblast cells. We identified that antioxidant induces phenotypic changes in transformed fibroblast cells. And these changes were associated with the inhibition of MMP and the induction of tissue inhibitor of metalloproteinase (TIMP) gene transcriptions.

In the experiment which creates a genetic competition by fusing two cells, one transformed and one normal, Harris and Watkins [1965] demonstrated that the phenotype that comes out in the fused cells was a normal cell. This suggested that the tumor suppressor gene must exist in normal cells [Harris et al., 1994]. We have shown that normal human heart fibroblast (HHF) cells produce MMP in response to serum condition [Tyagi et al., 1995b]. We tested the hypothesis that the induction of MMP by the serum responsive factor is mediated through an antioxidant mechanism in normal HHF cells. Through this process we have shown a possible molecular linkage between the redox-sensitive mechanism in fibroblast cells and the gene transcription of MMP and TIMP. The same transcription expands on the notion of oxidative stress as being an important regulatory signal in the expression of MMP and TIMP in the pathogenesis of tumorigenesis.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS), normal rabbit serum, minimum essential medium with Earle's salts (MEM), collagen and laminin coated culture plates, and Hanks' balanced salt solution all were obtained from Collaborative Research (Bedford, MA). Trypsin was obtained from GIBCO (Gaithersburg, MD). Collagenase was obtained from Worthington (Freehold, NJ). Reduced (GSH) and oxidized glutathione (GSSG), PDTC, NAC, L-ascorbic acid, gelatin (porcine skin, 300 bloom), casein, fibronectin, Triton X-100, SDS, and 1, 10 phenanthroline (Phen) were obtained from Sigma (St. Louis, MO). Prestained electrophoresis protein standards were obtained from Bio-Rad (Richmond, CA). Six well plates and their inserts were obtained from BioCoat Corp., Collaborative Biomedical Products, Bedford, MA.

Cell Culture

Normal human heart fibroblast (HHF) cells were isolated as described previously [Tyagi et

al., 1995b]. Isolated cells were platted at the end of several 10-min digestion periods on 100-mm culture dishes in MEM containing 10–15% FCS. The cell cultures were incubated for 2 h at 37°C in an incubator and then were humidified with 90% O_2/10%CO_2.

The HHF cells were cultured on collagen-coated and uncoated plates in medium that was supplemented with 20% normal rabbit serum, 0.1% collagen suspension (Vitrogen 100, Celtrix, Santa Clara, CA), 5% fetal bovine serum, 4.5 mg/ml glucose, gentamycin and fungizone (10 μg/ml), and 2 mM glutamine. For most experiments, cells were washed two times with serum-free Dulbecco's modified Eagle's minimum essential medium (MEM) prior to the experimental treatment. Cultures were routinely checked for the presence of mycoplasma [Chen, 1977] which has been shown to stimulate MMP level [Kluve et al., 1981]. Cardiac fibroblast cells were transformed by polyoma virus middle T antigen as described by Kaplan et al. [1986].

Serum Glutathiones, PDTC, NAC, and Ascorbic Acid Induction of MMP and TIMP

HHF cells were cultured until confluent in MEM with 20% FCS on Lab-Tek (Naperville, IL) Permanox chamber slides (Nunc, Naperville, IL) or on 60-mm culture dishes. Confluent cells were deprived of serum for 48 h. Cells were cultured without and with 100 μg/ml (~0.3 mM) GSH or GSSG, PDTC, NAC, and ascorbic acid in the serum-free medium for 24 h. Analysis of MMP and TIMP activity released in the MEM was carried out by zymography and by reverse zymography, respectively.

Electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with or without reduction by the method of Laemmli [1970]. After electrophoresis proteins were stained with silver nitrate or Coomassie Brilliant Blue R 250.

Collagenolytic/Gelatinolytic Activity

Matrix metalloproteinase activity in the gel was measured as described previously [Tyagi et al., 1993b]. Denatured type I collagen, gelatin, fibronectin, or casein was added to standard Laemmli [1970] acrylamide polymerization mixtures at a final concentration of 0.5 mg/ml under non-reducing conditions. The gel was scanned for lytic band intensity, photographed, and dried for permanent records. The activity was then measured under identical amount of total protein loaded onto the gel.

Anti-Proliferative Activity of Antioxidants

Normal human heart fibroblast (HHF) and polyoma virus transformed fibroblast cells were grown in 5% serum [Tyagi et al., 1995b] with (100 μg/ml) and without reducing agents GSH, PDTC, NAC, and ascorbic acid. In order to characterize the anti-proliferating activity of GSH and PDTC, the cells were counted by measuring acid phosphatase activity as described by Connolly et al. [1986]. For acid phosphatase assay, cells were grown in 96-well plates at densities between 10^2 and 10^6 cells per well. After removing the medium and washing with PBS, p-nitophenyl phosphate (10 mM) was added. The plates were incubated at 37°C for 2 h. The reaction was stopped with the addition of 0.1 N NaOH. The color development was measured at 405 nm using Microplate Reader (Molecular Devices, Menlo Park, CA). The blank contains PBS. For each assay, the nonenzymatic hydrolysis of substrate was determined by incubating wells that did not contain cells. The background value was subtracted from all experimental values. A standard plot was developed based on cell number and OD measurements at 405 nm. From this plot experimental values of cell numbers were obtained. Control cells contain serum and PBS. Each experiment was carried out in triplicates. Mean ± SD are reported. We use acid phosphatase activity as the marker of cell number in a Microplate Reader without detaching the cells from surface. This assay provides similar results as measured by hemacytometer on trypsinized cells. After the treatment of GSH, transmission micrograph of cell culture was obtained to identify apparent phenotypic changes in the cell population.

Reverse Zymography

To identify proteinase resistant band, reverse zymography was performed as described by Granelli-Piperno and Reich [1978]. The concentrated condition medium from normal and transformed fibroblast cells was loaded onto SDS-substrate reverse zymographic gels as described [Tyagi et al., 1993a]. After washing with Triton X-100 the gels were incubated at 37°C in the presence of trypsin activated condition medium of cell culture for 1–2 h. In the activated me-

dium 1 mM PMSF was added to inhibit trypsin. The gels impregnated with activated culture medium metalloproteinases were then incubated overnight in substrate buffer, stained, and destained as described [Tyagi et al., 1993a]. The medium contains several proteinases that partially degrade the substrate within the gel under these conditions. This condition does not allow the inhibitors to degrade.

Northern Blot (mRNA) Analysis of MMP, TIMP, and β-Actin

Total RNA was isolated from 1×10^6 cells using 4M Guanidine thiocyanate buffer [Churgwin et al., 1979]. RNA was quantitated at 260 nm absorbance. The purity of total RNA was assessed by absorbance ratio (260/280 nm) of 2.0. Twenty micrograms of total RNA were denatured in a formamide/formaldehyde solution at 65°C for 15 min and samples were then resolved on denaturing 1% agarose gel. The gel was transferred to nitrocellulose filter where it was prehybridized in a buffer containing 50% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardts, 50 mM $NaHPO_4$, and 100 μg/ml denatured sperm DNA at 42°C for 4 h. Blots were then hybridized for 16 h at 42°C with [α-^{32}P]-dCTP random prime labelled cDNA. The membrane was washed in 0.1 standard saline citrate plus 0.1% SDS at 42°C for 1 h and then exposed to X-ray films at −70°C for 24 h. The plasmid containing fibroblast collagenase (MMP-1) cDNA was obtained from American Type Culture Collection (ATCC, Rockville, MD). MMP-1 probe was 2.05 kb Hind III and Sma I fragment from human MMP-1 cDNA. MMP-2 probe was 2.119 kb EcoRI fragment from human MMP-2 cDNA. The plasmid containing TIMP-1 cDNA probe was obtained from Synergen Corp, Boulder, CO. TIMP-1 probe was 0.7 kb EcoRI fragment of a human TIMP-1 cDNA. TIMP-2 probe was 0.791 kb EcoRI and XbaI fragment of a human TIMP-2 cDNA was obtained from ATCC. A 1.1 kb EcoRI fragment from human β-actin cDNA was used as an internal control.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

Confluent normal HHF and transformed cells were treated with 100 μg/ml of GSH, PDTC, or NAC in serum-free medium for 24 h. Nuclear proteins were purified by a modification of Dignam et al. [1983] protocol. Briefly, after washing with PBS, cells were centrifuged and the cell pellet suspended in 500 μl buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT). After recentrifugation, the cells were resuspended in 80 μl buffer A containing 0.1% Triton X-100 by gentle pipetting. After incubating for 10 min at 4°C, the homogenate was centrifuged and the nuclear pellet was washed once with buffer A and resuspended in 70 μl of buffer C (10 mM Hepes, pH 7.9, 25% [v/v] glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT). The suspension was incubated for 30 min at 4°C followed by centrifugation at 20,000*g* for 10 min. The resulting supernatant was stored at −70°C until further use. Protein concentration was determined by the Bradford [1976] method. To minimize proteolytic degradation of nuclear proteins, buffer contained 1 mM PMSF. The oligonucleotide containing human collagenase promoter [Angel et al., 1987] sequence (5′-AAGCATGAGTCAGACACCTCTGGC-3′) was synthesized. A double-stranded oligonucleotide containing the collagenase promoter was prepared by annealing complementary synthetic oligonucleotide. All nucleotide were prepared by the University of Missouri-Columbia DNA core facility. Radio labeled double-stranded DNA was made by annealing an oligonucleotide complementary to the 3′ end of the sequence and extended with DNA polymerase and 50 μCi of [^{32}P]dCTP and unlabeled dATP, dGTP, and dTTP followed by the addition of 0.5 ml unlabeled dCTP. Unincorporated nucleotide were removed by column chromatography over a Sephadex G-50 column. Cold double-stranded DNA was made identically except that unlabeled dCTP was substituted for labeled dCTP. The DNA binding reaction was performed at 30°C for 15 min in a volume of 20 μl, which contained 2–5 μg of nuclear extract, 225 μg/ml BSA, 1×10^5 cpm ^{32}P-labeled probe (~1 ng), 0.1 mg/ml poly(dI-dC), 15 μl binding buffer (12 mM Hepes, pH 7.9, 4 mM Tris, 60 mM KCl, 1 mM EDTA, 12% glycerol, 1 mM DTT, and 1 mM PMSF). Samples were subjected to electrophoresis on a 6% 1 × Tris-glycine-polyacrylamide gels.

Co-Culture Experiment

Co-culture was carried out as described previously [Guarda et al., 1993]. Normal HHF and transformed fibroblast cells were grown in confluence. HHF cells (1×10^6 cells) were grown in inserts (BioCoat). The insert contains 0.45 micron pore in the bottom to exchange fluid be-

tween two cell types. The transformed cells (1×10^6) were grown in 60-mm disk. Once both HHF and transformed cells were confluent, the inserts containing normal HHF cells were gently placed into wells containing transformed fibroblasts under aseptic conditions and co-cultured for 24 h at 37°C in a 5% CO_2 atmosphere. Cells were removed and RNA was isolated and analyzed by Northern analysis.

RESULTS

Anti-Proliferating Effect of Anti-Oxidant in Human Heart Normal and Transformed Fibroblast Cells

To explore whether the serum activated fibroblast cell proliferation through an oxidation-dependent mechanism, normal HHF and transformed fibroblast cells were exposed to serum in the presence and absence of 100 μg/ml (~0.3 mM) reduced glutathione (GSH), PDTC, and ascorbic acid for up to 5 days. The cells were given fresh medium every day by changing fresh medium from day 0 to day 5. Serum induced HHF and transformed fibroblast proliferation (Fig. 1). There was no difference in proliferation in the presence and absence of 100 μg/ml oxidized glutathione (GSSG) which was added to the serum (data not shown), which suggests serum contains an oxidative environment. The addition of GSSG has not further proliferative effect on normal and transformed fibroblast cells. Transformed fibroblast cells proliferate more rapidly than normal HHF cells in response to serum, suggesting a greater degree of proliferation of polyoma transformed fibroblast cells. The growth curves of HHF and transformed fibroblast cells are shown in Figure 1A. In the presence of GSH and PDTC, serum induction was inhibited by over 50% and ascorbic acid has no effect under these conditions (Figure 1). The data demonstrate that the proliferation by serum occurs through a thiol antioxidant-sensitive regulatory step. Apparently GSH inhibits more transformed fibroblast cell growth than normal HHF cells, suggesting that transformed fibroblast are more sensitive to anti-oxidants.

Phenotypic Changes in Transformed Fibroblast Cells Are Associated With Anti-Oxidant

Transformed fibroblast cells grow rapidly to a higher cell density in minimum serum condition which suggests mitogenicity of these cells to serum components. We have demonstrated that transformed fibroblast cell growth was reduced by anti-oxidant (Fig. 1). When normal fibroblast cells are transformed by oncogenic products their morphology and growth characteristics are profoundly altered. To examine whether the change in growth of transformed cells is associated by phenotypic changes, we cultured transformed fibroblast with and without 100 μg/ml GSH in serum for 72 h. As shown in Figure 2, transformed fibroblast cells grow and develop pseudopodia (a characteristic of invasive and synthetic phenotypes) in response to serum conditioned or serum containing oxidized glutathione (data not shown). In the presence of the reducing environment, there was decrease in transformed fibroblast cell growth and number. Also, the treatment with GSH changed the apparent shape of the cell from the proliferative/synthetic phenotype to quiescent (contractile) phenotype (Fig. 2). This suggests that phenotypic changes are associated with the effect of antioxidant on transformed fibroblast cells.

Suppression of Matrix Metalloproteinase by Anti-Oxidants in Transformed Fibroblast Cells

To explore whether anti-oxidant sensitive proliferation and phenotypic changes in normal and transformed fibroblast cells are associated with MMP expression, we analyzed MMP activity in these cells after GSH, PDTC, and ascorbic acid treatments by zymography (Fig. 3). In response to serum factors normal and transformed cells produce MMP. We cultured these cells in the presence of 100 μg/ml GSH or PDTC or ascorbic acid for 24 h and replaced the medium with serum-free MEM after 24 h. The MMP activity in the medium was measured. The results suggest that MMP expression was suppressed by GSH and PDTC and not by ascorbic acid treatments, indicating thiol containing anti-oxidant sensitive MMP expression in fibroblast cells. The synthetic and invasive phenotypes of transformed cells were related to the release of high amount of MMP and its inhibition by antioxidants (Fig. 3). In vitro GSH and PDTC may inhibit MMP activity by blocking the active site residues in the enzyme via cysteine switch mechanism. We, therefore, removed the medium containing GSH and added fresh medium without GSH or PDTC for 24 h and measured MMP activity in this medium.

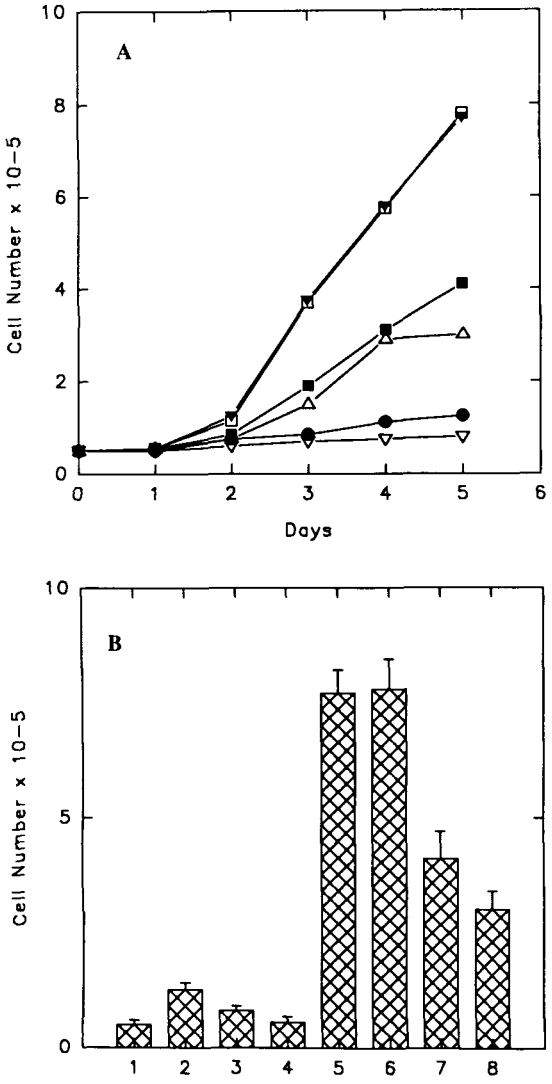


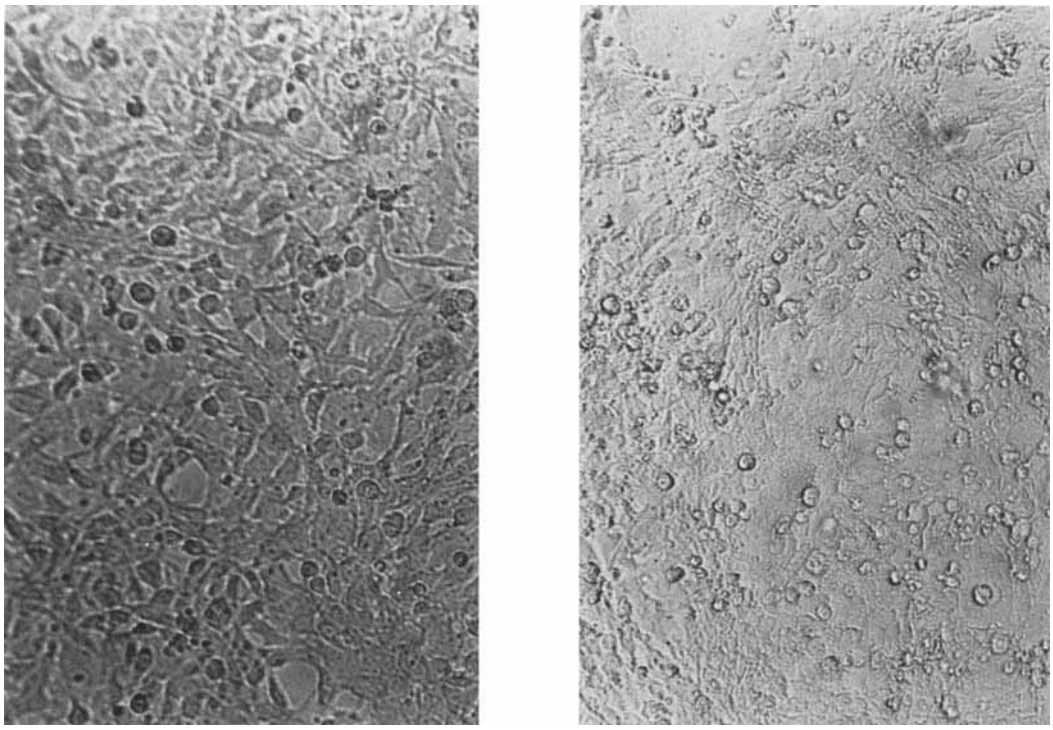
Fig. 1. Effect of redox-state on normal human heart fibroblast (HHF) and transformed fibroblast cells growth: Cell density was estimated using acid phosphatase assay. **A:** HHF were seeded at 5×10^4 cells/well in 5% serum on day 0. Test media were added on day 1 and cultures refed with fresh media every second day. ●—● HHF in 5% serum; ▽—▽ HHF in 5% serum plus 100 μg/ml (~0.3 mM) GSH; ▼—▼ transformed fibroblast in 5% serum; □—□ transformed fibroblast in 5% serum plus 100 μg/ml ascorbic acid; □—□ transformed fibroblast in 5% serum plus 100 μg/ml GSH; △—△ transformed fibroblast in 5% serum plus 100 μg/ml PDTC. **B:** Histographic presentation of normal and transformed fibroblast cell growth. (1) Normal HHF cell at day 0 in 5% serum; (2) normal HHF cell growth for up to day 5 in 5% serum; (3) normal cell growth for up to 5 days in 5% serum plus 100 μg/ml GSH; (4) transformed fibroblast cells at day 0 in 5% serum; (5) transformed fibroblast cells grown up to day 5 in 5% serum; (6) transformed fibroblasts grown for up to 5 days in 5% serum plus 100 μg/ml ascorbic acid; (7) transformed fibroblasts grown for up to 5 days in 5% serum plus 100 μg/ml GSH; (8) transformed fibroblasts grown for up to 5 days in 5% serum plus 100 μg/ml PDTC. Each bar represents the average number of cells obtained from three independent experiments. Mean ± SD are reported. The results suggest that GSH and PDTC reduce transform cell proliferation and ascorbic acid has no effect.

Suppression of Tissue Inhibitor of Metalloproteinase in Polyoma Transformed Fibroblast Cells

Regulation of MMP activity can occur at the level of gene expression which includes transcription and translation, at the level of activation, or at the level of inhibition by tissue inhibitor of metalloproteinase (TIMP). Perturbations at any of these points can theoretically lead to alterations in the proteinase-antiproteinase balance in matrix remodeling. To examine whether elevated MMP expression in transformed fibroblast cells is due in part to repression in TIMP level, we analyzed TIMP level in transformed fibroblast by reverse zymography and compared it with normal HHF cells. As shown in Figure 4, the collagenase resistant band was at a decreased level in transformed fibroblast cells as compared to normal HHF cells. The elevated expression of MMP (Fig. 3) and reduced level of TIMP (Fig. 4) may support the idea that MMP play an important role in the invasiveness of transformed fibroblast cells.

Anti-Oxidant GSH Inhibits Serum Induction of TIMP mRNA and Induces MMP mRNA in Normal Human Heart Fibroblast Cells

To investigate whether the induction in MMP and TIMP expression by anti-oxidant in HHF cells is at the gene level, we measured MMP and TIMP genes expression by Northern blot analysis in HHF cells by GSH (Fig. 5). HHF cells were exposed to 100 μg/ml GSH. As shown in Figure 5, serum oxidative conditions induce basal expression of MMP-1 and MMP-2. However, in the presence of GSH both MMP-1 and MMP-2 expression were increased significantly. This suggests that anti-oxidants have stimulatory effects on MMP gene expression and oxidative environment has a suppressive effect on MMP in normal fibroblast cells. Similarly, TIMP-1 and TIMP-2 levels were measured as a function of anti-oxidant treatment. The level of TIMP-2 was higher as a basal condition. However, under anti-oxidant conditions, TIMP-2 expression was suppressed (Fig. 5). After the treatment of anti-oxidants the level of TIMP-1 was slightly increased. Under the identical condition of GSH treatment the internal control, β-actin gene, did not change. Similar results were obtained with PDTC (data not shown). These results suggest that anti-oxidant suppressed TIMP-2 expression and induced MMP expression at the transcription level in normal HHF cells.



A B

Fig. 2. Inhibition of transformed fibroblast cell growth by GSH: Cells were seeded in 5% serum. After 24 h, medium was replaced with fresh medium containing 5% serum without (**A**) and with (**B**) 100 μg/ml (~0.3 mM) GSH. After 72 h light transmission micrograph was obtained. Reduction in cell number and changes in sprouting phenotype in transformed fibroblast cells were observed after GSH treatment.

Anti-Oxidant GSH Inhibits Serum Induction of MMP mRNA and Induces TIMP mRNA in Transformed Fibroblast Cells

Polyoma transformed fibroblast cells are invasive and malignant in phenotype. To explore the elevated expression of MMP in transformed fibroblast through an oxidation-dependent pathway, transformed fibroblast were exposed to serum in the presence or absence of anti-oxidant 100 μg/ml GSH for up to 24 h. In response to the serum, MMP-1 and MMP-2 mRNA levels were increased while TIMP-1 and TIMP-2 mRNA levels were suppressed in transformed fibroblast cells. Therefore, as shown in Figure 6, both MMP-1 and MMP-2 were suppressed at the gene level by anti-oxidant. The level of TIMP-1 and TIMP-2 mRNA were elevated by the treatment of GSH in polyoma transformed fibroblast cells. The level of internal control β-actin did not change under these conditions, which suggests no significant effect of anti-oxidant on β-actin expression. Similar results were obtained with PDTC (data not shown). These results suggested a molecular linkage between an anti-oxidant sensitive transcriptional regulatory mechanism and an MMP and TIMP gene transcription. These experiments expand on the notion of oxidative stress as an important regulatory signal in the pathogenesis of tumorigenic cells.

Reducing Agents Induce Metalloproteinase Expression in Normal HHF Cells by Activating DNA Binding Protein Activity to a Collagenase Promoter Site

To explore whether GSH regulated MMP gene transcription through collagenase promoter activity (and binding to transcriptional regulatory factor) nuclear extracts from treated and untreated normal HHF and transformed fibroblast cells were assayed for DNA binding activity to a double-stranded oligonucleotide probe containing the collagenase promoter elements located at positions −77 and −54 [Angel et al., 1987]. The probe sequence (5′-AAGCATGAGTCAGACACCTCTGGC-3′) was chosen from collage-

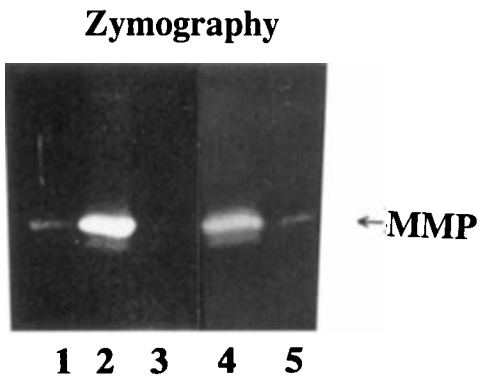


Fig. 3. Zymographic analysis of MMP expression in cells treated with or without PDTC, GSH, and ascorbic acid: (1) Normal HHF (1×10^6) cells were grown to confluence in serum and starved in serum-free MEM for 24 h. (2) Transformed fibroblast (1×10^6) cells were grown to confluence in serum and starved in serum-free MEM for 24 h. (3) Transformed fibroblast (1×10^6) cells were grown to confluence in serum containing 100 μg/ml GSH and starved in serum-free MEM for 24 h. (4) Transformed fibroblast (1×10^6) cells were grown to confluence in serum containing 100 μg/ml ascorbic acid and starved in serum-free MEM for 24 h. (5) Transformed fibroblast (1×10^6) cells were grown to confluence in serum containing 100 μg/ml PDTC and starved in serum-free MEM for 24 h. The MMP activity in the medium was measured by loading identical amounts of total protein (100 μg) onto each lane. The results suggest inhibition of MMP expression by GSH and PDTC and not by ascorbic acid.

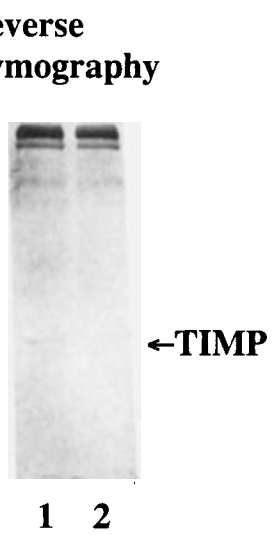


Fig. 4. Reverse zymographic analysis of TIMP: In condition medium of normal HHF and transformed fibroblast cells, the collagenase resistant band was identified in SDS-PAGE (12%) substrate-gel-matrix. (1) Medium from normal HHF; (2) medium from transformed fibroblast cells. Identical amounts of total protein (50 μg) were loaded on to each lane. Band at the top also indicated the same amount of total protein in the gel in these two lanes. The results suggest that TIMP band was at a reduced level in transformed fibroblast cells.

nase promoter [Angel et al., 1987]. This sequence has been used by Tina et al. [1992] and showed that binding to this sequence increased after serum induction in smooth muscle cells. We therefore use this sequence to identify the role of specific collagenase promoter sequence element in inhibition of serum-induced fibroblast collagenase expression by antioxidants. As shown in Figure 7, the complex band represented transcription factor binding activity in response to GSH, as well as PDTC and NAC in HHF cells, which indicates that structurally distinct antioxidant may stimulate a common, oxidation-sensitive regulatory step that affect MMP gene transcription. The specificity of protein-DNA complexes was determined by competition binding experiments. Competition with 20- and 100-fold excess cold oligonucleotide probe sequence resulted in 60 and 100% inhibition of binding, respectively. These results demonstrate that a transcription factor binding activity is induced by treatment of reducing agent in HHF cells. This suggests that the induction of MMP gene transcription (Fig. 5) is mediated by a transcription factor activity in the nucleus of normal HHF cells. There was no binding of the probe in the transformed cell nuclear extracts. This may suggest alteration of trans acting elements in transformed cells as compared to normal cells. These studies suggest that repression of MMP expression in polyoma transformed cells may be mediated through different repressor mechanism than in normal HHF cells.

Normal HHF Induces TIMP-1 in Polyoma Transformed Fibroblast Cells

To determine whether TIMP-1 could be stimulated in transformed cell, co-culture experiments were carried out using normal HHF cells in the upper chamber and transformed fibroblast cells in the lower chamber. These chambers were connected with a 0.45 micron porus bottom of insert. This allowed free exchange of medium between the cell cultures without mixing the cell types. The transformed cells by themselves produced very little TIMP-1 (Fig. 6). After 24 h of co-culture with normal HHF cells, the tumor cells produced TIMP-1 at the gene transcription level (Fig. 8). The levels of TIMP-2, MMP-1, and MMP-2 were not affected by co-culture under these conditions (data not shown). These results demonstrated that under cell separation condition but exchange of medium between two different cell types, the cells in the top

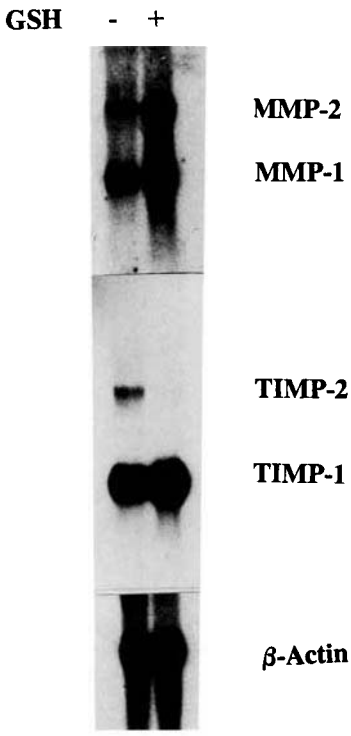


Fig. 5. Induction of TIMP-2 mRNA by serum is selectively inhibited by the antioxidant GSH. For 24 h confluent normal HHF cells (1×10^6) were grown in serum-free medium with and without the treatment of 100 μg/ml (~0.3 mM) GSH. The cells were collected and RNA was isolated. Northern blot (mRNA) analysis of MMP, TIMP, and β-actin was carried out. Total RNA was separated by agarose gel, transferred to nitrocellulose membrane, hybridized with cDNA probe for MMP-1, MMP-2, TIMP-1, TIMP-2, and β-actin. Identical amounts of total RNA were loaded onto each lane. The size of TIMP-1 transcript was 1.5 kb, TIMP-2 was 2.4 kb, MMP-1 was 2.5 kb, and MMP-2 was 3.5 kb. MMP-1 and MMP-2 mRNA are induced by reducing agent GSH.

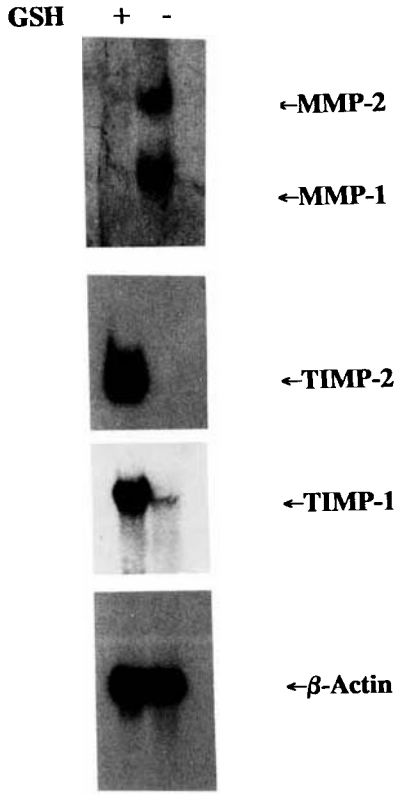


Fig. 6. Induction of TIMP-1 and TIMP-2 mRNA by the antioxidant GSH in transformed fibroblast cells. For 24 h confluent transformed fibroblast cells (1×10^6) were grown in serum-free medium with and without the treatment of 100 μg/ml (~0.3 mM) GSH. The cells were collected and RNA was isolated. Northern blot (mRNA) analysis of MMP, TIMP, and β-actin was carried out. Total RNA was separated by agarose gel, transferred to nitrocellulose membrane, hybridized with cDNA probe for MMP-1, MMP-2, TIMP-1, TIMP-2, and β-actin. Identical amounts of total RNA were loaded onto each lane. The size of TIMP-1 transcript was 1.5 kb, TIMP-2 was 2.4 kb, MMP-1 was 2.5 kb, and MMP-2 was 3.5 kb. MMP-1 and MMP-2 mRNA are suppressed by reducing agent GSH.

insert produce factor(s) which in turn induce TIMP-1 gene transcription in the cells of bottom well.

DISCUSSION

In response to serum induction, cardiac fibroblast cells activate concurrently with the expression of the metalloproteinase and tissue inhibitor genes [Tyagi et al., 1995b]. Here we show that this activation was regulated by a mechanism sensitive to antioxidants. However, the molecular mechanism underlying this activation is not fully understood nor is it known whether, and to what degree, these genes are activated by a common, or gene-specific, regulatory mechanism. Oxidants play a very significant role in cellular transformation and its metabolic function [Schreck and Baeuerle, 1991; Sckreck et al., 1991]. Transformation of fibroblast cells by oncogene products induce massive changes in the gene expression [Angel et al., 1987; Denhardt et al., 1989]. Imai and Takano et al. [1992] clearly demonstrated that the level of collagenase dramatically increases in the mortal fibroblast cells after T antigen-transformation. They also showed that SV40 T antigen might participate in a trans-acting machinery which seemed to be involved in the drastic changes of collagenase expression. To understand the effect of thiol (PDTC, GSH, and NAC) and non-thiol (ascorbic acid) reducing agents on normal and transformed fibroblast cells, we demonstrated specific induction of MMP expression in normal fibroblast and inhibition of MMP ex-

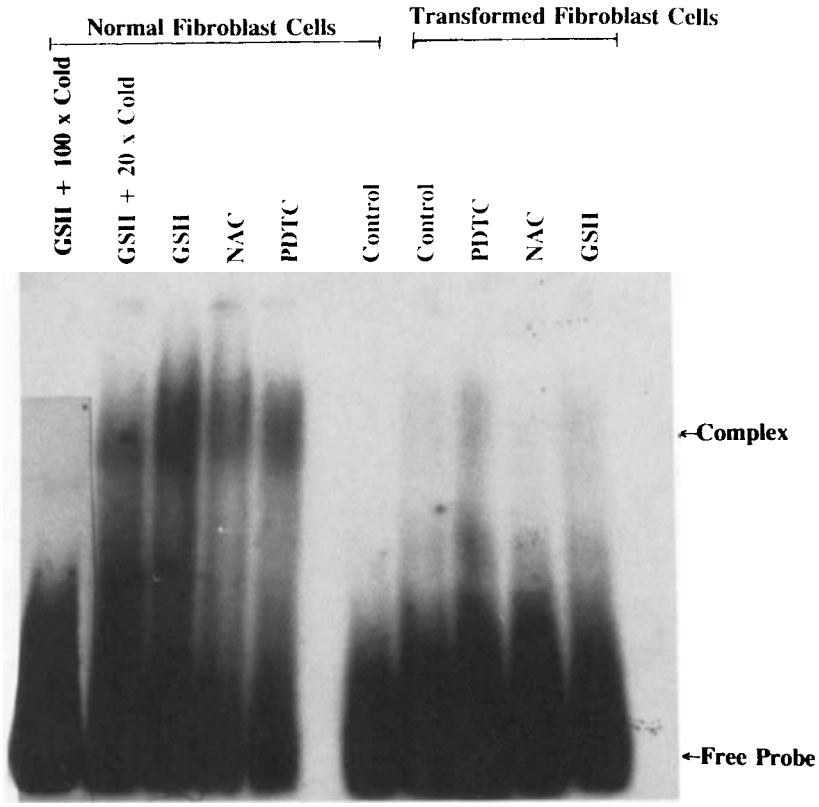


Fig. 7. GSH, PDTC, and NAC induce MMP expression by activating nuclear transcription factor activity of collagenase promoter in normal HHF and not in transformed fibroblast cells: Confluent normal or transformed fibroblasts were treated or untreated (control) with GSH, PDTC, and NAC (100 μg/ml) in serum-free medium for 24 h, respectively. Nuclear extracts were prepared. Ten micrograms of nuclear extract was incubated with a double-stranded ^{32}P-labeled collagenase promoter sequence and size fractionated on 6% native acrylamide gels, and exposed to autoradiography film. Extract from cells treated with GSH were incubated with radiolabeled oligoprobe in the absence or presence of 20 and 100 × cold probe.

pression in transformed fibroblast cells by thiol reducing agents. The ascorbic acid has no effect on both cell growth and MMP expression (Figs. 1, 3, and 7). After GSH treatment, in normal HHF cells TIMP-2 was suppressed and both MMP-1 and MMP-2 were induced at the gene levels. In transformed fibroblast cells both MMP-1 and MMP-2 were suppressed and both TIMP-1 and TIMP-2 were induced. The response to reducing agents in normal and transformed fibroblast is heterogeneous in the sense that GSH induces MMP expression in normal HHF and inhibits MMP expression in transformed fibroblast cells. Leco et al. [1992] have examined the expression of TIMP-1 and TIMP-2 mRNA in normal and ras-transformed murine fibroblasts. They showed that both the TIMP-1 and TIMP-2 mRNA were inducible by serum in normal cells. In oncogene transformed fibroblast cells both the TIMP-1 and TIMP-2 mRNA are not induced by serum whereas they are induced in normal cells, suggesting that TIMP-1 and TIMP-2 are differentially regulated in normal and transformed fibroblast cells.

Effect of antioxidant on cellular proliferation indicated that normal HHF cells as well as transformed fibroblast cells were limited in their growth in the presence of reducing agent GSH and PDTC (Figs. 1 and 2). The inhibitory effect of antioxidant was much greater on transformed fibroblast than on normal HHF cells. In normal HHF cells proliferation by serum and/or oxidative conditions may be related to the expression of a TIMP-like molecule, which is induced by serum/oxidative conditions. Both the TIMP-1 and TIMP-2 have been shown to be mitogenic against fibroblast cells [Nemeth and Goolsby, 1993; Hayakawa et al., 1992]. In normal HHF cells TIMP-2 expression was inhibited by antioxidant treatment. Our results clearly show that normal and polyoma transformed fibroblast cells are different in their response to serum elements (Figs. 5, 6) and demonstrate that the transformed phenotype can be reverse to nor-

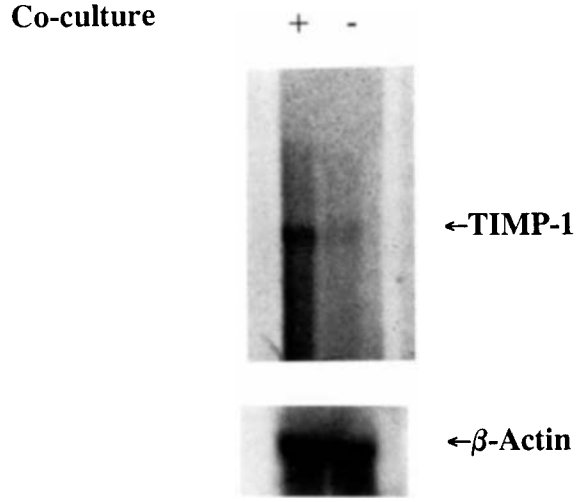


Fig. 8. Effect of normal fibroblast on transformed fibroblast cells: Induction of TIMP-1 in transformed fibroblast cells by co-culture (+) with normal fibroblast cells: Cells were grown to confluent in separate culture. Normal HHF cells grown in insert were placed in a 60 mm disk containing transformed fibroblast cells. The insert contains 0.45 μm pores. Co-culture in serum-free medium was continued for 48 h. Equal numbers of normal and transformed fibroblast cells (1×10^6) were used in the top and bottom inserts and wells, respectively. Cells were collected and RNA was isolated. The membrane was blotted using TIMP-1 cDNA probe. Control represents transformed fibroblast cells without co-culture (−). The size of TIMP-1 transcript was 1.5 kb. The β-actin was used as internal control. TIMP-1 was induced in the transformed fibroblast by co-culture with normal fibroblast cells.

mal by the treatment of antioxidant. The mechanism of proliferation in transformed fibroblast cells may be different than normal cells in the sense that TIMP is more suppressed in polyoma transformed fibroblast cells (Fig. 6) than in normal cells (Fig. 5) and may be related to increase MMP activity of tumorigenic cells [Ponton et al., 1991]. Protective effect of antioxidant on cellular proliferation has been shown by Diplock et al. [1994]. Burdon et al. [1990] have demonstrated that certain active oxygen species, e.g., hydrogen peroxide, were stimulatory in promoting the growth of transformed and immortalized cells. The antioxidant reverts this growth promoting effect of oxi-species.

Invasive and metastatic phenotypes of transformed cells are associated with overexpression of MMP and the reduction of TIMP levels [Alexander and Werb, 1989]. Our results suggest that polyoma transformed fibroblast has a low level of both TIMP-1 and TIMP-2 and a significantly elevated level of MMP-1 and MMP-2. By reverse zymography we demonstrate the reduction of functional TIMP level in transformed fibroblast cells (Fig. 4). McIlhinney and Hogan [1974] have shown the inhibitory role of proteinase inhibitors in normal and polyoma transformed cell growth. The role of MMP in synthetic phenotype of transformed fibroblast cells was identified by measuring MMP activity in these cell by zymography. Figure 3 indicates that MMP activity was elevated in transformed cells but not in normal HHF cells. Normal cells have basal activity. This activity of transformed cells was suppressed by antioxidant via secondary signal from cell membrane to cytosol. This is based on the fact that 24 h after the removal of GSH or PDTC, MMP activity was still repressed in transformed fibroblast cells. Serum contains a number of growth factors including FGF, PDGF, IGF, and others, all of which effect MMP and TIMP expression in fibroblast cells. *Inhibition* of TIMP by antioxidant in normal HHF cells indicates that there is a suppression effect of these growth factors.

Central to an understanding of the mechanism by which an oxidative signal regulates MMP and TIMP gene transcription, we have demonstrated that antioxidants activate nuclear transcription factor activity. Our studies indirectly suggest that GSH functions through mechanisms related to its thiol antioxidant properties. In support of this, the structurally unrelated, thiol antioxidant PDTC and NAC also effectively induced the activation of gene transcription via increased promoter binding activity (Fig. 7).

Cell-specific, transformation-specific, and serum-specific factors may both play roles in modulating extracellular matrix proteinase and inhibitors gene transcription. Indeed, we observe no effect of antioxidant at the nuclear transcription factors activity in polyoma transformed fibroblast cells (Fig. 7). This raises the possibility that serum conditions may activate MMP and TIMP genes through distinct molecular mechanisms distinguishable by their sensitivity to antioxidants. These studies suggested that cell-specific and/or transformation-specific transcriptional regulatory factors may also play a role in regulating MMP and TIMP transcription in normal and transform fibroblast cells.

Induction of fibroblast 92 kDa gelatinase/type IV collagenase expression by direct contact with metastatic tumor cells have been demonstrated in a mix-culture model [Himelstein et al., 1994]. The stimulation of TIMP-1 at the protein level in tumor cell by normal fibroblast cells has been

demonstrated [Nabeshima et al., 1994]. However, these studies were carried out in mixed culture of two cell types. Our studies represent co-culture of separated cells by a porous stratum which allows exchange of medium without mixing the cell types. We demonstrated that the normal fibroblast secreted a factor which stimulates TIMP-1 in tumor cells (Fig. 8). The identification of this factor remains to be elucidated. We did not observe induction of TIMP-2 or suppression of MMP-1 and MMP-2 under these conditions. These results support the idea that TIMPs may be tumor suppressors which are present in the normal cells but absent in the transformed cells.

We demonstrated that antioxidants induce the metabolic changes in transformed fibroblast cells. In normal fibroblast cells antioxidants induce the expression of MMP. In transformed fibroblast cells antioxidants reduce MMP expression. After the treatment with antioxidants TIMP level was repressed in normal cells where as it was induced in transformed cells at the gene transcription level. The mechanism for MMP repression by antioxidants in transformed fibroblast cells did not indicate activation of collagenase promoter. In normal fibroblast MMP activation by antioxidants is mediated through collagenase promoter activity. In co-culture model normal fibroblasts induce TIMP-1 expression in transformed cells. These results suggested that an antioxidant therapy may benefit against tumorigenesis.

In normal HHF cells MMP activity was also effected by antioxidant but not to the extent of transformed fibroblast cells. Although, an effect on a redox mechanism is inferred, this study does not identify the specific redox biochemistry mediating antioxidants regulatory effect. However, our studies indirectly suggest that thiol-antioxidants function through the mechanism related to its thiol group properties. Whether these effects are due to redox reactions involving direct modification by an oxygen radical or through intracellular glutathione-dependent oxidation-reduction reactions is not yet known. In support of glutathione-dependent redox reactions, other effective thiol reagents increase the intracellular levels of glutathione [Perchellet et al., 1987]. The determination of cellular permeability of antioxidants and their half-lives under tissue culture conditions are the critical parameters to be determined in order to develop a relationship between oxidative stress in vivo vs. in vitro. Such studies are in progress.

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